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Growth Analysis and Plant Hormone Studies
in Apple (Malus sylvestris Mill.)

A thesis submitted in partial fulfilment of the
requirements for the degree of Doctor of Philosophy

at

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Massey University
Palmerston North
New Zealand

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To My parents, Soon Ja, Jin Soo, Si Young, and Jin Hyung.

Since we are assured that the all-wise Creator has observed the most exact proportions, of number, weight and measure, in the make of all things; the most likely way therefore, to get any insight into the nature of those parts of the creature, which come within our observation, must in all reason be to number, weigh and measure.

"God has not only comprehended the dust of the earth in a measure, and weighed the mountains in scales, and the hills in a balance, Isai. Xl. 12." (Hales, 1727).

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AbstractPart I

Growth analysis studies

Previous data on gravitational effects on shoot growth and flowering have been inconsistent. Attempts have been made to investigate shoot growth and flowering on shoots with a $3/8$ phyllotaxis in 90 cm and 150 cm laterals. These were bent at different times to the horizontal or to a pendulous position in apple varieties Red Delicious and Granny Smith on MM 106 rootstocks grown under a semi-intensive system on a commercial orchard in the major apple growing area of Hastings. The four treatments comprised: horizontal or pendulous bending during the dormant period, at petal fall, second cover stage and with normal vertical laterals as controls.

Horizontal bending increased total shoot growth and flowering relative to the vertical controls in the 90 cm treatment in both varieties. There seemed to be a tendency to decrease total shoot growth when the time of bending was later in the season and no differences in flowering occurred among the horizontal bending treatment. On the other hand shoot growth was relatively constant in all treatments in the 150 cm treatment. A very significant increase in flowering, however, was found in the petal fall pendulous bending. In the dormant period pendulous bending there was a slight effect on the flower promotion relative to the verticals.

The production of laterals and flower buds was always more pronounced on the upper side of the bent shoots, with an intermediate on the flanks, and greatly inhibited effects on the lower side, indicating a steep linear relationship from the lower to the upper during the dormant period treatment in all experiments. Generally, the percentages of shoot growth and flower production were increased from the dormant period bending to the petal fall, second cover and to the vertical control.

The greatest increase in shoot length and increased percentage of flowering in all experiments were found in the apical whorl zone, and these further decreased from the 1st to the 2nd, and to the 3rd whorl; this was the case for shoot growth and flowering in the first whorl was not increased due to the inherent properties in Red Delicious. The shoot growth and flowering at the different whorls in 150 cm length laterals bent pendulously in Red Delicious showed a quadratic relationship due to the longer shoots in the apical and the arch position on the shoot when bent at the 5th whorl in all treatments. But at the 5th whorl flowering was reduced considerably, because of substantial lateral growth.

In order to describe the growth relationship between shoot volume and total leaf area an index based on the ratio of vegetative and reproductive responses was established e.g. vegetative 10.83 and reproductive 19.80-24.40.

The relationship of shoot growth and flowering are discussed in terms of a hormone balance theory.

AbstractPart II

Plant hormone studies

In order to establish a ratio of different plant hormones for an understanding of physiological phenomena, appropriate extraction procedures are required for especially apple leaves which are rich in phenolic compounds and other inhibitors. Therefore extraction procedures and purification were examined using ^{14}C -IAA and ^3H -zeatin.

Loss of ^{14}C -IAA during extraction procedures was due to a high pH in the aqueous phase during solvent partitioning. The final recovery of ^{14}C -IAA was 3.8% at pH 8.0 and 81.1% at pH 2.5 through solvent partition and column chromatography. ^{14}C -IAA was chromatographed on a silica gel-celite column and a Sephadex LH-20 column, giving 80% recovery in 30 ml elution volume around the main peak and 90% recovery in 20 ml elution volume around the main peak respectively. Nearly 100% recovery from a Sephadex G-10 column was obtained. 50-57% recovery of ^{14}C -IAA was obtained in cellulose thin layer chromatography at the Rf of IAA, and no loss of ^{14}C -IAA occurred during 3 days storage in a dark cabinet.

The partition coefficient of ^3H -zeatin at pH 8.3 was 13.12 with ethyl acetate and 0.488 with n-butanol; at pH 2.5, 108-89 with ethyl acetate and 16.73 with n-butanol. Backwashing can recover ^3H -zeatin from ethyl acetate phase which was partitioned at pH 2.5. 80% recovery of ^3H -zeatin in the first 1,000 ml was obtained from Sephadex G-10 and Dowex 50 W x 8. 88.6% recovery of ^3H -zeatin could be obtained in a 20 ml peak using Sephadex LH-20 eluted with 95% EtOH containing 0.001 M HCl. The behaviour of ^3H -zeatin was studied in paper chromatography and cellulose, DEAE cellulose and silica gel, thin layer chromatography, about 82-60% of ^3H -zeatin the Rf of ^3H -zeatin being recovered.

Four series of plant hormones were determined from apple leaves by ethyl acetate partitioning, Sephadex G-10 column, silica gel-celite column and cellulose thin layer chromatography of acidic fractions containing auxin-, gibberellin-, and ABA-like substances, and by butanol partitioning, Sephadex G-10, Sephadex LH-20, and DEAE cellulose thin layer chromatography for cytokinin-like substances from basic fractions. Possibly two kinds of auxin-like substances were found and possibly GA_9 , GA_4 , GA_5 , GA_1 , or GA_3 and GA_8 -like substances were eluted from a silica gel-celite adsorption column. Several groups of cytokinin-

like substances were obtained from Sephadex LH-20 column chromatography, possibly zeatin, zeatin-riboside and other cytokinins were found in apple leaves.

Based on the estimation of each plant hormone from thin layer chromatography, a relative plant hormone index was established, i.e., Relative Auxin Activity Index, Relative Inhibitor Activity Index, Relative Gibberellin Activity Index, and Relative Cytokinin Activity Index, representing 6.59, 1.04, 2.64, and 8.16 respectively, the hormone giving the highest ratio being considered the dominant hormonal factor at that stage of development.

GLC techniques were also studied for plant hormone analysis, using 3% OV-1 and NAA, IAA, IPA, GA₁, GA₃, GA₄, GA₅, GA₇, GA₉, GA₁₃ and ABA markers to establish retention times and detector response at the 2.5 ng level. N.O.-bis(trimethylsilyl)trifluoroacetamide (BSTFA) together with Trimethylchlorosilane (TMCS) silyl reagents produced the best peak heights for IAA, IPA, GA₃ and GA₁ but reduced the ABA peak by half and the GA₉ peak by 20%.

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Abbreviations

ABA	Abscisic acid
Alar	Succinic acid 2,2-dimethyl hydrazide
AW-DMCS	Acid washed Dimethyldichlorosilane
Axs	Auxins
BSA	(bis(trimethylsilyl)acetamide
BSTFA	N.O.-bis(trimethylsilyl)trifluoroacetamide
BuOH	n-butanol
CKs	Cytokinins
CHCl ₃	Chloroform
CH ₃ Cl	Methyl chloride
DC-11	Silicone grease
EDTA	Ethylenediamine tetraacetic acid
EtOH	Ethyl alcohol
Epon 1001	Exoxy resin
GA	Gibberellin
GAs	Gibberellins
GLC	Gas liquid chromatography
HCl	Hydrochloric acid
HIEFF=8BP	Cyclohexane dimethanol Apipate
HMDS	Hexamethyldisilazane
IAA	Indolyl-3-acetic acid
IAAp	Indolyl-aspartate
ICA	Indolyl-3-carboxylic acid
ILA	DL-3-(3-indolyl)lactic acid
IPA	3-(3-indolyl)-propionic acid
IPA	6-(3-methyl-2-butenylamino)adenosine (pp. 207)
msIPA	6-(3-methyl-2-butenylamino)-2-methylthioadenosine
IpyA	Indolepyruvic acid
I	Iodine
Ibs	Inhibitors
K ₂ HPO ₄	Potassium phosphate
KI	Potassium iodide
KOH	Potassium hydroxide
LSD	Least Significant Difference
MAAW	Methyl acetate:Acetonitrile:Ammonium hydroxide:water
MeOH	Methyl alcohol
NAA	Naphthalene acetic acid
Na ₂ CO ₃	Anhydrous sodium carbonate

Na HCO ₃	Sodium bicarbonate
NaOH	Sodium hydroxide
Na ₂ SO ₄	Anhydrous sodium sulphate
Na ₂ S ₂ O ₅	Sodium pyrosulfate
NH ₄ OH	Ammonium hydroxide
OV-1	Methyl silicene
PYR	Pyridine
PVP	Polyvinylpyrrolidone
QF-1	50% trifluoropropyl methyl silicone
SE-30	Methyl silicone
SE-52	Methyl silicone
TLC	Thin layer chromatography
TMCS	Trimethylchlorosilane
TNH ₂	Tryptamine
TOH	Tryptophol
t-RNA	transfer ribonucleic acid
TTP	Tryptophan
UC-W98	Unknown % vinyl methyl silicone
V	Volume

CHAPTER 1
Introduction

Bending techniques together with training fruit trees have been used in practice in orchards for many centuries in Europe for inducing precocious flowering and stimulating yields. The techniques used, particularly the Spindlebush, the Palmetta, and the New Zealand modified central leader systems produced remarkable results. There are however many contradictions in the literature describing vegetative and reproductive growth under different environmental conditions and with different methods used.

In the last two decades many research workers have been engaged intensively on the problems of gravitational effects on woody plants. The morphological responses of woody plants under the gravity influences have been termed 'Gravimorphic'. The morphological responses achieved by bending include the inhibition of outgrowth of buds, changes in the habit of shoot growth, the formation of reaction wood, and the induction of flowering. The relationship between the physiological mechanisms involved in apical dominance and gravity effects is far from clear. Current theories on factors involved in growth and developments embrace the existence of a delicate balance between various plant hormones.

There are no clear data on the orientation of buds and shoots along the laterals in the bending treatments. There are also no data on growth relationship in terms of volumetric considerations in relation to leaf area so that the vegetative and reproductive growth is not understood. There are many methods used available to analyze plant hormones. However, there are no methods described which allow for effective determination of hormones from one sample, in particular; auxins, gibberellins, cytokinins, and abscisic acid. Therefore it has been impossible to determine interacting gradients existing in the various hormones likely to be involved in gravitational effects.

The present investigation therefore had several objectives (a) to analyse the growth and reproductive responses of shoots exposed to a range of gravimorphic stimuli when applied to apple trees under orchard conditions and (b) to establish effective techniques for the determination of various hormone groups extracted from the same samples.

Initially it was intended to extend the programme to include the analysis of the hormones in samples obtained from apple trees subjected to gravimorphic treatments. This latter has been excluded on account of the time factor.

CHAPTER 2

Review of Literature

2.1. Historical outline of gravimorphic studies

As early as the seventeenth and eighteenth century French gardeners practised the training of fruit trees with bending techniques. The bending techniques for controlling the growth, flowering, and fruiting of fruit trees were introduced into England and several experiments on the effect of bending on flowering in fruit trees were carried out. Knight (1803, 1813) outlined the effect of gravitation on growth and flowering as follows: "I however consider gravitation as the most extensive and active cause of motion, in the descending fluids of trees;... No power at all had been fatal; and powers sufficiently to strong wholly to counteract the effects of gravitation, had probably been in a high degree destructive. And it appears to me by no means improbable, that the formation of blossoms may, in many instances, arise from the diminished action of the returning system in the horizontal or pendulous branch." Knight (1813) recommended training bearing branches by bending rather than ringing as follows: "I believe, gravitation to be the beneficial agent in this case; by tight and long continued ligatures, or by training the bearing branches almost perpendicularly downwards. It greatly increased the disposition in the tree to bear fruit, without injuring its general health, and because it occasions a degree of vigour to be every whorl almost equally distributed". Knight (1813) also produced plenty of mulberries by training branches horizontally on 25 years old trees.

In 1831 Lindley widely recommended one of the following orchard practices to improve the quality of some fruits and the extent of fruiting. The practices were (1). ring barking; (2). bending the branches downwards; (3). training; (4). the use of different kinds of rootstocks (Sax. 1962).

At the turn of the last century German plant physiologists studied polarity in plant growth. Early this century a number of research workers studied the bending effects on flowering of fruit trees in Europe and America. Lundergardh in 1960 wrote about his early studies (1913) on gravitational effects on the form of trees. External factors such as geotonic phenomena and gravitational effects combine to form a suitable shaping of the plant. He cited the example of side shoots which grow out abundantly from the upper side of plagiotropic laterals and grow more than those on the lower side. If the laterals were arched the

morphotropic factors were able to supplement the geotonic induction in the arching laterals.

Champagnat (1954) who worked with apple trees described the outgrowth of lateral shoots from the upper side and the inhibition of buds on the lower side of the branches. The strong growing shoots from the upper side of the laterals were called 'Gourmand shoots' (Champagnat, 1954 b). The outgrowth of the lateral buds was regulated by 'inherent properties' and 'inhibition actuelle' in the horizontal and arched laterals (Champagnat, 1954 b).

In 1958, Wareing and his co-workers studied the physiological basis of the gravitational effects on shoot growth and flowering in fruit trees. Previously, many research workers had drawn attention to the various effects of gravity on plant growth. Wareing and Nasr (1958) introduced the term 'Gravimorphism' to describe the effect of gravity on the extension of shoot growth and flowering and the interaction of apical dominance (Wareing and Nasr, 1961), as well as the many other effects of gravity in various fruit trees and other species (Longman and Wareing, 1958 and Longman, et al, 1965). Longman (1960) suggested that gravity is an environmental factor which affects all plants but differs from many other factors in that it is constant in direction and intensity. The magnitude of the gravitational effect may differ among different plant organs.

Since Wareing and his co-workers in 1958 first established the concept of gravimorphism in plants, there has been much literature discussing gravimorphic effects in other woody plants.

In 1962, Sax reviewed growth and flowering in the juvenile stage in relation to 'Gravimorphism'. In 1964 Mullins reviewed historical, physiological, and practical aspects of the gravitational effects on growth and flowering in fruit trees. Mullins (1964, 1967) described gravimorphic phenomena which are independent of any directional influence of gravity. He raised problems of terminology associated with gravimorphism. As examples he cited the use of the term 'Morphases' by Borgström (1939) to describe the outgrowth of laterals from the highest point of the arch in bent shoots and roots and Bücher's (1906) use of the term 'Camptotrophism' to describe the reaction of bending on the cortical cells of certain herbaceous plants. Mullins (1964) proposed 'Campylomorphism' to describe the reaction of bending on the cortical cells of certain herbaceous plants and to describe the morphogenetic reactions of plants to the effects of bending and which have a physiological basis. Vigorous shoots which grow from buds in the region

of a bending are determined primarily by the act of bending (i.e., Campylomorphism), suggesting a regulation via the action of growth substances in the xylem, but it is suggested that the location on the stem which can be bent and subsequently produce 'gourmand' shoots is determined by gravity.

2.2 Bending techniques in general orchard practice

In orchard practice vigorous upward branches in young fruit trees grafted on to vigorous rootstocks or seedlings do not exhibit early flowering. Horticulturists have observed profuse flowering, good quality fruits and high yield in the early years on the horizontal or pendant branches (Lorette, 1925; Page, 1939; Bryner, 1943; D(Unster), 1943; Kemmer et al, 1943; Champagnat, 1954; Militue et al, 1965; Constantinescu et al, 1965, Tromp, 1967).

An old system of apple and pear tree training based on bending the branches of fruit trees (Page, 1939) and has given excellent results as regards yield, and economy of space (D(Unster), 1943). Fey and Wirth (1944) indicated that the popular method of pruning (Spindlebush) in Germany, is to tie down the lateral shoots horizontally and to refrain from summer-pruning until they are about ten years old whereas the dwarf pyramid trees are summer pruned from the second year.

Hilkenbaumber (1949) experimented on the pruning of Spindlebush apples in commercial orchards. In the case of Cox's Orange Pippin both tying down and summer-pruning produced higher yields than winter pruning. Jonkers (1966) illustrated some recent developments in apple and pear production in the Netherlands, involving adaptation of pruning and bending to the varietal characteristics in a hedgerow system. In discussing gravity effects in apple trees, Mullins (1967) enumerates many modern systems of training apple trees involving bending of branches and says "With a system such as the Spindlebush, the Palmetta, and the New Zealand modified Central Leader, upright branched are deliberately bent into inclined or pendulous positions."

2.3. The effect of gravity on flowering

2.3.1. Precocious flowering

Woody plants generally require a certain size, age, or morphological changes in leaves before flowering from seedlings or graftings is possible. This period is called the 'seedlings juvenile phase' for seedlings, or the 'clonal juvenile phase' for grafted trees. A number of early horticulturists tried to shorten this juvenile phase by using several techniques including bending treatments to induce

precocious flowering from seedlings and young grafted fruit trees (Park, 1969).

Knight in 1803 claimed that tying down branches brought about heavy fruiting and two decades later Williams (1820) induced earlier fruiting of pear seedlings by training the branches into a horizontal or pendant position. Shanks (1922) also brought young pear trees into bearing 2-3 years earlier by tying down the branches instead of using the current technique of severe pruning (Longman, et al, 1965). Mclean (1940) obtained flower buds on a two-year old sweet cherry Prunus avium which was tied into loops to produce the mechanical effect of the bending. Matthews and Mitchell (1957) produced a great density of male and female flowers in nine-year-old Japanese and hybrid larch by training as espaliers (Longman, et al, 1965).

The successful induction of precocious flowering by tying down the branches has been reported in young apple trees (MacDaniels and Heinicke, 1925; Hilkenbaumer, 1960) and in pear trees (MacDaniels and Heinicke, 1925).

2.3.2. Apple trees

Apple trees usually bear fruit on the terminal spurs derived from laterals grown the previous year. However, in young vigorous trees of certain varieties many axillary and terminal buds on the current shoots are initiated as flower buds.

No flowering was initiated in the first year horizontal or vertical shoots with apple rootstock No 3436 (Wareing and Nasr, 1958). On the other hand, a marked increase in the number of flower buds formed in the horizontal trees occurred in the second year (Wareing and Nasr, 1958). The same trend was found in potted Golden Delicious on M IX rootstock in which the horizontal treatment was attained by keeping branches horizontally for the whole season or for only the early or late part of the season (Tromp, 1968). In cherry rootstock (Mazzard F 12/1) no flowering was initiated either in the horizontal or vertical trees during the first year treatment (Wareing and Nasr, 1958). However, after a subsequent year with the same treatments, flowering was stimulated a little on nonrotated horizontal cherry trees, but not in the vertical and rotated horizontal trees. Rotation of trees treated horizontally reduced flowering both in apple and cherry trees. In plum and blackcurrant, gravity had no effect on flowering (Wareing and Nasr, 1958).

Militiu, Negrila, and Lupescu (1965) reported that 8 apple varieties bent over to four semi circles 2 years after planting in the

orchard, yielded earlier and better in the five subsequent years, than did the untreated trees.

The apple variety Cox's Orange Pippin has been used for studies of gravitational effects by a number of research workers. With one year old trees, Longman, Nasr, and Wareing (1965) doubled flowering in the whole horizontal potted trees compared with the whole vertical trees. Poll (1966) inclined one year old apple trees from the vertical to 45° and 90° and found that the larger angle increased flowering. Tromp (1967 b) obtained 51.1% of flowering in the horizontal rotated trees and 10.9% from the vertical trees. Only the four upper most shoots were allowed to grow during his experiments. Mullins (1964) did not obtain a significant difference in flowering of the potted horizontal, vertical, and in vertical trees. It should be noticed in his experiments that 90% of flower buds were borne in the axillary buds in the current one year shoots. Although Mullins (1964) did not find flowering to be increased in the horizontal trees, the distribution of flowering in these indicated a significant physiological phenomena affected by gravity. Profuse flowering was observed in the apical region of shoots in the horizontal laterals and flowering progressively decreased towards the basal shoots which had a longer growing season. The intensity of lateral flowering was negatively correlated ($r = 0.72$) with the duration of shoot growth.

Hilkenbäumer (1960) reported differential responses to the effects of bending in different varieties. Young trees of the apple varieties Goldpaumer and Golden Delicious had yields stimulated by tying down the branches. No yield responses however occurred in the apple varieties James Grieve and Olden Burg. MacDaniels and Heinicke (1925) obtained highly successful bearing and earlier cropping from vigorous and upright habit varieties such as Northern Spy and the pear variety Keiffer by using bending techniques. Conclusive bending effects on increasing flowering in young apple trees were reported by Chandler (1965) Srivastava (1938) Newmann (1962 a) and Mullins (1967).

Efendiev (1949) reported that trees with bent branches and those bound with wire produced greater flowering than untreated trees. In the established trees of Cox's Orange Pippin, tying down the branches increased flowering in East Germany (Newmann, 1962 a), but no effects of bending the branches were demonstrated in Holland (Jonkers, 1962). Bending one-year-old upright shoots in mature apple trees did not stimulate yields (Gardner, 1917). Bailey (1927) reported in America that tying down of branches was not a fundamental orchard practice.

2.3.3. Silviculture

In Wisteria sinensis Sweet, McLean (1940) induced early flowering and fruiting by the looping of the woody stem. The flowering of young Japanese larch is markedly influenced by the position of the branches in relation to the gravitational field (Longman, 1960). This has been demonstrated by fixing branches as follows: (1) control branch fixed in their existing position, (2) control branch unfixed, (3) branch tied approximately horizontal, and (4) branch fixed down to approximately 135° from the vertical. The flowering on the horizontal and tied down branches was remarkably distinguishable from that of the control, although there were a slightly different responses between the treated branches. However, in birch Longman, Nasr, and Wareing (1965) could not increase flowering with gravimorphic treatments except on ringed trees.

2.4. The effect of gravity on vegetative growth

2.4.1. Extension of shoot growth

The extension of shoots grown under the influence of gravity has been investigated by several workers but the results are variable.

In experiments using a single leading shoot as the terminal shoot, marked reduction of extension growth occurred in the horizontal treatments. Studies were done on cherry, apple, plum, blackcurrant (Wareing and Nasr, 1958, 1961), apple (Neumann, 1962 b), and Japanese Larch (Longman and Wareing, 1958). Reduced extension shoot growth under both rotated and non-rotated horizontal treatments was due both to smaller internode numbers and to reduced internode length in cherry and plum, but only to reduced internode length in apple and blackcurrant (Wareing and Nasr, 1958, 1961). Mullins (1965 b) confirmed that the reduction of internode in the terminal shoots of the horizontal trees, reduced terminal shoot growth by 30% when compared with the length of terminal shoots.

Tromp (1967 b) reported that the mean shoot extension when only the four upper most shoots in the stem were left to grow was reduced from 162 cm in the vertical to 86 cm in the horizontal orientation in one year old Cox's Orange Pippin trees on M IX. The 50 % reduction of shoot growth in the horizontal position was confirmed in Golden Delicious (Tromp, 1968) and in Cox's Orange Pippin (Tromp, 1970) both on M IX.

Sternberg and Kulikova (1957) reported a 73 % reduction in length of total shoots in the horizontal laterals from 6-year-old

apple trees Antonovka when tied on May 17, and on June 26. This was due to an 85% reduction of total internode length. Husabø (1966) found that vegetative growth of the apples decreased as the angle between the main stem increased to 30° , 60° , 90° and 120° on the young trees of Ingrid Marie, Red Gravenstein, and Red Savastaholm, but vegetative growth in Moltke pear variety was greater when the branch angle was 120° .

Kato and Ito (1962) studied the gravitational effect on shoot growth in the branches of 7-year-old McIntosh Red apple trees. In the invertical shoots, the shoot growth was terminated at about 17 cm on 20th June. In the horizontal shoot, the shoot growth was terminated at about 26 cm on the 30th June. On the other hand, the vertical laterals of the shoot continued to grow until they reached about 55 cm at the end of July. Secondary growth often occurred.

Mika (1969 a) carried out gravimorphic studies on one-year-old Belle de Boskoop and Wealthy at orchards in the moderate growing conditions of central Poland. The total shoot growth from the lateral arms bent horizontally or inverticaly was less than the corresponding growth of the lateral arms which grow naturally at about 45° angle. The growth of the central leader on the tree with the horizontal lateral arms was greater than on the control trees. When total shoot growth per tree was compared between the bending trees, there was no significant difference.

Without restricting shoot numbers, Mullins (1964, 1965 b) reported total shoot growth extension showed no significant difference between horizontal, vertical, and invertical trees grown in pots. Poll (1966) confirmed the results obtained by Mullins (1964) using the same variety and age on M IX potted at an angle of 45° or 90° from the upright. The results obtained by Poll (1966) were assessed by Mika (1969 a) in the field with the same variety.

2.4.2. Outgrowth of buds

When buds receive a new orientation according to inclination of laterals, the buds on the upper side of the laterals are stimulated to grow out, whereas the buds on the underside are inhibited. Vöchting (1878) showed that in willow the outgrowth of lateral buds was greatly affected by the different angles from the vertical. The budbreak occurs commonly on the upper side of laterals and inhibition on the underside (Reed and Halma, 1919; Halma, 1923, 1926; Gardner, 1925; Champagnat, 1954; Wareing and Nasr, 1961; Smith and Wareing, 1964; Mullins, 1964, 1965 a, 1967). Smith (1962) also reported that the bud orientation determined

earliness of bud break in the horizontal shoot. The earliest bud growth occurred in the upper side of the stem and next to the flank of the stem, while the buds on the lowerside did not grow at all.

Smith and Wareing (1964) investigated the physiological basis of the gravitational effect in the bud-break of Osier willow. They trained the Osier willow into loops of a 'U' form. Both concave and convex forms were used. The rate of bud-break in the loops was counted from 8 various regions after 12 days. They found that there was no effect of mechanical strain on the rate of bud-break. Bud-break was more rapid on the tension rather than the compression side in the concave loop, but the opposite case held for the convex loop. That is there were a tendency for bud-break to occur on the upper side of both the concave and convex loops.

Smith and Wareing (1964) examined the effect of the angle of inclination and gravity on bud-break in Salix viminalis, using angles from 0,15,30,60,90,120,150, and 180 degrees to the vertical upright, imposed for 21 days. Complete inhibition of bud-break on the lower side of the laterals occurred on laterals at 90°. Bud inhibition increased as the angle increased from 0 to 90°, and from the horizontal direction to 180 degrees as angle increased the bud-break increased. Therefore, the following conclusion can be drawn from the data of Smith and Wareing (1964); viz. The rate of bud-break on the lower side of the lateral shoots is proportional to the angle between the horizontal and the direction of shoot.

The presence of buds on the arched region has some physiological role in inducing the different pattern of outgrowth of lateral buds. Smith (1962) reported that in willows the vigour of bud outgrowth at the apical inverted region was promoted by disbudding at the arched region. Even the presence of lower side buds on the arched region inhibited the production of long shoots from the apical inverted region. The long shoot from the lower side of the lateral could not compete with the shoots from the upper side of the arched lateral. This different pattern of growth for the buds on the lateral may be a correlative effect. Smith (1962) suggested that the inhibition of vigorous outgrowth in the apical region could be due to monopolization by the buds on the top of the loop for nutrients and growth substances produced from the roots.

Mullins (1964, 1971) compared the outgrowth of shoots of basal groups among the distal, middle, and proximal regions apart from the apex group of the horizontal stem, and found a marked basipetal gradient, i.e. the longest shoot always grew on the upper side of the basal region. The vigour of shoot growth in these groups was affected by the position of the basal group of buds. The more the bud group was

located close to the root, the more the length of the basal laterals and the total shoot per bud group were produced. Smith and Wareing (1964) conducted experiments in which Salix viminalis was trained into two consecutive full loops or arches. The marked inhibition of lateral shoot growth from the stem apical region (Wareing and Nasr, 1961) was confirmed by them. However, they induced rooting at the base of the loops, and thereby relieved this inhibition of shoot growth by providing either nutrients or deionized water. They suggested that the roots synthesize substances essential for shoot growth and this can be translocated through the xylem. The maintenance of optimum shoot growth is dependent upon processes taking place within the root itself. Therefore they assumed that the effect of the roots on shoot growth is indeed mediated through the synthesis of one or more substances essential for shoot growth. They defined a term, 'the root factor' to describe the active substances involved. These substances are analogous to 'caulocalines' postulated by Went (1938), and may therefore be growth hormones. Finally they indicated that monopolization of the postulated 'root factor' by laterals on the upper side of the arch may be important in the establishment of patterns of branch dominance. In another experiment, Smith (1962) also demonstrated that among two consecutive loops the shoots on the first arch dominated other shoots unless the shoots on the first arch were removed. Thus shoots may monopolize essential substances stored in the stem. Halma (1926) suggested that inhibition of shoot growth on the lower side may be due to the accumulation of inhibitory growth substances on the lower side.

2.4.3. Distribution of shoots on the lateral

Mullins (1964) reported that lateral shoots on horizontal, vertical, and invertical apple trees of the Cox's Orange Pippin variety showed a distinct seasonal pattern of shoot growth. Mullins (1964, 1965 b) suggested that lateral shoots grow from both the apical and the basal region. From early season until mid-summer most shoots grow in the apical region, but after mid-summer the shoot growth occurs in the basal region of the trunk continuing until late in the year in the vertical, horizontal, and inverted stems. The relative vigour of the apical and basal region shoots is mutually influenced because as the basal shoots attain rapid growth after mid-summer, while the growth of apical region shoots drops accordingly. The pattern of shoot length on the stem showed the marked basipetal gradient which occurs towards the basal region in the horizontal trees. This gradient was less marked in both the vertical and invertical trees. In the horizontal trees, the

longest shoots developed from the lower-most buds on the upper side of stems. In the inverted trees, the longest shoots developed from those buds which were a few nodes distal to the base of stem.

Gunawardene (1965) reported that the orientation of trees may affect the development of lateral branches on peach seedlings. In the vertical trees, the lateral shoots developed first from the centre of the shoot and then progressively towards the apex. On the other hand, the inverted trees developed lateral shoots firstly from the centre and then towards the base of the stem. After the trees were placed horizontally, shoot development started from the centre and continued further towards the base.

2.4.4. Shoot position on outgrowth of lateral buds

Champagnat (1954) stated that a bud in the highest position will develop and dominate the growth of other buds. Therefore buds on the arched part of the stem become the physiologically terminal buds.

Wareing and Nasr (1961) trained shoots in various positions and studied the lateral growth in the following regions:
 (1) the proximal region, between the root and the summit of the arch,
 (2) the distal region, lying between the summit of the arch and the sub-apical region, and (3) the apical region which comprises the apical few centimetres below the tip of the first year shoot. They found that the orientation of the apical region of the main stem affects very slightly the outgrowth of laterals from the basal region. However, the orientation of the remainder of the stem could affect primarily the outgrowth of laterals. Outgrowth occurred primarily in the proximal region where the stem was arched from the vertical position. The strongest lateral on the arched part of the stem obtained an apical dominance, thereby producing the greatest growth. Therefore, the apical dominance of the terminal shoots of the main stem in the arched stem was lost.

Wareing and Nasr (1961) reported that shoot growth in a willow loop was greatly reduced at the apical region and vigorous shoots occurred at the proximal region of the roots. When the looping was rooted, shoot growth from the apical region was equal to that of the vertical trees. These results were confirmed by Smith and Wareing (1964).

Wareing and Nasr (1961) concluded that the vigour of lateral outgrowth which was greater in the arched and horizontal laterals was due to the following factors: "(1) some mechanism is involved whereby nutrients are diverted to the highest upward directed meristem, (2) other factors being equal proximity of laterals to the roots appears

to confer an advantage."

2.4.5. The interaction between apical dominance and effect of gravity

The outgrowth of shoots varies with the horizontal or vertical orientation of the laterals to the main axis. In general apical dominance is related to the outgrowth of shoots from earlier grown laterals in woody plants. Gravitational effects and apical dominance may determine the growth of lateral shoots on the main axis. Wareing and Nasr (1961) investigated the interaction between apical dominance and gravity with apple, plum, and cherry. They experimented with lateral shoots trained in both horizontal and vertical positions. The horizontal training of shoots reduced growth more in the upper shoots than in the lower ones. When three laterals were trained horizontally the individual shoot growth was similar. Apical dominance in tree form was not found in Prunus species. However, when all the shoots were trained horizontally differences in shoot growth were apparent. The upper most shoots gained apical dominance, and thereby achieved the greatest growth. These workers concluded that when upper most shoots are oriented vertically the apical dominance is fully manifest. However, when the upper most shoots were trained horizontally and the second lateral was trained vertically, the second lateral now became a leading shoot and dominated growth. When the first and second laterals were trained vertically and the third lateral was trained horizontally, the growth of the third lateral was greatly reduced. These conditions stimulated growth in plums, but not in apple (Wareing and Nasr, 1961).

2.5. Gravitational stimulus

2.5.1. Rotation effect

A common phenomenon in inclined shoots is the inhibition of buds on the lower side and promotion of buds on the upper side. This mechanism is not yet fully understood.

The theory of bud inhibition (Thimman, 1937) by an auxin was suggested by Wareing and Nasr (1961). They found that the inhibition of buds on the lower side of laterals was due to the transverse migration of auxin from the upper to the lower side of laterals under the gravitational effect. In horizontal shoot, the growth substance might be unevenly distributed between the upper and lower sides because of the gravitational effect. Buds on the under side are inhibited by a high concentration of auxin, whereas growth of buds on the upper side is induced by the low concentration.

Indirect evidence for bud inhibition by some growth substances

has been shown by rotation experiments. Halma (1926) studied the effect of gravity on the bud inhibition in Chinese lemon (Citrus medica). Buds on the lower side of horizontal laterals were not inhibited by weekly rotations of 180 degrees.

Mullins (1964, 1965 a) investigated in apple rootstock Malling Crab C the inhibition of buds on the lower side of laterals by splitting them in the horizontal plane and inserting a non-porous plastic material which prevented substances from migrating between the upper and lower sides. He found that there was no migration of inhibitory substances from the upper to the lower side, so that all buds on both the lower and upper sides could produce long shoots. On shoots split vertically all the buds on the lower side remained dormant. He suggested that the release of a bud inhibitor from the lower side of the vertically split horizontal lateral was not due to translocation of some inhibitory substances under the effect of gravity. These phenomena were found by Mullins (1964) in further rotation experiments. He found that there was no bud inhibition on the lower side of laterals in the apple trees which were grown horizontally and rotated 180 degrees each day so that the gravity effect was counteracted. Shoots grew from the lower side but did not show any geotropic curvatures and formed well-developed lateral shoots along the trunk. Shoots from both the upper and lower sides grew symmetrically.

However, Smith and Wareing (1964) reported that the separation of the upper and lower sides by aluminium foil, in the horizontal shoots of Salix viminalis, did not induce bud growth on the lower side within 12 days, although in the control 90% bud-break occurred during that time.

2.5.2. Distribution of nutrient, assimilates, and enzymes

Few investigators have studied the effect of gravity on the distribution of nutrient and assimilates in plants. Dickson and Samuels (1956) found that looping in apple rootstocks disrupted the basipetal transport of ^{32}P from the leaves. Moorby (1959) examined the ^{32}P distribution in the loops of birch by autoradiography. The highly active ^{32}P was present in the axillary buds between the point of injection and the top of the loop, but there was very little radioactivity present in the shoot apical region. Smith and Wareing (1966) injected ^{32}P in the base of the stem and examined its redistribution in bent and vertical shoots in seedling trees of Betula pubescens. ^{32}P was well distributed in the apex of laterals and the leader on the vertical trees. However the greatest amount of ^{32}P accumulated in the apex of the lateral which occupied the highest position of apical dominance through bending or arching the stem. Carlson (1962) found the higher K content in the leaves

of the trees grown in the inverted position compared with the vertical in unbranched 1-year-old Northern Spy, Red Delicious apple, Bartlett pear, and Schmidt cherry trees.

Mika (1969 b) studied the effect of gravity on the distribution of ^{14}C -assimilates in the active, moderately active, and mature shoots of the apple variety Jonathan on M IX and Bancroft on Antonovka. Under active growth, ^{14}C -assimilates were mainly translocated to the shoot apex when $^{14}\text{CO}_2$ was applied on a single leaf. However there was a slight difference between the bent and vertical shoots. ^{14}C -assimilates, being accumulated in the apex, did not move down to the bent stem, while in the vertical trees the ^{14}C -assimilate accumulated comparatively more in the lower stem when $^{14}\text{CO}_2$ was applied to a single leaf. Under moderate growth ^{14}C -assimilates moves both in the direction of the shoot apex and the root in the inverted trees. However, the ^{14}C -assimilates moved mainly into the shoot apex in the inverted trees but mainly were transported downward to the base of shoots and roots in the vertical laterals and markedly high activity was counted in the roots. In slowly grown and non-growing apple shoots, the ^{14}C -assimilates moved down to the stem and roots from the treated leaf. The highest concentrations of ^{14}C -assimilates were found in the bent section. The roots of the vertical plant had much more activity than did the bent stem in the tree.

Halma (1926) found that the activity of catalase was greater on the lower side of the horizontal Chinese lemon (Citrus medica).

Palmer and Halsall (1969) found that GA stimulated polar transport of ^{14}C -IAA in the vertical shoot and reduced the inhibitory effect on polar transport in the horizontal shoot.

2.5.3. Growth substances

Intensive work on geotropism in coleoptiles and hypocotyls of Helianthus has shown that lateral translocation of auxin plays a substantial role in the magnitude of geotropic response in the plant tissues (Gillespie and Briggs, 1961; Gillespie and Thimann, 1963; Goldsmith and Wilkins, 1964).

Several workers (Lyon, 1965, 1968) found by bioassay that a 40 to 60% ratio of auxin was present in the upper to lower sides of stems for geotropic curvature to occur; e.g., in epicotyle of Phaseolus and Vicia, and hypocotyls of Helianthus and Lupinus. The 40-60 ratio of auxin content using the radioisotope ^{14}C -IAA applied to corn coleoptiles (Gillespie and Thimann, 1961; Goldsmith and Wilkins, 1964) was recovered in an agar block from the upper and lower halves. Lyon (1965, 1968) obtained similar results in immature plants from a clone of Coleus blumei

Benth using radioassay techniques on tissues from curved stems.

In woody plants gravity may influence the movement of auxin-like substances and inhibitory substances from the upper to the lower side of horizontal laterals. This effect has been demonstrated by several workers.

Onaka (1949) reported that the diffusible auxin content was two to four times higher in the lower part than the upper part of inclined stems of Pinus thumbrigii Parl. He used the Avena coleoptile curvature test.

Necessary (1958) studied the promotor and inhibitor substances from the upper and lower side in the horizontally growing branches of Populus tremula L. and Pinus silvestris L. Necessary (1958) assumed that β -IAA was the major growth substance, although he did not identify the other growth substances and stimulatory substances were abundant in the lower side of the horizontal while inhibitory substances in the upper side.

Westing (1960, 1962) investigated the asymmetric distribution of a substance extracted with Salkowski reagent in horizontally displaced Easter white pine leaders. Westing found that an indole compound other than IAA comprised 60% of the total in the lower halves of horizontal leaders. The application of naphthalene acetic acid prevented the transverse redistribution.

Growth substances from bark and buds on the horizontal and vertical stems were compared, using the coleoptile cylinder test in ether extraction (Newmann, 1962 b). Newmann (1962 b) found that there were three promotors at Rf. 0.1-0.4 and two inhibitors at 0.45-0.75 and 0.8-0.95 in the horizontal apple laterals. Leach and Wareing (1967) found that the ratio of auxin content on the upper and lower sides in the disbudded one year old horizontal stem was about 40:60 for ^{14}C -IAA in Populus robusta. This was confirmed by bioassay with the combination of oat mesocotyls and wheat coleoptiles.

An inhibitor, probably abscisic acid, was greater in the lower side of Populus robusta (Leach and Wareing, 1967) and Blackcurrant by El-Antably (Leach and Wareing, 1967). Borkowska (1973) found two inhibitors identical to phloridzin and ABA in the extracts from bark and young shoots of Cortland apple trees kept in a horizontal position for 6-12 days during bud burst. The phloridzin content was higher in the upper side of the shoots, whereas the ABA content was the same in the upper and lower sides.

Leach and Wareing (1967) found no differences in the gibberellin content of the upper and lower side of the horizontal

stem of Populus robusta, using the lettuce hypocotyl assay.

Hillman (1968) found that the greatest content of ^{14}C -kinetin accumulated in the buds of the upper side of the horizontal shoots and the apex of arched birch (Betula pubescens). On the other hand nutrients such as ^{14}C -adenine, ^{14}C -urea, ^{14}C -sucrose, or ^{32}P -phosphate were not distributed differentially between the upper and lower buds (Wareing, 1970).

Kato and Ito (1962) studied the physiological function of shoot growth in vertical, horizontal, and inverted shoots, by determining the auxin and gibberellin-like substances in seven-year-old McIntosh Red apple trees. They found that the auxin content of the terminal bud of vertical vegetative shoots was greater than that for other treatments. The auxin content in horizontal shoots tended gradually to decline from 20th June to 10th August (Northern hemisphere) and this tendency was more acute in the inverted shoots. No changes of auxin contents showed in vertical shoots up to 15th July but considerable increases occurred before the second flush growth of the shoots in the early part of August.

Kato and Ito (1962) bioassayed the gibberellin-like substances in the terminal buds from vertical, horizontal, and inverted shoots. They found many more gibberellin-like substances and less anti-gibberellins in the vigorous vertical shoots than the horizontal ones. The reduced growth of shoots, however, contained only very small amounts of gibberellin-like substances. Consequently the reduced growth of shoots was likely to result in less quantities of the native gibberellin-like substances.

Gunawardene (1965) investigated growth substances from the leaves in the horizontal, inverted, and vertical peach trees using the method either of tip excision or an aluminium cap on the apex. He found the active auxin level to be around Rf 0.5 and minor active growth factors around Rf 0.87. Inhibitors were around Rf 0.62. In the plant with intact apices, the active auxin level was much higher in the upright plants compared with the inverted plants. In the horizontal plants the active auxin level was not detected, but the inhibitory compound existed at Rf 0.5. However there was a minor active compound at 0.25 in the horizontal plants. In upright plants, the existence of an inhibitory substance masked the minor active compound at Rf 0.25. In the aluminium capped plant the auxin content did not show differences between the vertical, inverted, and horizontal tree. However, the auxin content in the horizontal trees tended to be higher. In excised shoots,

regardless of orientation of shoots, the auxin content was low in all the treatments.

2.5.4. Anatomy of wood and gravitational effect

Internal specific activities or redistribution of growth substances under the gravitational stimulus may have a physiological, chemical, and anatomical role in the inclined branches. The differential cambium activity on the upper and lower sides of the horizontal laterals under the gravitational effect induced pronounced different characteristics in the formation of reaction wood in the different species (Priestley and Tong, 1927). As a common phenomenon, cambium activity is greater in the lower side of the horizontal lateral in Gymnosperms, thereby forming reaction wood, called compression wood. On the other hand, in Angiosperm reaction wood forms in the upper side of horizontal laterals and is called tension wood. The formation of reaction wood is due to a major physiological function of redistribution of growth substances under the gravitational effect (Wardrop, 1964; Westing, 1965).

Several forest research workers have studied reaction wood because it has several commercially undersirable characteristics, e.g., harder, denser, weaker, or more brittle than the remainder of the wood. It is important to understand the mechanism of formation of reaction wood in the inclined stems because physiological functions in relation to anatomical and chemical differences are closely associated with the gravitational effect.

Recently Wardrop (1964) reviewed literature pertaining to experiments on the reaction wood in Angiosperms and Westing (1965) has reviewed literature on compression wood in Gymnosperms. Westing discussed both Gymnosperms and Angiosperms in relation to physiological phenomena, and concluded that auxins are a crucial factor in controlling the physiological functions which evoke the initiation and differentiation as well as subsequent development and maturity of the wood. Kozlowski (1971) has also summarized reaction wood both in Angiosperm and Gymnosperm.

A brief summary for reaction wood (Wardrop, 1964; Westing, 1965; Kozlowski, 1971) is as follows:

2.5.4.1. Compression wood

Compression wood differs from surrounding wood in that the tracheids are uniformly round, thick walled, and in many cases have a 30% reduction in diameter. The tracheid is surrounded by intercellular spaces containing mucilaginous substances which lignified. Compression wood contains 20 % less cellulose than the remaining wood and

which is less crystalline. The non-cellulosic polysacchrides are similar to normal wood except for galactan which is more than twice as great in compression wood. Average lignin content is 28% higher in compression wood. Two-thirds of the total lignin in the wood is deposited in the middle lamella and primary wall. The lignin has somewhat different chemical properties with fewer methoxyl groups, although the basic skeletal structure is not different. Peroxidase activity may be necessary to control the level of auxin and the polymerization of coniferyl alcohol to lignin.

2.5.4.2. Tension wood

The formation of tension wood may be due to a low quantity of auxin in the upper side of the inclined stem in Angiosperms (Cronshaw and Morey, 1965; Leach and Wareing, 1967). Anatomical changes are apparent in tension wood with fewer and smaller vessels in reaction wood. Vessel diameters in tangential and radial directions are less. In the apple shoots bent pendulously, there are fewer vessels and these are less porous than in the normal wood of upright shoot (Christoferi and Giachi, 1964). Average fibre length is usually greater in tension wood. However, in Populus, fibres are shorter than in some other species. These differences are due to a differential radial growth of the stem, depending on the eccentricity of radial growth by the increased duration or rate of division of the cambium.

The cell wall in normal wood consists of S_1 , S_2 , and S_3 layers. In tension wood these are termed the gelatinous layer and referred to as G_1 , G_2 , and G_3 . In some cases an additional layer G_4 develops. The shape of the cell wall is determined by the replacement of S_2 and S_3 with the gelatinous layer in tension wood. The fibres within the G layers consist mainly of cellulose, orientated almost parallel to the fibre axis with fewer and smaller pit chambers and the channels are slit like. The basic pattern of lignification is the same in the tension wood. However, the amount of lignin in the fibres of the whole cell wall is less in the tension wood than normal wood. Tension wood lignin has a lower methoxyl content and a lower ratio of syringyl to guaiacyl nuclei. The mechanical association of lignin with the other cell wall constituents was different in reaction wood. Tannin content is high in the upper side of horizontal stems (Scurfield and Wardrop, 1962). In tension wood the xylem on the upper side of horizontal apple laterals was darkly stained by tannin acid / ferric chloride / sodium bicarbonate, although the exact nature of the dark staining of the tension wood fibre walls is not clear (Mullins, 1964).

2.5.4.3. Phloem

Although the basic pattern of anatomy in phloem development on the upper and lower side of laterals does not change, reaction phloem occurs in some species resulting in asymmetric phloem development. This special phloem development is very clear in Lagunaria pattersonii and Pittosporum undulatum but not as apparent in Eucalyptus spp (Scurfield and Wardrop, 1962). Furthermore in Eucalyptus spp, the normal sequence of sieve tubes, companion cells, parenchyma containing tannin layer, and fibres were reported more often in the upper side of laterals. The thick cell walls usually develop in the upper layer of phloem (Scurfield and Wardrop, 1962).

Mullins (1964) found that sieve tubes in the upper and lower side of the horizontal Northern Spy apples on M IX rootstock did not change. However the special phloem band developed as secondary phloem fibres on the upper side of laterals. This band occurred only in the bark of the upper side of horizontal laterals. He found a preponderance of crystals in the phloem of the lower side of horizontal apple laterals.

The ratio of wood to bark thickness has been related to the degree of dwarfness of apple rootstocks. A smaller ratio of bark and xylem in the scion stem occurs in vigorous rootstocks (Colby, 1935; Beakbane, 1952; Mosse, 1952). McKenzie (1954) found that there were very large differences in the bark percentage of shoots and branches, between vertical and horizontal trees e.g., the bark percentage tended to increase in stems of the more inclined slow-growing tree, and was correlated with the slow growth rate. Mullins (1964) confirmed this result in Lord Lambourne and Cox's Orange on M IX, e.g., the bark percentage was less in the vigorously growing upright laterals as compared with the slower growing pendulous laterals while a slight effect on total thickness exhibited in the horizontal laterals.

2.6. The outgrowth of shoot and shoot growth (Apical dominance)

The induction of bud outgrowth by breaking the inhibited growth has been discussed under apical dominance and correlative growth. Discussion of the whole aspect of apical dominance is beyond the scope of this study.

The term apical dominance has several meanings and will be defined in this thesis as follows: 'Apical dominance' embodies the inhibitory influence of the apex of the dominant shoot, or the apical part on the growth and orientation of lateral buds, branches, leaves, rhizomes, and stolons (Phillips, 1969). 'Apical control' suggested by Brown, Mcalpin, and Kormanik (1967) is used to describe the influence

Fig.1. Speculative flow chart representing the integration between biochemical and physiological phenomena in the whole plant.

The speculative flow chart is to aid an understanding of general principles at the molecular level of biology as they link with physiological and biochemical phenomena in the whole plant.

The spiral-symbolism represents 'Dialectic' of Hegel's philosophy which could be interpreted with a 'balance theory' of the ancient oriental philosophy (The 'Yang' (+) and the 'Um' (-)) as applied to cause and effect in plants.

The circle which is divided equally and in perfect balance represents the 'balance theory' which applies to a balanced metabolism in plants not only between carbon and nitrogen metabolism and free and bound hormone metabolism or in themselves but also between carbon/nitrogen and hormone metabolisms.

The leaves assimilate CO_2 from the atmosphere by the process of photosynthesis and thus supply carbon skeletons for further synthesis using water and mineral elements obtained such as nitrogen from the soil by the root system. This process is represented in Fig.1 by carbon/nitrogen metabolism forming biochemical pathways such as the citric acid cycle, pentose-phosphate shunt, and the urea cycle which are leading to the synthesis of amino acids, and purines and pyrimidines, the basic units of proteins and nucleic acids respectively.

The upper central spiral circle represents the development and direction of each metabolic step as the consequence of reciprocal cause-effect relationship in feedback systems.

In a modern concept of plant physiology, plant hormones are postulated as controlling growth and differentiation phenomena such as apical dominance, flowering, dormancy, and senescence. The left upper side of the circle symbol represents a hormonal balance which interacts with carbon/nitrogen metabolism to control plant growth and development.

In order to keep a balanced state of plant hormones there is an interaction between not only different plant hormones and metabolism but also between metabolism or conjugate forms of hormones as follows. Plant hormones act as a buffer between excesses and deficits of metabolites in the process of growth and development and activators of enzyme systems. It is well known that there are many kinds of conjugate or bound forms of hormones. In IAA and carbon metabolism the presence of IAA induces conjugates of IAA such as IAA-glucose, IAA-arabinose, and IAA-inositol, and in IAA and nitrogen metabolism, IAA-aspartate, IAA-glutamate, and bound auxins, which exist as IAA-protein and ascorbigen. It is also possible that IAA may also be incorporated in r-RNA, sRNA, and DNA. Gibberellins and abscisic acid form glycosides in plant tissues while cytokinins are generally 6-substituted adenine derivatives, present either as the free base or with a ribose moiety attached to the 9-position. The cytokinin activity is dependant on both the structure of the molecular by the substitution of side-chain and modification of the purine ring, possibly giving an important role in nucleic acid metabolism.

Plant growth and development primarily depend on gene activity and protein synthesis, the end result being either vegetative growth or reproductive growth. The lower part of the chart represents possible relationships between nucleic acid synthesis, protein synthesis and hormonal balance in differentiating between two critical developmental stages. $\frac{\text{CKs}}{\text{AXs}} \text{Ibs}$ and $\frac{\text{GAs}}{\text{AXs}} \text{CKs}$ in the right lower side in the section of reproductive growth symbolize a particular balanced quantitative relationship between the plant hormones. For example the relationship between cytokinins, auxins, and inhibitors, as symbolized $\frac{\text{CKs}}{\text{AXs}} \text{Ibs}$ indicates that flower initiation could require an optimum quantity of cytokinins together with minimum quantity of IAA to lead to initiation of flowering but inhibitors must be reacted in order to suppress cytokinins, GAs, and IAA, which induce vegetative growth, and to favour flower initiation. $\frac{\text{GAs}}{\text{AXs}} \text{CKs}$ indicates that cytokinins are important plant hormone at the time of flowering initiation. Gibberellins tend to inhibit flowering while in contrast vegetative growth requires gibberellins together with auxins and cytokinins, represented as a ratio $\frac{\text{AXs}}{\text{CKs}} \text{GAs}$ and $\frac{\text{AXs}}{\text{GAs}} \text{CKs}$.

The flow of plant hormones is closely correlated with the meristematic activity in the tissue, where the majority of plant hormones such as auxins, cytokinins, and gibberellins are produced.

Inhibitors can either stop growth or influence its direction depending on the ratio of other hormones.

AXs = Auxins, GAs = Gibberellins, CKs = cytokinins, Ibs = Inhibitors,

ABA = Abscisic acid, un = as yet unidentified plant hormones

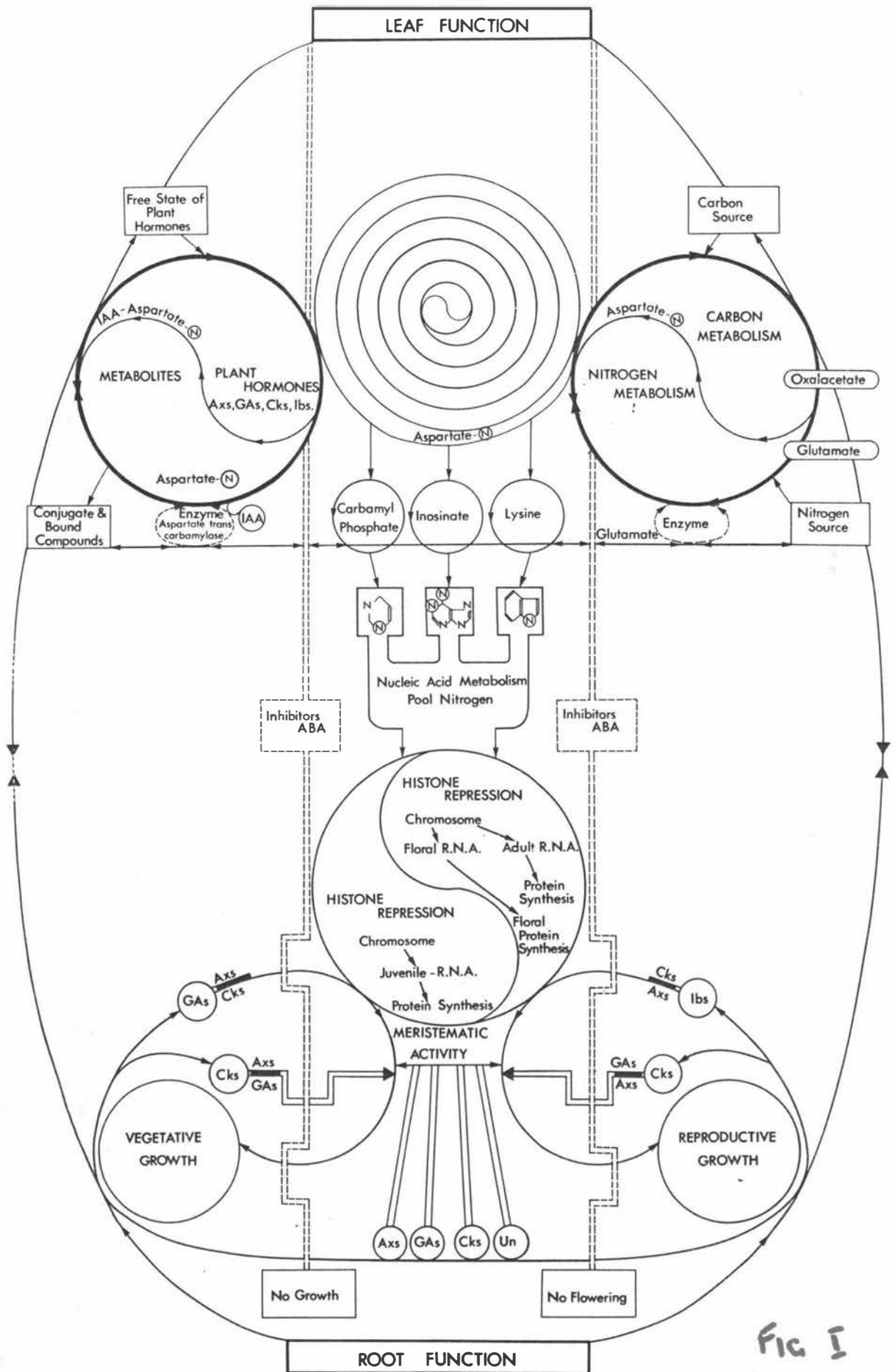


FIG I

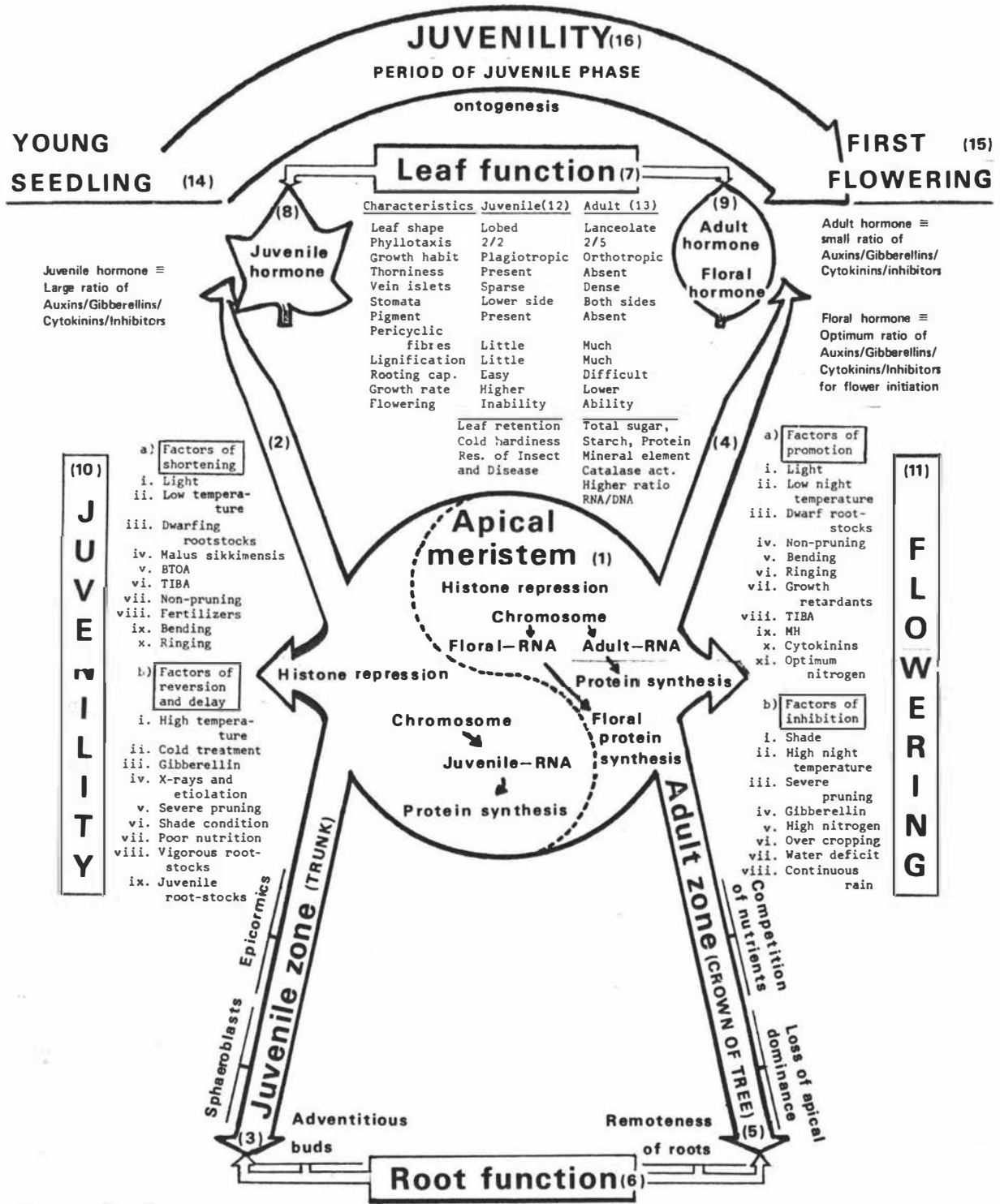


Fig. 2.
SPECULATIVE SCHEME OF JUVENILITY AND FLOWERING

Fig. 2. Speculative scheme of juvenility and flowering.

I. Introduction

From the survey of juvenility and some viewpoints of flowering in woody plants, a speculative scheme linking juvenility and flowering has been devised in an attempt to summarise the issues raised and to present an integrated concept.

1.1.1 The relationship between the leaf, root, apical meristem and juvenile zone in the juvenile plant (Left hand side of Fig.2)

Seeds produced by the adult plant contain various reserve materials which are available to the plant during germination and the early stages of plant growth when special juvenile characters are present. When the seed germinates, roots develop by using the supply of reserve foods from the cotyledons, and the products of the embryonic apical meristem. These developing roots absorb mineral nutrients and water, which are supplied to the apical meristem, cotyledons, new leaves, and other parts of the plant (2). The root is one site for the synthesis of amino acids, proteins, RNA, and growth substances such as gibberellin, cytokinins, and other shoot growth substances. In a number of alkaloid containing plants the alkaloid or its immediate precursor is produced in the roots. The function of the root may be dependent on, or be influenced by, the translocation from the shoot of metabolites such as the sugars and the B vitamins. The function of metabolism in the roots (6) of seedlings or juvenile rootstock may differ as compared with that of clonal varieties. Some products from the juvenile rootstock or seedling roots may affect the ability of the apical meristem to maintain its juvenile character. As a plant grows, the degree of response to products from roots by the apical meristem may be dependent on the amount of products received from the roots, due to the complexity of the branch systems, which represents a remoteness from the roots.

The differentiated juvenile leaves in the apical meristem manifest and maintain a juvenile character in the leaf function (7 & 8) as shown in Fig. 2. These juvenile leaves (8) and other parts of the plant (2) and trunk (3) and roots (6) in the juvenile phase may produce the "juvenile hormone" which comprises a large gradient of auxins/gibberellin-like substances/cytokinins. In fact, the development of the plant is controlled by the interaction of auxins, gibberellins, cytokinins, inhibitors, and nutrients with one another (Nitsch and Nitsch, 1967a & b). In plant hormones, a rise or fall in concentration of one affects the response caused by the others (van Overbeek, 1966). Therefore, for the development of the juvenile phase in a plant, optimum ratios of auxins to gibberellins, auxins to cytokinins, and gibberellins to cytokinins are required. In the juvenile phase these optimum ratios are such as to produce a large gradient of auxins/gibberellin-like substances/cytokinins. In other words, the juvenile hormone and other metabolites, which are produced in the juvenile leaves (8) and other plant parts (2, 3 & 6), are transferred to the apical meristem (1) in which the differentiation of the organs takes place, thereby manifesting the juvenile characteristics in the juvenile phase.

1.1.2 Apical meristem (1)

The apical meristem is shown in the centre of Fig. 2. The dotted line in the centre distinguishes between the apical meristem of the juvenile and adult stages, representing a turn "on" and "off" of gene action to produce protein synthesis needed in each growth phase. It is reasonable to assume that the apical meristems differ in each stage. In the juvenile stage the "juvenile hormone" in this meristem as shown in left side of the dotted line may suppress production of those histones which in turn suppress production of "juvenile-RNA" needed in the juvenile stage of growth to initiate the differentiation of juvenile leaves. Therefore, the apical meristems act as agencies for the differentiation of organs and the synthesis of some products including growth substances, derived from nutrients; and as receiving agencies for the complex of growth substances from root, leaves, shoots, and other plant parts. This process of differentiation in the apical meristem is affected both by the products mentioned above and by the

products from the apical meristem itself, thus complementing gene action. The feedback system between the apical meristems (1), and the other parts of the plants (2, 3, 4, 5, 6 and 8) produces a system of reciprocal cause and effect relationships in the process of the plant development towards the adult stage. This is illustrated by the cause and effect relationship between the five lobed juvenile leaf (8), other parts of plant (2), and the root function (6) which operate via the apical meristem (1).

1.1.3 Juvenile zone (3 and 6)

The juvenile zone in trees refers to that part of the trunk near the ground, the oldest limb, and the root zone, where sphaeroblasts, epicormics, and adventitious buds on a root can be produced. The latter have some juvenile characteristics. Epicormics are actually buds which have not sprouted in young seedlings, possibly due to apical dominance at an early seedling stage and to the accumulation of some inhibitors. Therefore, the apical meristem of epicormics, from which the dormant buds are sprouted at the adult stage, is physiologically young, being similar to apical meristems in the young seedlings, but is chronologically old. Sphaeroblasts originate from true adventitious buds which have a property of a juvenile character in ontogenesis. The whole root system of seedlings can be assumed to be a juvenile zone, because adventitious buds occur.

1.1.4 Factors affecting the duration of juvenility (10)

1.1.4.1 Shortening of the juvenile phase

The feedback system between the apical meristems and other parts of the plant such as juvenile leaves, roots and shoots is maintained during the juvenile phase. This feedback system may be accelerated by some biochemical changes through some control measure in some species. The juvenile phase, therefore, can be shortened by light control, low temperatures, the use of dwarfing rootstocks or apomictic seedlings from *Malus sikkimensis*, benzothiazole-2-oxyacetate, triiodo-benzoic acid, non-pruning, application of fertilisers, bending and ringing. The effect varies according to the species and intensity of juvenility.

1.1.4.2 Reversion or lengthening of the juvenile phase

Reversion from the adult to the juvenile stage can be made by using high temperatures, cold treatment, X-ray, etiolation, juvenile rootstocks, severe pruning, and application of gibberellin in certain species. Lengthening of the juvenile phase can be induced by shade conditions, poor nutrition and vigorous rootstocks.

1.2 The relationship between the leaf, root, apical meristem, and adult zone in the adult plant (Right hand side of Fig. 2)

Between the onset of differentiation at the embryonic stage and the adult stage, at which flower bud formation can be initiated, the apical meristematic cells divide repeatedly. Biochemical changes may take place in the apical meristem during this period. Before the tree reaches a given size as an adult, we may assume that complicated metabolites exist in the whole plant between the juvenile and adult stages. For example, carbohydrates, nitrogenous compounds, and some growth substances may be synthesised and accumulated in the other plant parts (4) after repeated cycles of seasonal change, thereby gradually changing the mode of action of differentiation in the apical meristems (1). These phase changes may be due to gradients of auxin/gibberellin-like substances/cytokinins/inhibitors, and nutrients. Therefore we may define the "adult hormone" as a small gradient of auxins/gibberellin-like substances/cytokinins which will maintain the adult phase, in contrast to the large gradient required to maintain the juvenile phase. This "hormone" would inhibit the synthesis of those histones which suppress the production of "adult-RNA", or at least would inhibit the synthesis of those factors which indirectly suppress "adult-RNA" production, as shown in the entire lanceolate adult leaf (9) and apical meristem (1) in right side of the dotted line. The adult-RNA synthesises proteins which are needed to differentiate the adult leaves in the apical meristem.

The concept of feedback system mentioned in the juvenile phase fits the adult stage, continuously producing a system of reciprocal cause and effect relationships.

1.2.1. Adult zone (crown of tree) (5)

As a tree becomes older, the branch systems become more complex. Hence, there develops a competition for nutrients, apical dominance is reduced or lost and geotropic effects are lost in the crown of trees (5). As the tree grows taller the roots become more remote. Therefore, the distribution of metabolic products and mineral nutrients absorbed from the roots (6) may be different from one position on the plant to another, including apical meristems. We may assume that a response of root to the apical meristems is different in the adult stage in which roots are more remote from the apical meristems than in the juvenile stage when the apical meristems are close to the roots.

Pruning in the crowns of trees renews the aged branches and consequently changes the required distribution of nutrients. This is similar to grafting an old bud on a rootstock because the old bud can grow vigorously, although an adult stage is maintained.

1.2.2. Flowering (11)

In the adult stage, we meet the further problem of the degree of flower initiation. Other factors are required to induce flowering in a tree. Although the crown of a tree is an adult zone (5), flowering will not always occur. As an extreme example, a certain period must elapse between vegetative growth and vigorous flowering, when the scion of a clonal variety is grafted on a vigorous rootstock. Therefore, we may define this period as "the clonal juvenile phase".

The initiation of flower bud formation in the apical meristem is a phenomenon involving the termination of the growth of vegetative shoots or spurs, although there are mixed flower buds in some species. This initiation of flower bud formation in the apical meristem (1) may be governed by a "floral hormone". This may be an optimum gradient of auxins/ribberellin-like substances/cytokinins which may inhibit the synthesis of those histones which suppress the production of "floral-RNA" (1) with which protein synthesis takes place in order to initiate flowering in the adult stage.

1.2.2.1 Factors affecting the promotion of flowering.

The degree of flowering in the adult stage of trees depends more or less on the spurs, shoots, or branches being positioned in the trees so as to maintain the optimum gradient of auxins/ribberellin-like substances/cytokinins and nutrients. In such a way, there are many factors which control and promote flowering in the adult stage. Environmental factors such as temperature, light and precipitation are important. For example, high temperature at night during the initiation of flowering inhibits flowering in apple. Practical measures such as i) the application of growth retardants, maleic hydrazide, triiodobenzoic acid, benzothiazole-2-oxyacetate, xanthin, and uracil, ii) the application of nitrogen fertilizers and irrigation, iii) pruning, including ringing, notching, bending branches downward, pinching growing shoots before the initiation of flowering, and iv) the effect of cropping; may all affect flowering.

1.2.3. Definition of juvenility (16)

The curved arrow (16) in Fig. 2 represents the progress of a plant from the young seedling (14) to the first flowering (15). This clockwise movement on the diagram from the seedling to the first flowering is brought about by gradual changes in differentiation in the apical meristems (1) indicating the feedback system in the whole plant. In woody plants there are many morphological and physiological changes during this period of ontogenesis. In the concept of readiness-to-flower, we define the length of the juvenile phase in woody plants as that period from the young seedling to the first flowering. Therefore, "true juvenility" in woody plants can be defined as the characteristics present during this time.

During the juvenile phase typical characteristics or factors, which may occur in a range of combinations, depending on species, are dimorphism of leaf shape,

phyllotaxis, anatomical changes, chemical and biochemical changes, production of pigment, leaf retention in winter, plagiotropic growth, easy-to-root properties, greater growth rate and thorniness. It is impossible to find or define characteristics which apply to all species. However, in some species, some differences can be found between the juvenile and adult stages. These are shown under the juvenile (12) and adult (13) characteristics in Fig. 2. The density of vein islets, the number of vessels and stomata, leaf thickness, the number of pericyclic fibres, lignification, the contents of total sugar, starch, protein, and mineral elements, the RNA/DNA ratio, and catalase activity increase in the adult stage in some species.

Finally, we may assume that these characteristics result from the differentiation occurring in apical meristems, in which the co-ordination of gene function with biochemical changes plays a major role, bringing about a phase change in the ontogenesis of woody plants.

It will be obvious from the above account and a study of the accompanying diagram that we have a long way to go in the attempt to reduce the physiological and biochemical explanation of juvenility to simple factors.

* The number in brackets is shown in Fig. 2.

exerted by the tree crown on the expression of excurrent and decurrent habits of individual trees. Apical dominance should be restricted to define the pattern of bud inhibition on currently elongating twigs. The branch forms in the one-year-old woody shoot are described as acrotony, mesotony, and besotony by Champagnat (1954 b, 1961). In woody plants, acrotony of shoot growth from the one year old lateral describes the ramification of branches from the most apical part of laterals; mesotony, from the middle part of laterals; and besotony, from the basal part of laterals.

Champagnat (1954 b, 1961) pointed out that apical dominance in woody plants differs from that of herbaceous plants but the physiological significance of apical dominance in current shoots in both herbaceous and woody plants (Phillips, 1969) is similar. 'Apical control' is best used for the outgrowth habit of woody plants from the one-year-old laterals. It is proposed in this thesis, therefore, to use 'Apicalism' to describe the correlative phenomena in growth habit affected by apical meristems, apical parts, and dominant organs in the plants under the control of a balance of the various plant hormones.

The mechanism of controlling 'apicalism' and vegetative and reproductive growth is shown in a speculative scheme in Fig.1. The release and inhibition of axillary bud growth appears to be due to the availability of cytokinins in the presence of IAA, GA, ABA, and other inhibitors. Growth is increased in the presence of a high ratio of available cytokinins to IAA, GA and ABA. This ratio can be increased by eliminating the source producing the latter or by affecting translocation, e.g., by decapitation and defoliation, notching, ringing, exposure to X-ray, and application of chemicals such as Alar, CCC, TIBA, MH, and Morphactins. These chemicals directly or indirectly prevent the biosynthesis of plant hormones so that IAA, GA, and ABA levels are reduced, or increase the available forms of metabolites, utilizing carbohydrates and nitrogen sources.

Recently there have been developments in the understanding of the specific roles of individual plant hormones and their interaction with other hormonal processes controlling plant growth and development. A hypothetical juvenile hormone or an adult hormone which may comprise different ratios of auxins, gibberellins, cytokinins, and abscisic acid in association with other inhibitors produced by the apical meristem, leaves, and roots may control conversion factors determining the change from the juvenile to the adult phases and vice versa in Fig.2 (Park, 1969). This plant hormone balance theory may explain also the mechanism of 'Apicalism'. The hormone-balance theory proposed

by Shein and Jackson (1971) suggests that the background level of hormones in the transpiration stream may have a specific role in controlling the growth processes. This role functions in association with plant hormones produced by the localized activities of plant organs. Woolley and Wareing (1972, a, b, c) also gave evidence that apical dominance is achieved by a delicate interaction between auxins, gibberellins, and cytokinins in Solanum andigena.

IAA is known as a plant hormone which inhibits axillary bud outgrowth when applied to decapitated stumps. On the other hand, gibberellins on the decapitated stump inhibit or promote apical dominance. That is GA with IAA on the stump further increases the inhibitory effects of IAA. However, generally the application of exogenous gibberellin to the intact plants inhibits axillary buds by promoting main stem elongation, although GA treatment promotes lateral bud growth when the plants are already subjected to conditions to abolish correlative inhibition (Phillips, 1969).

A remarkable phenomenon in apical dominance is that the lateral bud can be released from apical dominance influence by applying cytokinins. However further extension of shoot growth requires either auxins, (Sachs and Thimann, 1967) or gibberellins. Therefore, the promotion of shoot growth from the totally inhibited bud requires two steps; (i) bud formation and (ii) extension of shoots (Jackson and Field, 1972; Woolley and Wareing, 1972 c). Cytokinins may interact with both IAA and gibberellins in controlling bud formation and shoot expansion. Bud formation requires a high ratio of cytokinins to IAA (Skoog and Miller, 1957). Shoot extension requires a good balance of gibberellin and cytokinins (Jackson and Field, 1972; Woolley and Wareing, 1972 a, b). A synergistic effect of GA with CK reveals that the release of IAA induced both the inhibition of lateral buds and the increase of subsequent growth under higher nitrogen levels (Catalano and Hill, 1969). In soybean plants (Glycine max Mer. cv. Flambeau), Ali and Fletcher (1970) also confirmed that the hormonal regulation of apical dominance changes as the plant develops. The results indicate that while the cotyledonary buds are at an active stage of mitosis GA alone can release the IAA induced inhibition of buds, but after mitosis had ceased, both GA and CK were required for subsequent bud growth. CK and IAA control the elongation of shoot growth induced by GA (Jackson and Field, 1972).

Abscisic acid can be synthesized by the buds (Arney and Mitchell, 1969; Tucker and Mansfield, 1972), and in young and mature leaves and has even been found in xylem (Davison, 1963; Lenton, Bowen, and Saunders, 1968). Woolley and Wareing (1972 c) suggested that apical

dominance in Solanum andigena may overcome an inhibitory effect such as of ABA on an axillary bud by CK (Tucker and Mansfield, 1972). Tucker and Mansfield (1973) have concluded that apical dominance may be mediated indirectly by IAA which induces the formation of ABA in the buds themselves, and the consequent high concentration of ABA inhibits bud growth.

Abbott (1970) showed that the scale removal gave better bud burst, blossom, and subsequent bud development in spring and induced a renewed growth flush from the bud in the current shoot. Roberts, Tomasovic, and Fushigami (1974) observed that bud scale removal brought about immediate elongation of the shoot axis and of needle primordia in young plants. The bud scale may have some physiological significance not only in the onset and duration of dormancy (Schneider, 1973) but also in correlative inhibition. The preliminary experiment in these investigations in 1975 to assess the function of bud scales in apple variety Sturmer Pippin showed that bud scale removal from the buds in January 15 in late summer induced bud burst and blossom under ample irrigation treatment as shown in Fig.3. Some vegetative terminal buds were also induced to burst, but no buds in which flowers were already initiated were induced to bud burst, although the buds were swollen. 75% of buds from which the scales were removed blossomed. This phenomenon could indicate that bud scales as well as flower primordia may prevent bud burst in summer when the prevailing environmental conditions are such that one could normally expect bud burst, e.g., heavy summer rains, the application of summer nitrogen, and early summer pruning. There is a possibility that cytokinins are available, then but not under drought conditions. This preliminary experiment suggested that the bud scales and primordia may have a function in correlative inhibition in plant growth and development, possibly producing IAA and ABA from them.

2.6.1. Availability of cytokinins

Utilization of active or available cytokinins depends upon an antagonism between cytokinins and IAA in the tissues (Wickson and Thimann, 1958; Sachs and Thimann, 1964, 1967; Davies, Seth, and Wareing, 1966; Ali and Fletcher, 1970). But the mechanism of any such antagonism is unknown. Woolley and Wareing (1972 a,b) found that the presence of IAA greatly stimulates a change of metabolism of cytokinins, the extent depending upon the different quantities of IAA: i.e., at a low IAA-status in the tissues BA-riboside, which is immobile, is metabolized predominantly, whereas at a high IAA-status compound C (a cytokinin-

FIGURE 3

A and B. Effect of bud scale removal on the bud burst at the 15th January.

A = Photograph taken on 22/2/75.

B = Photograph taken on 29/2/75.

C and D. Effect of heavy rain after drought season on the bud burst.

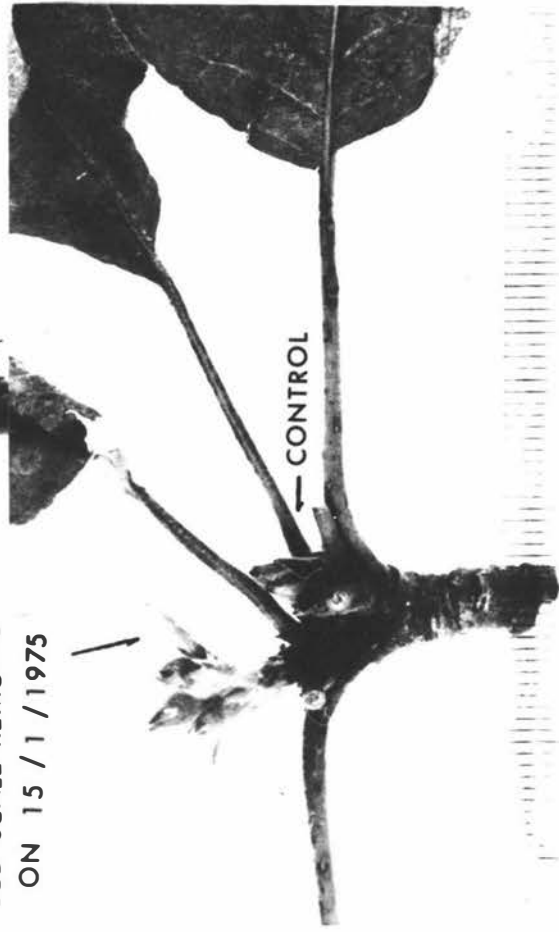
C = Bud bourse of Granny Smith,
photographed on 13/3/70.

D = Spur buds of Sturmer Pippin,
photographed on 13/3/70

A



BUD SCALE REMOVAL
ON 15 / 1 / 1975



B cm 1 2 3 4 5 6 7 D

C



cm

like substance) which is mobile, is metabolized predominantly. The distribution of cytokinins and their metabolites are greatly affected by the presence or absence of IAA, (See, Fig.1) providing evidence for the predominantly and acropetal transport of compound C by the presence of IAA and the acropetal and basipetal movement of BAP and immobility of BA-riboside by the absence of IAA. Therefore they concluded that IAA has not only a direct influence on the transport of cytokinins but also indirectly on the distribution of cytokinins through the change in cytokinin metabolism. In view of the above results they concluded that the inhibitory effects of IAA on the lateral buds from which the root-produced cytokinins can cause release, is due to the inhibition of cytokinin accumulation in the buds by the presence of IAA, diverting the distribution of cytokinins to other organs.

In controlling the level of IAA in the plant tissues, cytokinins could induce control via IAA-oxidase. Lee (1971) found that the optimum concentration of 0.2 μ M kinetin increased the total activity of IAA-oxidase and repressed the development of isoenzymes A_5 and A_6 in Tobacco callus cultures. Lee showed that the activity of IAA oxidase varied at the different concentrations of cytokinins, presumably increasing or decreasing IAA oxidase isoenzymes A_5 and A_6 due to the action of different concentrations of cytokinins at the site of RNA synthesis.

The buds themselves may produce some cytokinins in Coffee arabica (Browning, 1973). The application of hadacidin, an enzyme inhibitor of adenylosuccinate synthetase, retarded bud development. Bud development inhibited by hadacidin was restored when cytokinins or aspartic acid were applied to the buds, but was not restored with the application of glycine, serine, and glutamic acid. Adenine had a slight effect (Lee, Kessler, and Thimann, 1974). Lee, Kessler, and Thimann (1974) suggested that lateral bud development appeared to be due to a block in the synthesis of cytokinin via the activity of adenylosuccinate synthesis linked with aspartic acid in the buds.

2.6.2. IAA conjugating system with aspartate

IAA can be conjugated (See, Fig.1) with various compounds so that most of them become complex compounds in the plant tissues; e.g., indoleacetyl aspartic acid (Andreae and Good, 1955; Andreae and van Ysselstein, 1956, 1960 a,b; Fang, Theisen, and Butts, 1959; Row, Sanford, and Hitchcock, 1961; Lantican and Muir, 1969), Indoleacetyl-glutamic acid (Andreae and Good, 1955; Andreae and van Ysselstein, 1956), 1-indole-3-acetyl-D-glucose (Zenk, 1961), IAA-arabinose and IAA-galactose, and IAA-inositol (Ueda, 1970). Kendal, Park, and Mer (1971)

found that the only metabolite in pea seedlings when treated with either ring- or carboxyl-labelled IAA was IAA-aspartate, although very small amounts of indole-3-aldehyde and other substances were found. Patrick and Woolley (1973) also found that the principle conjugate metabolites of IAA-aspartic acid were detected within 9-12 hours, and included two minor compounds such as Indole-3-aldehyde and IAA-glucose, when a physiological level of labelled IAA was applied on the decapitated stems of Phaseolus vulgaris. Lantican and Muir (1969) found that dwarfism of the dwarf pea variety 'Little Maruel' as compared with the normal pea variety 'Alaska' grown in light, is probably due to the greater ability of IAA to conjugate with aspartate rather than in the synthesis of IAA by the apex. No effect of GA on the formation of indole acetyl aspartate occurred per se in the induction of the indoleacetyl aspartate synthetase.

The conjugation of IAA with aspartic acid takes place extensively in active growing tissues; i.e., at root tips (Andreae and van Ysselstein, 1960 a) and at the apex of peas (Morris, Briant, and Thomson, 1969), in 1 cm of stem (Diffusate 50.6%; extractable, 93.0%) and five consecutive 1 cm stem sections (diffusate, 6.2% and extractable, 66.9%), and from the decapitated stem stumps after application of 0.1% ^{14}C -IAA (Patrick and Woolley, 1972). Excised root tips lost the ability to conjugate IAA with aspartate, whereas in the presence of IAA, the ability of inducing IAA-aspartate was restored (Andreae and van Ysselstein, 1960). Therefore the fate of IAA-aspartate is closely related to the activity of IAA production and the presence of IAA in the tissues (Kang, Newcomb, and Burg, 1971; Goren, Bukovac, 1973). The quantitative balance of IAA and IAA-aspartate in the tissue over time may be of physiological significance (Morris, Briant, and Thomson, 1969; Ryago and Breen, 1974). Ryago and Breen (1974) obtained and concluded that IBA inhibitor IAA oxidase or increases available IAA by b-oxidation of IBA. In fact IAA-aspartate is stable because it is not destroyed by IAA-oxidase enzymes (Andreae and Good, 1955).

Key and Ingle (1968) found that the incorporation of ^{14}C -IAA and ^{14}C -2,4-D into rRNA, sRNA, and DNA, to be probably due to hormones and the reutilization of the ^{14}C -nucleic acid synthesis. This process was also recognized by Bendana et al. (1965). Davis and Galston (1971) postulated that indoleacetyl aspartate could become attached to tRNA aspartate giving a compound similar to formylmethionine-tRNA, which might have a function as a protein chain initiator. However, they could not obtain any IAA-aspartate binding to tRNA better in vivo or in the labelled lower molecular weight fraction using aspartate- ^{14}C in Pisum

sativum, var. Alaska and dwarf bean Phaseolus vulgaris. However, Kobayashi and Yamaki (1972) suggested that ^{14}C -IAA can be bound with some kinds of sRNA after 30 minutes. At the saturated condition, ^{14}C -IAA could be incorporated by not only a genesis of sRNA binding ^{14}C -IAA, but also a de novo synthesis of the sRNA molecule and some with other nucleotides of sRNA molecules.

Many aspects of enzyme activities in the plant tissues are influenced by treatment with IAA and some synthetic auxins, indicating increased or decreased activities of enzymes (Glasziou, 1969; Marcus, 1971). Aspartate transcarbamylase (Carbamylphosphate; **1-aspartate** carbamyltransferase) is now known to play an important regulatory role in pyrimidine biosynthesis by catalyzing aspartate + carbamyl phosphate = carbamyl aspartate (Ureidosuccinate) + inorganic P in the higher plants (Johnson, Niblett, and Shively, 1973). The activity of aspartate transcarbamylase is stimulated by 2,4-D in etiolated cowpea hypocotyls, thereby increasing the nucleotides (Johnson, Niblett, and Shively, 1973).

The conjugation systems of IAA with aspartic acid and their possible physiological roles in the plant are known as (i) detoxification of excess free IAA (Andreae and Good, 1955, 1957; Andreae and van Ysselstein 1956; Zenk, 1961, 1964; Patrick and Woolley, 1972), (ii) a temporarily stored form of IAA and aspartate, thereafter both can be used for several sequences of nitrogen metabolism as shown in a speculative scheme (Fig.1) and for several possible types of metabolism in IAA utilization, i.e., the process of IAA conjugation with aspartate precedes the incorporation of IAA into protein. (iii) prevention of oxidation of IAA and excess of aspartate.

2.6.3. A significant role of aspartate

Recently the physiological significance of several amino acids has been investigated in higher plants. Dunham and Bryan (1968, 1969) have studied a series of aspartate mechanism metabolisms associated with the growth and development of the multicellular plants from gemmalings of the liverwort Marchantia polymorpha. Among 31 different amino acids, 13 amino acids at concentration below 1 mM are able to prevent its growth in several cases, in which aspartic acid is closely related to a biosynthetic pathway; e.g., lysine, threonine, and methionine. This inhibition of growth by lysine and threonine synergistically can be only partially reversed by their removal although the inhibition is prevented by a low concentration of methionine or its metabolic precursor, homoserine (Dunham and Bryan, 1969). The initial effect of lysine-threonine might be a concerted (multivalent) feedback inhibition of

aspartokinase activity which could lead to reduced methionine biosynthesis (Dunham and Bryon, 1969). Wong and Dennis (1973) confirmed that in the growth of Lemna minor inhibition by lysine and threonine could be overcome by methionine. The mechanism of growth inhibition by lysine and threonine appeared to be due to the activity of aspartokinase isolated from Lemna minor under the feedback inhibition of lysine and threonine. This indicated that the inhibition of aspartokinase by lysine-threonine induced a lack of methionine synthesis which is required for protein synthesis (Wong and Dennis, 1973). However, the inhibitory effects of amino acids differ among the different species. The control of enzyme systems in Lemna minor by amino acids is due to aspartokinase activity, and is not regulated by the nitrate reductase in tobacco cells cultured with nitrate as nitrogen source, threonine, isoleucine, methionine, and aspartate were inhibitory to growth and this inhibition may be due to the repression of nitrate reductase formation (Filner, 1966). In maize roots, threonine, serine, cysteine, and aspartate inhibited homoserine dehydrogenase (Bryan, 1969). Aspartate is known to enhance the inhibition of homoserine dehydrogenase and can attribute to a multienzyme complex between aspartokinase and homoserine dehydrogenase (Bryan, 1969). Aspartate, glutamate, and gibberellin released larger amounts of α -amylase in the embryoless barley endosperm (Galsky and Lippicott (1971 a,b)), but acetate and citrate inhibited GA_3 -induced α -amylase (Paleg, 1960).

Dunham and Bryan (1971) proposed a sequential series of metabolic events in protein synthesis through the aspartate related pathway. The inhibition of protein synthesis, therefore, is due to an accumulation of (i) pathway products such as lysine and threonine, (ii) aspartate, (iii) glutamate, and (iv) ninhydrin positive compounds. In the systems of compartmentation of metabolites (Oaks and Bidwell, 1970), the mechanism of feedback systems of inhibition among the metabolites by particular enzyme systems may exist to control a balance of metabolism (See.Fig.1), thereby inducing growth and development in plants. To IAA then can be attributed a physiological role in which an excess or deficit of either IAA or aspartate at the active metabolite site may lead to the formation of specific functions for plant constituents, depending on the physiological needs of developing tissues or organelles.

CHAPTER 3
Materials and Methods

3.1. Organisation of field experiments

Field experiments were carried out on a commercial orchard in the Hastings district, a major apple producing area approximately 100 miles from the University campus.

The varieties used were Red Delicious and Granny Smith both grafted on to MM 106 rootstocks and grown under the semi-intensive system (row spacing 16', spacing in the row 12'). The two varieties were planted in two blocks each with four rows of 66 trees per row. Initially (July 1970) 45 trees of each variety were selected at random after excluding trees forming guard rows. At this time the trees were three years old, were well grown and had been trained according to the central leader system (McKenzie and Mouat 1963).

3.2. Application of treatments

3.2.1. General

In July 1970 one-year-old uniform lateral shoots on the four first tier fruiting arms (See Fig. 4-A, B, C, D) situated within 45 cm of the central leader stem were selected and labelled. Each of these laterals was 'headed back' to a fixed length of either 90 cm (Exp. 1 and 3) or 150 cm (Exp. 2). The selected lateral on each branch was allocated at random within each tree to one of three or four treatments (described for each experiment below) and preparations were made for constraining either to the horizontal or below the horizontal if this was required by the allotted treatment.

After the blossom period in the spring of 1971 the laterals were examined and those laterals with dead buds at any shoot position were excluded. The randomised block design with a block corresponding to a tree was abandoned at this point. Therefore number of replications was reduced to 10, 8, or 7 for individual experiments.

3.2.2. Experiment 1

The apple variety used was Red Delicious. Twenty nine consecutive bud positions numbered from the apex were identified for study. The four treatments, each replicated 10 times were:

T₁ Horizontal bending of the lateral at the dormant period
(26 Aug. 1970)

T₂ Horizontal bending of the lateral at the petal fall stage
(26 Oct. 1970)

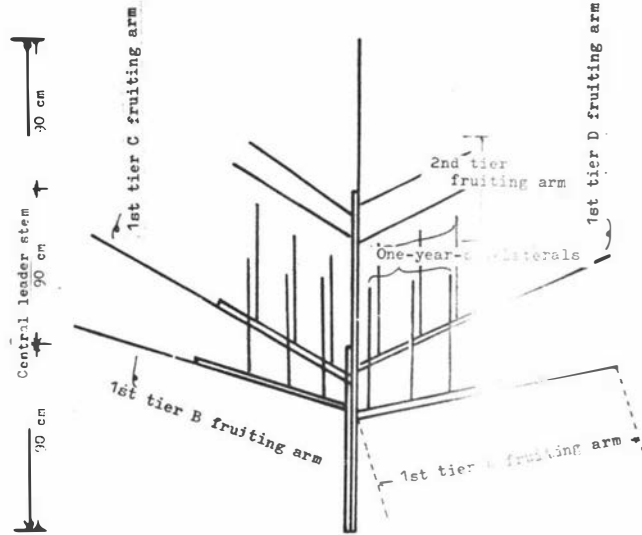
FIGURE 4

Experimental trees and definition for orientation and classification of whorl groups

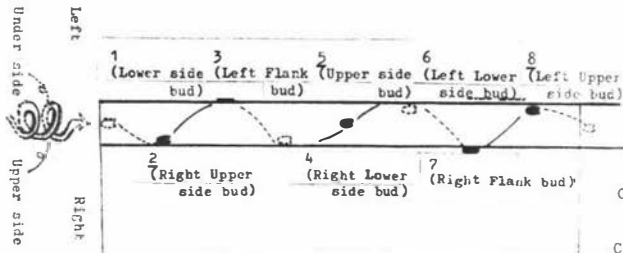
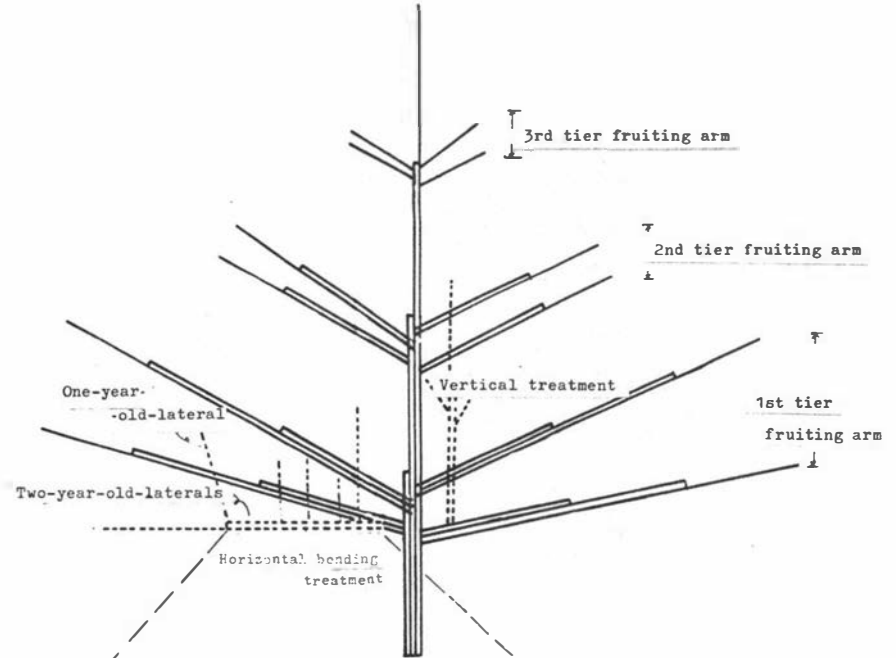
- A. 3 year old tree
- B. 4 year old tree
- C. Definition for orientation of the shoots
in the whorl group
- D. Lateral shoot; classification of position,
orientation, and whorl group

A. 3 YEARS OLD TREE

- 1 year old wood
- ▨ 2 year old wood
- ▧ 3 year old wood
- ▩ 4 year old wood



B. 4 YEARS OLD TREE



C. DEFINITION FOR ORIENTATION OF SHOOTS IN THE WHORL GROUP

Class of shoots (PO)
 Order of shoots (OR)
 Class of whorl groups

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29
4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8
Apical (A)					1st (W ₁)								2nd (W ₂)								3rd (W ₃)							

D. LATERAL SHOOT CLASSIFICATION OF POSITION OF SHOOTS IN THE WHORL GROUP

T₃ Horizontal bending of the lateral at the second cover stage
(26 Nov. 1970)

T₄ Control treatment: normal vertical lateral.

3.2.3. Experiment 2

The apple variety used was Red Delicious and bending treatments were applied in the same manner as for Experiment 1. In this experiment, however, the laterals were brought to a pendulous position approximating 135° from the vertical lateral. Forty five consecutive bud positions numbered from the apex were identified for study on the longer laterals (headed back to 150 cm) in this experiment.

The four treatments, each replicated 8 times, were:

T₁ Pendulous bending at the dormant period (22 Aug. 70)

T₂ Pendulous bending at the petal fall stage (22 Oct. 70)

T₃ Pendulous bending at the second cover stage (22 Nov. 70)

T₄ Control treatment, normal vertical lateral.

3.2.4. Experiment 3

The apple variety used was Granny Smith and the design was similar to that for Experiment 1 except for the omission of one of bending treatments and the reduction of the number of replications to 7. The treatments were :

T₁ Horizontal bending at the dormant period (24 Aug. 70)

T₂ Horizontal bending at the petal fall stage (24 Oct. 70)

T₃ Control treatment, normal vertical lateral.

3.3. Measures of shoot growth

3.3.1 Shoot diameters

At the time of heading back the one year laterals in July 1970 the diameters of these laterals were measured at 5 cm from the proximal end and at 30 cm intervals distally. In the cross section of each lateral at these positions the maximum diameter and the diameter at right angles to it were measured. The position at which measurements were made were labelled so that measurements could be repeated in August 1971. Similar measurements were also made on new one year laterals at that time.

3.3.2. Leaf areas

Leaf areas were obtained by assessment using a transparent plastic plate marked out in known areas in the average shape of leaves. 30 most appropriate sizes were selected based on experimental

sampling. The selected leaf areas were obtained using a planimeter.

To confirm the accuracy of using transparent plastic plates described above, actual leaves were subject to the same treatment, so that data were analyzed statistically. The T-test gave no significant difference between the mean of leaf areas from leaf samples and a matched leaf area on the plate. Therefore the plastic plates were used with confidence to measure leaf areas in these studies. Total leaf areas were obtained from an individual leaf size on the shoots of the treated laterals.

3.3.3. Ratio of total leaf area to total increment of shoot volume

Generally plant growth studies have been based on leaf area and total dry weight. In this study, however, leaf areas and volume of shoots were obtained for the studies of growth relationships, because of the impossibility of obtaining total dry weight on trees of this size and on account of the deposition of structural resources and food resources in shoots.

A ratio of total leaf area to total increment of shoot volume (R) was determined using the following formula:

$$R = \frac{\text{Total leaf area}}{W_2 - W_1}$$

Where W_1 is an initial volume of one year old lateral at the beginning of experiment, and

W_2 is total volume of a two year old lateral and the new growth of one year old laterals from it.

Shoot volumes were calculated by the formula for an ellipsoid (Pease, 1968).

In order to confirm the use of the ellipsoid formula, two comparisons were made, i.e., an actual volume of shoot and a calculated volume of the shoot were compared. 92 one year old laterals were grouped according to size and were headed back to 90 cm from the base. The width and narrowest diameters were measured at the same positions that is, at 30 cm intervals. The volume of each 30 cm was calculated by the ellipsoid formula. The actual volume was measured by a water displacement technique. The differences between the methods were analyzed statistically by the paired T test applied to a paired set of data (Snedecor and Cochran, 1967). The T value obtained from 92 samples was 0.4805. There was thus no evidence of difference between

the methods. Therefore the increment of shoot growth in these studies was determined by calculation using the ellipsoid formula.

3.3.4. Shoot length

In experiment 1 the shoot length at each of the 29 bud positions were made only in August 1971 (after leaf fall).

3.4. Statistical analyses

3.4.1. Classification of bud positions (See Fig.4)

The three terms, position, whorl, and orientation, which have been used in the present investigation to describe the site occupied by a bud on an experimental lateral are defined as follows:

Position has been used to mean the numerical order of buds on a lateral numbered proximally from the apical bud. Twenty nine positions were labelled and studied in Experiments 1 and 3. A further sixteen proximal positions were included in Experiment 2.

Whorl has been used to specify a group of buds falling in the $3/8$ phyllotactic arrangement of leaves on apple shoots (Roach, 1938). In Experiments 1 and 3 the twenty nine positions were grouped into an apical whorl and three sub-apical whorls. In Experiment 2 the additional sixteen positions form a further two distal whorls. Each apical whorl included five bud positions (positions 1 to 5) and each sub-apical whorl eight bud positions (positions 6 to 13, positions 14 to 21, positions 22 to 29 etc).

Orientation has been used to identify the location of a bud within each whorl. Orientations 4 to 8 were identified in an apical whorl and 1 to 8 in a sub-apical whorl. The spatial arrangement of buds of different orientation was important where laterals were constrained away from the vertical. Under these conditions orientations 2, 5 and 8 were specified buds on the upper side of the lateral (Designated 'upper' underlined), orientations 1, 4 and 6 were buds on the lower side (designated 'lower') and orientations 3 and 7 were buds on the flank of the lateral shoot (designated 'flank').

3.4.2. Analysis of variance models

A number of possible linear models which could explain the growth responses of individual buds were considered. The first model selected was:

$$Y_{ijk} = m + t_i + e_{ik} + p_j + (tp)_{ij} + e'_{ijk} \quad (\text{model A})$$

Where any observed response of an individual bud was the sum of m , the general mean;

t_i , the fixed effect of a treatment applied to all buds on a given lateral ($i=1, \dots, 3$ or 4);

p_j , the fixed effect of the position of any bud on any lateral ($j=1, \dots, 29$ or 45);

$(tp)_{ij}$, the interaction between the fixed effects of t_i and p_j ;

and two random elements, e_{ik} and e'_{ijk} , each from a population with zero mean and constant variance. The two population variances were assumed to be estimated by the sample variance between replications within treatments ($k=1, \dots, 7$ or 8 or 10) and the sample variance between replications within treatments by positions respectively.

Model A omits any term for blocks because the final choice of experimental material (See 3.2.1) disassociated replications and trees which were the original 'blocking unit'. Superficially Model A resembles the model for a split plot of nested design, but it differs in one essential respect: the apparent subplots are not allotted at random within the apparent main plots (lateral arms) subjected to the same bending treatment. It follows that the second assumed random element (e'_{ijk}) is not a random element and the sample variance between replications within treatments by positions is not an appropriate denominator for the calculation of F ratios for the effects of position by treatment. Whereas a conservative test of the significance of the effect of position would be provided by using the sample variance for positions by treatments as a denominator, there is not any test of the significance of the biologically important interaction of treatment and position.

Model A had a further practical disadvantage. Whereas from the sample estimate, e_{ik} appeared to be homoscedastic for the variables, particularly after transforming responses to a logarithmic scale, e'_{ijk} was markedly heteroscedastic on both the original and transformed scales for measurements of shoot length.

The second model examined was (Model B):

$$Y_{ijkl} = m + t_i + e_{ik} + w_j + o_l + (wo)_{jl} + (tw)_{ij} + (to)_{il} + e'_{ijl} \quad (\text{Model B})$$

The new terms in Model B are defined as follows:

w_j is the fixed effect of a whorl, o_1 the fixed effect of orientation and $(wo)_{j1}$ the interaction between the fixed effects of w_j and o_1 ($j= 1, \dots, 3$ or 5) ($i=1, \dots, 8$). These three terms collectively define the effect of position ascribed to p_j in Model A.

$(tw)_{ij}$ is the interaction between the fixed effects t_i and w_j and $(to)_{i1}$ is the interaction between fixed effects t_i and o_1 .

The remaining part of the interaction term $(tp)_{ij}$ in Model A appears in Model B as the random element e'_{ij1} which is computationally identical with the interaction between treatment, whorl, and orientation.

Model B assumed that there was no biologically important interaction between the three fixed effects and that this part of the overall interaction between treatment and position was an appropriate denominator for computing sample F ratio for position (= whorl + orientation + the interaction of whorl and orientation) and for the remainder of the interaction of treatment and position (treatment by whorl + treatment by orientation). A practical advantage of Model B was that e'_{ij1} appeared to be homoscedastic on the appropriate transformed scale (logarithmic or square root) for any variable.

3.4.3. Orthogonal subdivision of main effects and interactions

Tests of specific hypotheses regarding the effects of treatments, bud positions and interactions between treatments and positions, including the independent effects of whorl group and orientation, were carried out by computing the mean squares for individual degree of freedom comparisons (Li, 1964). The comparisons chosen were orthogonal and in specific instances the multipliers used were orthogonal polynomial coefficients (Fisher and Yates, 1958). Subdivision of interaction sums of squares was achieved using multipliers formed as a Kronecker product of the multipliers for the main effects concerned in the interaction.

Table 1. Effect of horizontal bending at various times on shoot length, total leaf area and number of leaves:
Experiment 1 Red Delicious - All 29 shoot positions

(a) Analysis of Variance

Source of variation	Degrees of freedom	DECEMBER SHOOT LENGTH ¹		JANUARY SHOOT LENGTH ¹		AUGUST SHOOT LENGTH ¹		TOTAL LEAF AREA ²		NUMBER OF LEAVES ³	
		Mean Square	F	Mean Square	F	Mean Square	F	Mean Square	F	Mean Square	F
Treatments	3	9137.1	8.66***	9591.3	6.03**	10948.8	6.49**	6411.7	0.58	394.25	1.94
T ₁ vs T ₄	1	25699.7	24.37***	28010.3	17.61***	32483.5	19.26***				
T ₂ vs T ₃	1	250.3	0.24	696.4	0.43	351.9	0.21				
T ₁ & T ₄ vs T ₂ & T ₃	1	1461.3	1.38	67.4	0.04	11.0	0.01				
Replications within treatments	36	1054.6		1590.2		1686.7		11134.3		202.97	
Positions	28	54734.9		68721.9		74158.4		125118.6		4520.04	
Positions x Treatments	84	1465.8		2117.4		2624.3		8913.7		228.79	
Positions x Rep. x Treat.	1008	569.7		897.0		989.6		3981.7		80.69	

(b) Means for bending treatments (10 laterals and 29 shoot positions)

	DECEMBER SHOOT LENGTH ¹	JANUARY SHOOT LENGTH ¹	AUGUST SHOOT LENGTH ¹	TOTAL LEAF AREA ²	NUMBER OF LEAVES ³
Dormant period (August) bending (T ₁)	48.41	53.71	56.28	141.2	27.72
Petal fall (October) bending (T ₂)	40.17	47.37	45.77	144.7	27.12
Second cover (November) bending (T ₃)	38.86	45.18	48.21	142.6	25.83
Vertical lateral (Control) (T ₄)	35.10	39.81	41.31	133.9	25.17

(c) Coefficients and mean squares for orthogonal polynomial equations relating shoot length to position on the lateral

	DECEMBER SHOOT LENGTH ¹		JANUARY SHOOT LENGTH ¹		AUGUST SHOOT LENGTH ¹	
	Coefficient ⁴	Mean Square ⁵	Coefficient ⁴	Mean Square ⁵	Coefficient ⁴	Mean Square ⁵
Dormant period bending (T ₁)						
Linear	-2.265	104101.6	-2.471	123939.1	-2.532	130138.7
Quadratic (after linear)	0.2105	50220.7	0.2307	60281.8	0.2320	60970.0
Cubic (after linear, quadratic)	-0.04520	86795.3	-0.05230	115093.3	-0.05381	121836.3
Quartic (after linear, quadratic, cubic)	0.006362	43708.4	0.006990	52767.7	0.007161	55378.4
Quintic (after linear to quartic)	0.0007208	2600.9	-0.001074	5779.7	-0.001078	5814.6
Petal fall bending (T ₂)						
Linear	-1.771	63724.6	-1.665	56275.4	-1.612	52762.3
Quadratic	0.2769	86830.7	0.3123	110507.0	0.3172	113944.4
Cubic	-0.04832	98232.0	-0.05300	118205.7	-0.05516	128031.5
Quartic	0.005716	35281.5	0.006166	41053.2	0.006393	44132.5
Quintic	-0.0009106	4151.5	-0.0001108	6151.2	-0.001155	6677.8
Second Cover bending (T ₃)						
Linear	-2.631	110563.9	-2.850	164932.2	-2.911	171998.7
Quadratic	0.2716	83540.6	0.2872	93450.1	0.2860	92676.8
Cubic	-0.04845	98769.6	-0.05427	123904.9	-0.05665	135013.1
Quartic	0.006838	50500.0	0.007782	65399.1	0.007976	68696.3
Quintic	-0.001162	6760.9	-0.001278	8172.1	-0.001209	7321.9
Vertical lateral (T ₄)						
Linear	-2.669	144590.4	-3.183	205662.1	-3.430	238786.4
Quadratic	0.3164	113381.2	0.3707	155633.5	0.4061	186792.9
Cubic	-0.05063	107846.2	-0.05972	150041.4	-0.06486	176993.1
Quartic	0.006784	49705.9	0.007917	67690.8	0.008714	81990.0
Quintic	-0.001208	7311.4	-0.001266	8020.0	-0.001568	12309.9

- Shoot length in cm, transformed by taking logarithms, 100 Log (L + 1.1).
 - Total area of the leaves born by a shoot in cm², transformed by taking logarithms 100 log (A + 1.1).
 - Number of leaves born by a shoot, transformed by taking square roots, 10 (N + 1)^{1/2}.
 - Given by $\sum_{ij} T_j / n \sum_{ij} W_{ij}^2$ where W_{ij} is a Fisher's orthogonal co-efficient, 1 = 1, 2, ..., 5 powers; j = 1, 2, ..., 29 positions; T_j is a total of shoot lengths at the jth position (n = 10).
 - Given by $(\sum_{ij} T_j)^2 / n \sum_{ij} W_{ij}^2$.
- *** = 0.001 > p, ** = 0.01 > p > 0.001, * = 0.05 > p > 0.01, + = 0.1 > p > 0.05, - = p > 0.1

Abbreviations:

- T₁ = Horizontal bending of the lateral at the dormant period.
- T₂ = Horizontal bending of the lateral at the petal fall stage.
- T₃ = Horizontal bending of the lateral at the second cover stage.
- T₄ = Control treatment, normal vertical lateral.
- W₁ = The first whorl.
- W₂ = The second whorl.
- W₃ = The third whorl.
- O = Orientation. Numerical numbers from 1 to 8 indicate the orientation of buds in the whorl.
- vs = Versus.

CHAPTER 4
Experimental Results

4.1. Shoot length section

Shoot lengths at different positions, orientation, and whorls which were affected by horizontal and pendulous treatments can be shown graphically and classified into three levels, i.e., under 0.3 cm, between 0.3-10 cm, and over 10 cm in Fig.6-A,B,C.&D; Fig.8-A,B,C;& D; Fig.9-A,B, & C. Unfortunately data were not able to be analyzed statistically by the classified levels referred to above, because the sample size was too small. Some conclusions, however, could be drawn and considered from the trends indicated in those figures.

Generally, the longer shoots (gourmand shoots) were produced from the upper side of horizontal laterals at the dormant period bending. However, the number of gourmand shoots was decreased when laterals were bent horizontally as the season progressed. The extremely acrotony type of shoot growth is shown in the 90 cm treatment in the vertical control in Red Delicious in Fig.8-A. However, the growth pattern of the shoot is different between the 90 cm of Red Delicious and Granny Smith as shown in Fig.6 and 9. In Granny Smith the number of shoots produced from the middle of lateral to the apex is seen in the vertical control (Fig. 9-C).

In Fig. 8 - D in Exp. 2, shoot growth from the lateral is more or less of similar form of total shown for **Granny Smith** in the vertical control. There was no restriction on the production of gourmand shoots on the fifth whorl at the position where the lateral was arched to bring about the pendulous direction in the dormant bending treatment. The number of gourmand shoots was therefore also decreased in the laterals bent pendulously in the later part of the growing season. This is the same result as shown in Exp. 1.

4.1.1. Experiment 1 (Red Delicious with 29 positions of laterals, horizontal bending)

Analyses of variance for the three measurements by time for shoot length are shown in Table 1., along with coefficients of orthogonal polynomial equations fitted to the means for each set of measurements for the 29 positions and the curves corresponding to the empirical equations are shown for each of the four treatments in Fig. 5. As shown in Table 1 and Fig. 5, the shoot length measured at December did not change very much after one month (in January) and even at the end of

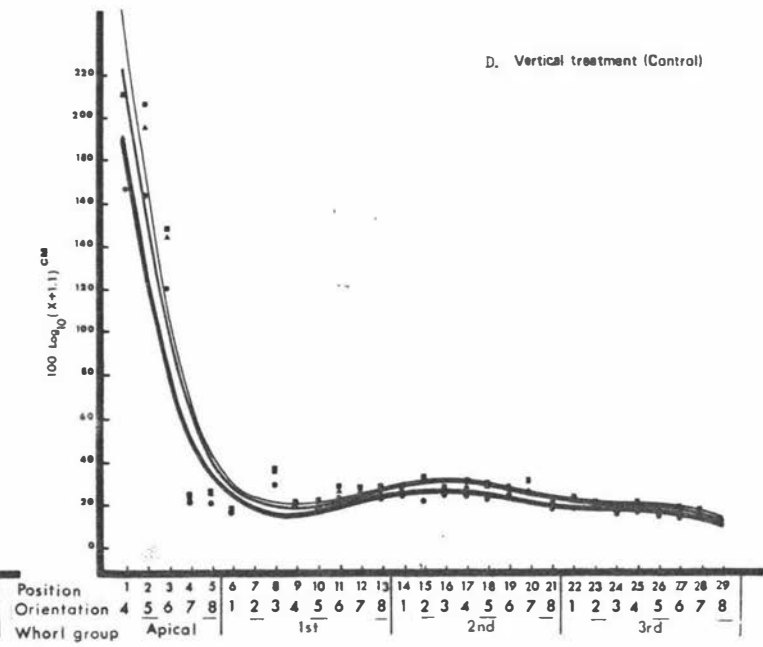
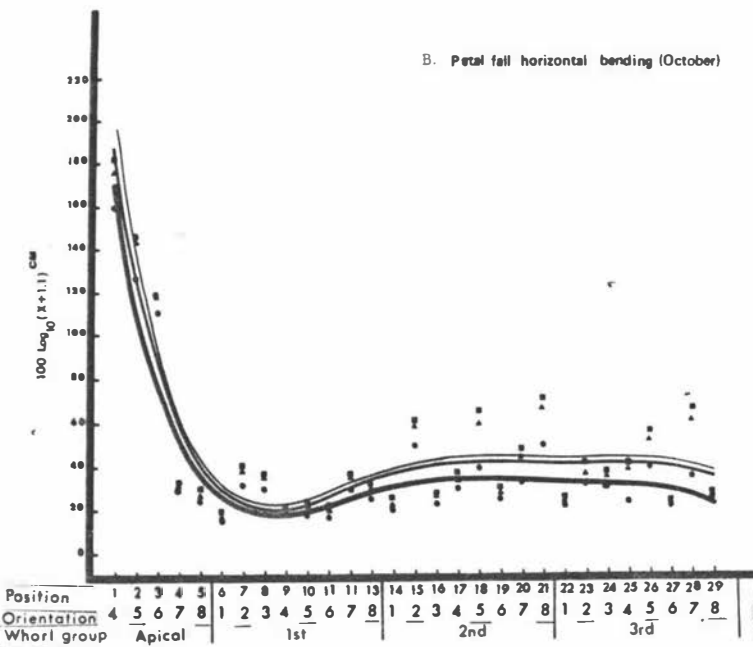
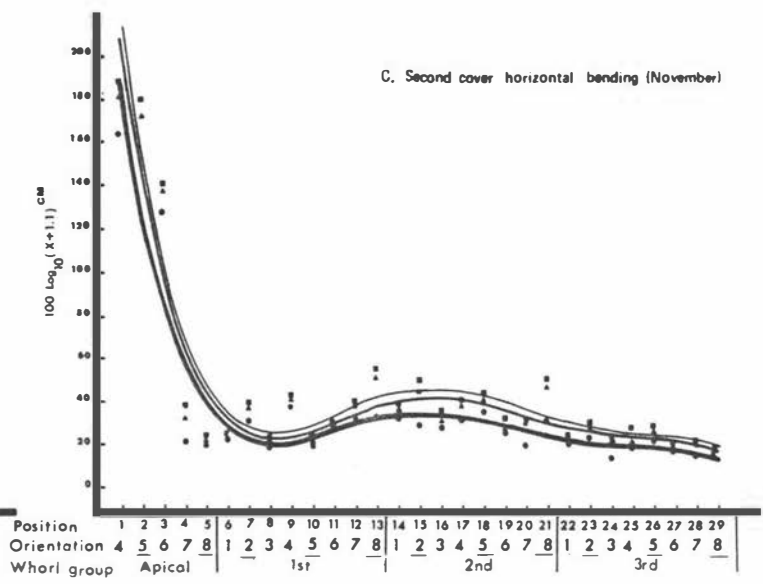
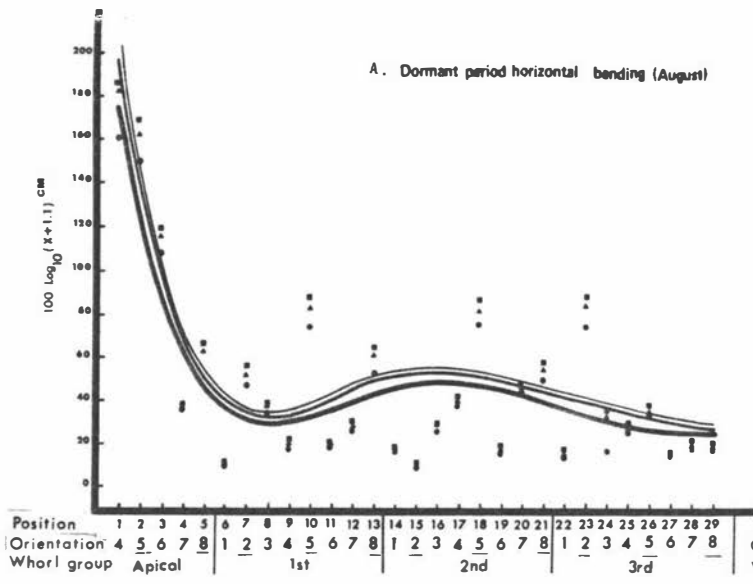
FIGURE 5

Seasonal change of shoot growth in horizontal and vertical treatments at different time.

- A. Dormant period bending (August)
- B. Petal fall bending (October)
- C. Second cover horizontal bending (November)
- D. Vertical treatment (Control)

The shoot length measured at December, January, and August. The different treatments were examined by fitting orthogonal polynomial coefficients (Fisher and Yates) up to the 5th power.

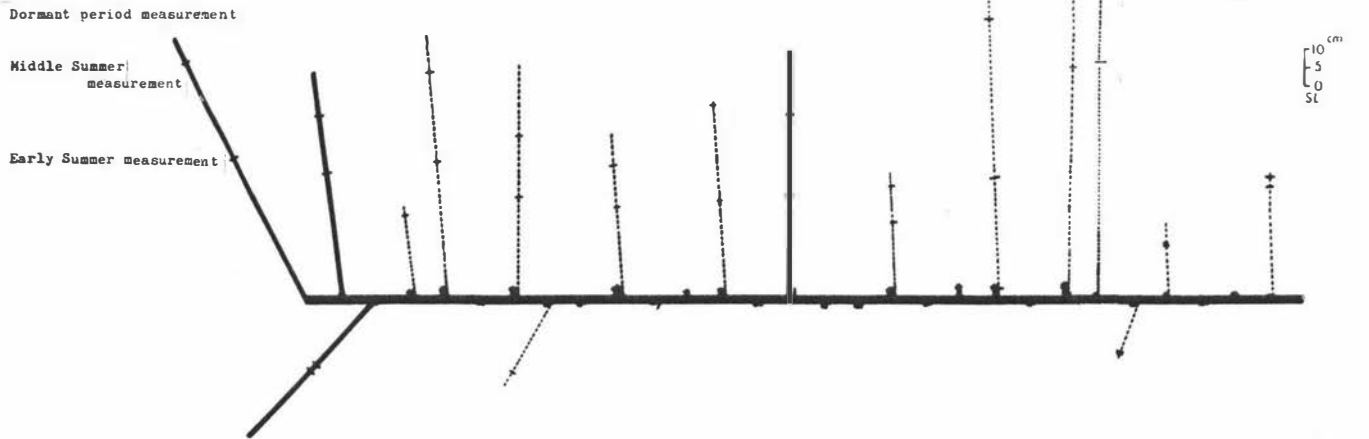
The 29 in the parenthesis indicates the 29 positions examined in the laterals. Shoot length were transformed by $100 \log_{10}(X + 1.1)$. The orientations 2.5, and 8 on the X-axis have been underlined to indicate the measurements on the upper side of lateral shoots.



● Actual mean In Early Summer measurement (December) ▲ Actual mean In Middle Summer measurement (January) ■ Actual mean In Dormant period measurement (August)
 — Predicted mean

A. Dormant period horizontal bending (August)

(Apple variety - Red Delicious)(29)

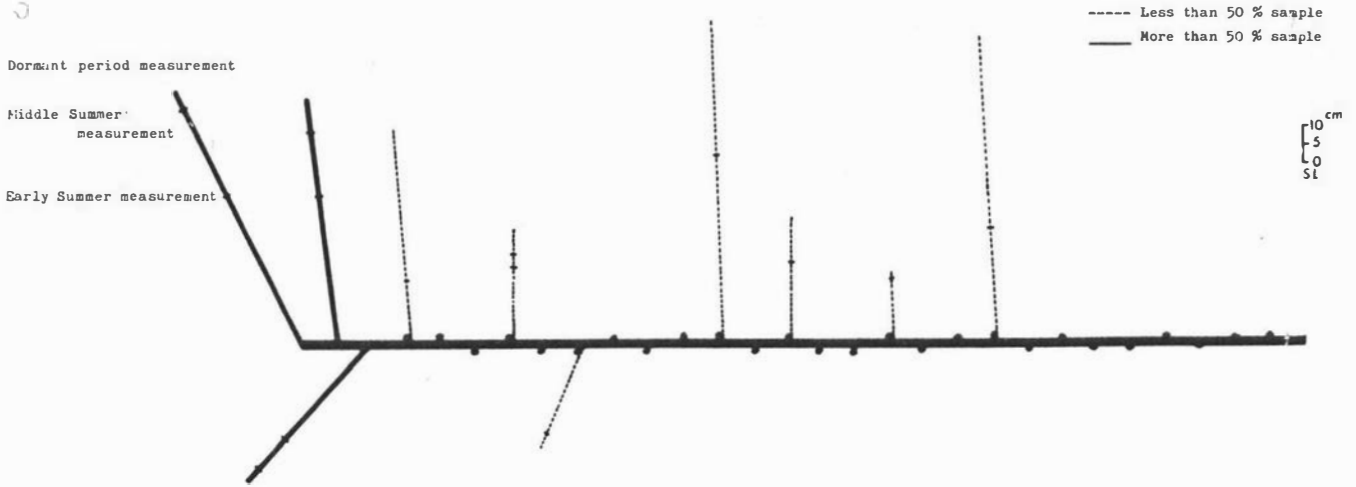


Position of shoots		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29		
Orientation of shoots		4	<u>5</u>	6	7	<u>8</u>	1	<u>2</u>	3	4	<u>5</u>	6	7	<u>8</u>	1	<u>2</u>	3	4	<u>5</u>	6	7	<u>8</u>	1	<u>2</u>	3	4	<u>5</u>	6	7	<u>8</u>		
Class of whorl groups		Apical					1st								2nd								3rd									
Shoot growth cm	Class of S4																															
	Under 0.3 cm	No	0	0	0	3	2	9	1	2	4	0	7	3	3	4	0	2	4	0	4	1	3	3	1	5	4	2	4	1	6	
	Mean	0.0	0.0	0.0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.0	0.2	0.2	0.2	0.2	0.0	0.2	0.2	0.0	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	
	S D	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
	% Fl	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	0.0	
	0.3 - 10 cm	No	0	0	6	3	6	6	1	8	7	6	6	5	7	7	6	4	8	5	6	6	9	8	7	6	4	5	7	6	9	3
	Mean	0.0	0.0	1.2	7.7	11.8	0.5	17.7	7.1	5.5	13.7	5.9	11.5	12.2	0.8	1.9	1.2	1.2	2.5	0.7	2.9	2.6	0.5	3.4	0.7	0.9	1.1	0.4	1.5	1.0		
	S D	0.0	0.0	0.4	1.3	2.0	0.5	2.2	1.0	0.9	2.3	2.0	1.6	1.7	0.8	1.9	1.2	1.2	2.5	0.7	2.9	2.6	0.5	3.4	0.7	0.9	1.1	0.4	1.5	1.0		
	% Fl	0.0	0.0	0.4	1.6	3.9	0.0	4.9	1.6	1.3	3.7	4.0	3.6	5.0	1.0	3.3	3.3	1.3	3.5	1.5	5.4	4.3	1.0	5.4	0.9	1.4	2.0	0.9	4.9	1.0		
	Over 10 cm	No	10	10	7	1	2	0	1	1	0	4	0	0	2	0	6	0	1	4	0	0	1	0	3	1	1	1	0	0	1	
Mean	776.5	583.0	331.7	24.0	135.0	0.0	60.0	25.0	0.0	167.0	0.0	0.0	100.5	0.0	63.8	0.0	12.0	128.0	0.0	0.0	95.0	0.0	244.0	115.0	15.0	14.0	0.0	0.0	31.5			
S D	77.0	58.3	47.4	24.0	67.5	0.0	60.0	25.0	0.0	41.8	0.0	0.0	50.3	0.0	15.4	0.0	12.4	0.0	0.0	0.0	95.0	0.0	81.3	115.0	15.0	14.0	0.0	0.0	31.5			
% Fl	100.0	96.0	30.0	10.0	0.0	0.0	0.0	0.0	0.0	30.0	0.0	0.0	6.0	0.0	10.0	0.0	10.0	30.0	0.0	0.0	0.0	0.0	20.0	0.0	10.0	0.0	0.0	0.0	10.0			
GT	776.5	583.0	332.9	32.3	147.2	2.1	77.9	32.5	6.3	186.7	7.3	12.1	112.9	0.6	39.0	1.0	1.9	142.2	0.5	2.6	11.6	4.1	264.4	118.7	20.4	22.4	5.5	13.3	35.6			
Mean	77.7	58.3	33.3	3.2	14.7	0.2	7.8	3.3	0.6	18.1	0.7	1.2	11.3	0.3	13.0	0.3	1.2	6.6	0.2	0.9	4.8	0.1	14.9	12.1	1.5	1.4	0.3	0.5	3.5			
% Fl	100.0	90.0	30.0	50.0	50.0	0.0	70.0	30.0	40.0	80.0	20.0	40.0	50.0	20.0	40.0	50.0	50.0	90.0	20.0	90.0	60.0	10.0	70.0	20.0	20.0	60.0	0.0	40.0	30.0			

Fig.6-A. Effect of horizontal bending at dormant period on shoot growth and flowering at different orientation, whorl, and class of shoots in 29 positions of Red Delicious (Exp.1).

From 10 replications, the lengths of the groups of shoots were classified as follows; < 0.3 cm, 0.3 - 10 cm, and > 10 cm. The shoot lengths measured in early December, January, and the end of season are shown by the bars. Shoot lengths < 0.3 cm are not presented in the diagram. 29 shoot positions were examined on the 90 cm laterals. The 2, 5, and 8 orientations are underlined to indicate the upper side of shoots on the horizontal laterals. The orientation in the whorl is followed by 3/8 phyllotaxis of apple shoots (Roach, 1939).

C. Second cover horizontal bending (November) (Apple variety - Red Delicious)(29)



Shoot growth cm	Position of shoots																												
	1					2					3					4					5								
	Apical					1st					2nd					3rd													
Under 0.3 cm	Class of whorl groups																												
	Orientation of shoots																												
0.3-10 cm	Class of whorl groups																												
	Orientation of shoots																												
Over 10 cm	Class of whorl groups																												
	Orientation of shoots																												
GT	Class of whorl groups																												
	Orientation of shoots																												
Mean	Class of whorl groups																												
	Orientation of shoots																												
S.D.	Class of whorl groups																												
	Orientation of shoots																												
% FI	Class of whorl groups																												
	Orientation of shoots																												

Fig. 6-C. Effect of horizontal bending at second cover stage on shoot growth and flowering at different orientation, whorl, and class of shoots in 29 positions of Red Delicious (Exp.1)

D. Vertical treatment (Control)

(Apple variety - Red Delicious)(29)

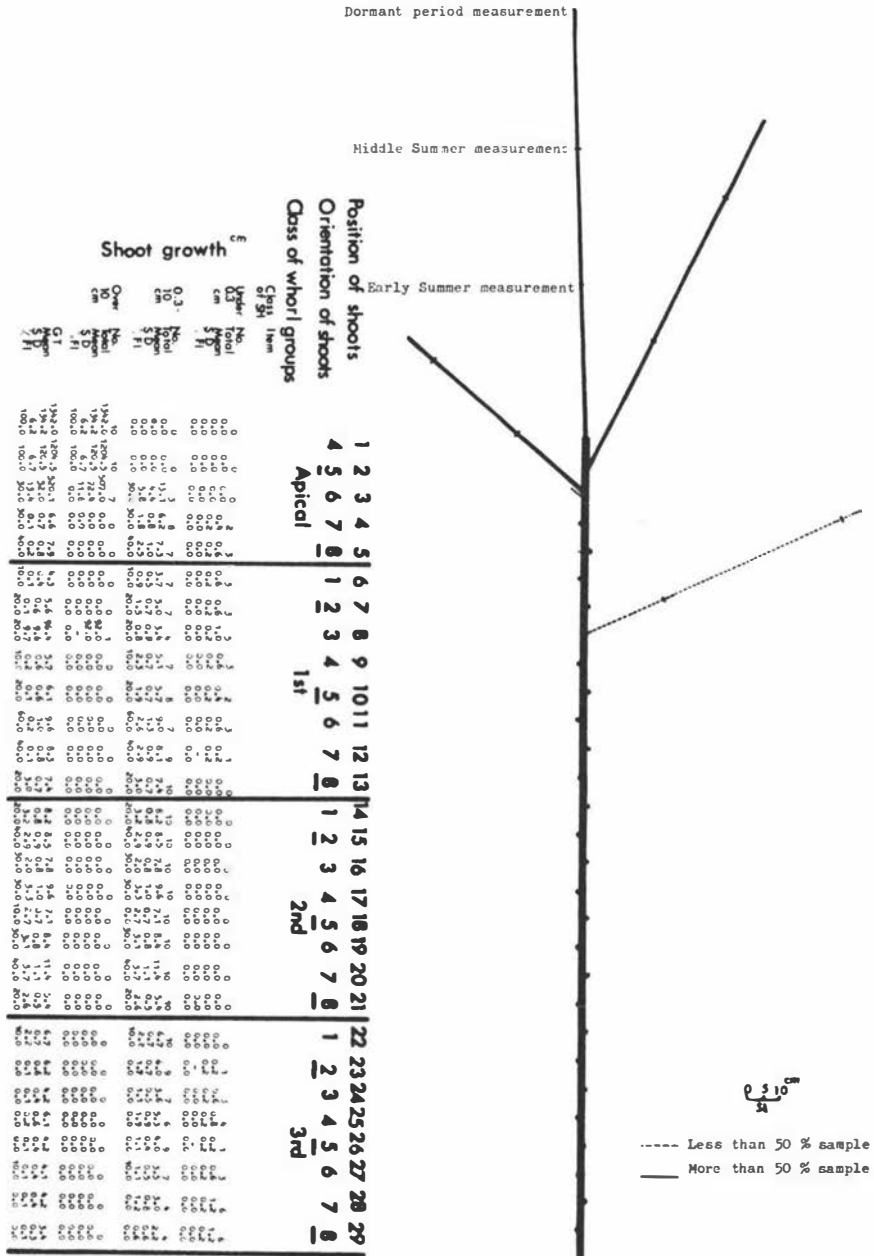


Fig.6-D. Effect of vertical control on shoot growth and flowering at different orientation, whorl, and class of shoots in 29 positions of Red Delicious (Exp.1).

TABLE 2. Effect of horizontal bending at various times on shoot length¹ and flowering² at the end of the growing season and at blossom: Experiment 1 Red Delicious
Analysis of variance for all whorls other than the apical whorl

Source of variation	Degrees of Freedom	Shoot length		Flowering	
		Mean square	F Ratio	Mean square	F Ratio
Treatments	3	16723.3	11.36***	2,5144	28.2112***
T ₂ vs T ₁	1	45965.9	31.24***	4,7403	53.2020***
T ₃ vs T ₄	1	2429.0	1.65	.0112	0.1257
T ₁ & T ₄ vs T ₂ & T ₃	1	1775.1	1.21	2.7916	31.3311***
Replications within Treatments	36	1672.0		0.0891	
Positions	27	5296.8	4.78***	0.4974	5.5825***
Whorls	2	11709.1	10.64***	3.4918	39.1823***
Linear response (W ₃ - W ₁)	1	1103.2	0.99	1.1500	12.9068***
Non-Linear response (2W ₂ - W ₁ - W ₃)	1	22494.9	20.29***	5.8336	66.4725***
Orientations	7	10614.5	9.57***	0.1920	2.1646
Upper vs lower (O _{2,5,8} vs O _{1,4,6})	1	60742.0	54.76***	0.5022	5.6431
Flank vs upper & lower (O _{3,7} vs O _{1,2,4,5,6,8})	1	443.7	0.40	0.1636	1.8761
Within upper: Linear (O ₂ - O ₃)	1	6028.7	6.06*	0.1426	1.6008
Within upper: Non-linear (2 O ₂ - O ₃ - O ₄)	1	124.9	0.11	0.1006	1.1291
Remainder	4	2087.1	1.89	0.1469	1.6467
Whorls x orientations	14	1769.0	1.54	0.2219	2.4896*
Treatments x Positions	69	2290.9		0.1664	
Treatments x whorls	6	2216.7	2.00*	0.5223	5.8608***
T ₁ vs T ₄ x Linear response	1	4.3	< 0.01	0.6980	7.8339**
x Non-linear response	1	1684.1	1.52	0.0318	0.3569
T ₂ vs T ₃ x Linear response	1	9868.7	8.90**	0.6676	7.4927**
x Non-linear response	1	1.7	< 0.01	0.4101	4.6027*
T ₁ & T ₄ vs T ₂ & T ₃ x Linear response	1	1465.2	1.41	1.2600	14.1414**
x Non-linear response	1	179.2	0.16	0.0664	0.7452
Treatments x Orientations	21	4676.2	4.22***	0.3934	4.4140***
T ₁ vs T ₄ x upper vs lower	1	61287.3	55.23***	3.4643	38.8810***
x flank vs upper & lower	1	3808.4	3.43*	0.0065	0.7300
x within upper: Linear	1	8648.6	7.79**	0.0071	0.0797
x within upper: Non-linear	1	30.7	0.03	0.4407	4.9461*
T ₂ vs T ₃ x Upper vs lower	1	3579.0	3.23*	0.3833	4.3019*
x Flank vs upper & lower	1	2379.7	2.15	0.0053	0.0595
x Within upper: Linear	1	470.7	0.42	0.1099	1.2334
x Within upper: Non-linear	1	1692.7	1.53	0.1311	1.4714
T ₁ & T ₄ vs T ₂ & T ₃ x upper vs lower	1	9406.4	8.48**	1.2727	14.2840***
x flank vs upper & lower	1	507.4	0.46	0.0377	0.4231
x within upper: Linear	1	5622.5	5.07*	0.0335	0.3760
x within upper: Non-linear	1	281.2	0.21	0.0010	0.0112
Remainder	9	65.1	0.06	0.2631	2.8774**
Treatments x Whorls x Orientations	42	1108.8		0.0891	
Positions x Replications within Treatments	828	906.7			

1. Shoot length in cm. transformed by taking logarithms, 100 Log (L + 1.1). 2. Flowering transformed by SQRT (X + 0.5) where X = No. of flowering.
***-0.001 > p, ** = 0.01 > p > 0.001, * = 0.05 > p > 0.01, + = 0.1 > p > 0.05, - = p > 0.1

Abbreviations:

- T₁ = Horizontal bending of the lateral at the dormant period.
- T₂ = Horizontal bending of the lateral at the petal fall stage.
- T₃ = Horizontal bending of the lateral at the second stage.
- T₄ = Control treatment: normal vertical lateral.
- W₁ = The first whorl
- W₂ = The second whorl
- W₃ = The third whorl

O = Orientation. Numerical numbers from 1 to 8 indicate the orientation of buds in the whorl.
vs = Versus.

of the growing season for all treatments. This indicates that most of the shoot growth was already terminated by December.

The empirical polynomial equations were similar for all treatments. The relationship between shoot length and position was essentially linear within the apical whorl and the negative slope was greatest for the control (T_4). The form of the equations and the extent of deviations of the means from the curves described by the equations showed greater differences between the treatments for the non-apical whorl positions.

The relationship between shoot length and position in the subapical whorls was investigated in greater detail in an analysis of variance of the August measurements (Table 2). In this analysis physiologically important comparisons have been shown separately within the major divisions of variance.

4.1.1.1. Effect of treatments

Horizontal bending during the dormant period (T_1) increased average shoot length (over all 24 non-apical positions) relative to the control (T_4). The effect of bending at the later growing stages of petal fall and second cover stage (T_2 and T_3) was not significantly different from the control. The effects of bending at petal fall (T_2) did not differ significantly from the effects of bending at the second cover stage (T_3).

4.1.1.2. Effect of position

When the apical whorl was excluded the overall effect of whorl group was small and was resolved by the analysis into a single significant effect: the average shoot length (taken over all treatments and eight positions within a whorl) was greater in the second whorl than in the first or third whorl. This is shown graphically in Fig. 7-A, for each treatment. With respect to the interaction of treatment and whorl, the only significant comparison was detected in T_2 vs T_3 x linear effect of whorl. This was reflected by the larger average shoot length in the third whorl of laterals bent at petal fall (T_2).

The overall effect of shoot orientation was resolved into two significant components: i.e., the shoots grown from the upper side of horizontal laterals were longer than those from either the lower or flank and within the shoots from the upper side of the laterals, there was a linear increase in length from 2 orientation through 5 orientation to 8 orientation.

These overall effects of orientation were not equal as would have been expected in all treatments. In the control the upper, lower,

FIGURE 7

The effect of bending for Red Delicious (Exp.1 and 2) and Granny Smith (Exp.3) on shoot growth and flowering at the whorl group and orientation.

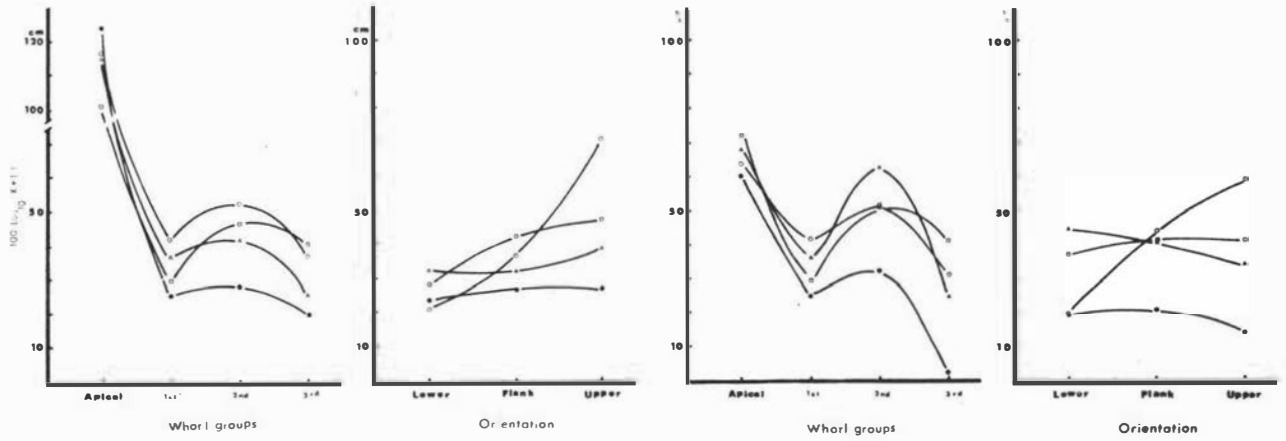
A. Red Delicious (29) in Experiment 1.

B. Red Delicious (45) in Experiment 2.

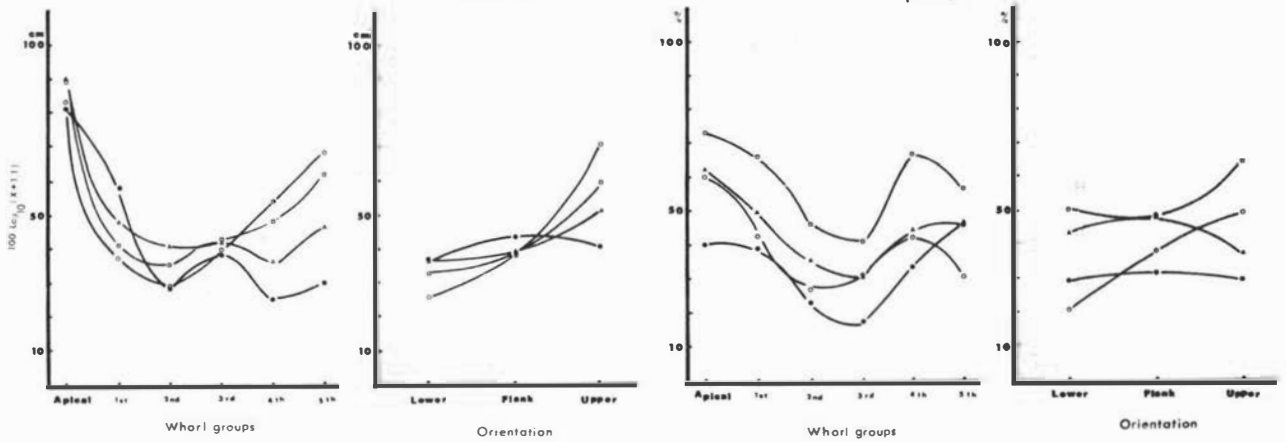
C. Granny Smith (29) in Experiment 3.

The means of each whorl group are derived from 50 shoots for the apical whorl, 80 shoots for the 1st, and 2nd, and 3rd whorl in Exp.1; 35 shoots for the apical and 56 shoots for the 1st, 2nd, and 3rd whorl in Exp.2; 40 shoots for the apical and 64 shoots for the 1st, 2nd, and 3rd whorl in Exp.3. The means of the lower orientation of the whorls are derived from 1, 4, and 6 orientation in the whorl originated from the 1st, 2nd, and 3rd whorl in Exp.1 and 3; and the 1st to 5th whorl in Exp.2, excluding the apical whorl.

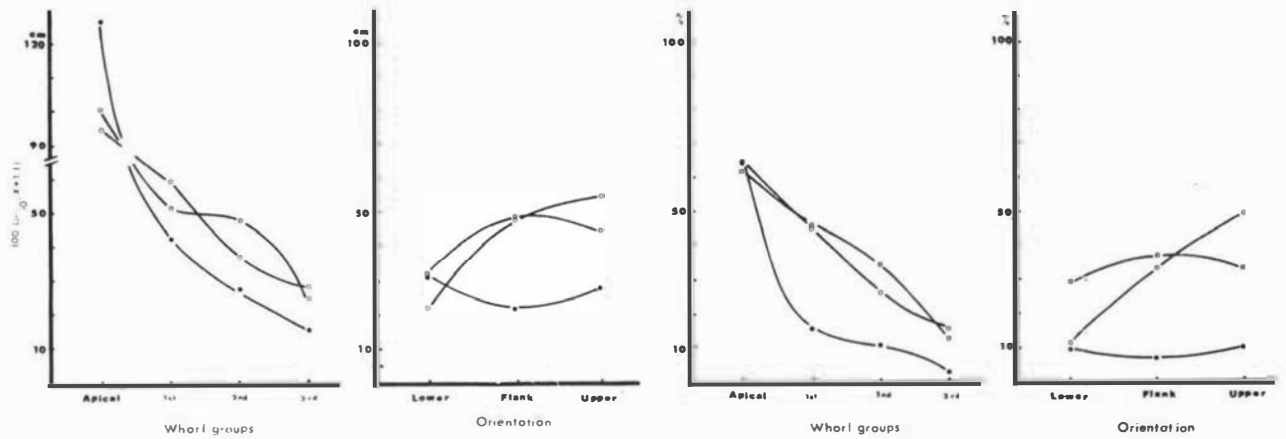
In such a way mentioned above, the means of flank orientation are derived from 3 and 7 orientation in the whorls and the means of the upper orientation are derived from 2, 5, and 8 orientation in the whorls.



A. (Apple variety - Red Delicious)(29) Exp. 1.



B. (Apple variety - Red Delicious)(45) Exp. 2.



C. (Apple variety - Granny Smith)(29) Exp. 3.

- — ○ Dormant period bending (August)
- △ — △ Petal fall bending (October)
- ▲ — ▲ Second cover bending (November)
- — ● Vertical treatment (Control)

SHOOT GROWTH^{CM}

FLOWERING %

Figure 7.

TABLE 3. Effect of pendulous bending at various times on shoot length¹ and flowering² measured at the end of the growing season and at blossom: Experiment 2 Red Delicious
Analysis of variance for all whorls other than the apical whorl

Source of variation	Degrees of Freedom	Shoot length		Flowering	
		Mean square	F Ratio	Mean Square	F Ratio
Treatments	3	1917.2	0.84 ⁻	2.0435	18.6792 ^{***}
T ₁ vs T ₄	1	4150.9	1.81 ⁻	0.3155	2.8839 ⁺
T ₂ vs T ₃	1	1281.4	0.56 ⁻	1.4482	13.2377 ^{***}
T ₁ & T ₄ vs T ₂ & T ₃	1	319.4	0.14 ⁻	4.3668	39.9159 ^{***}
Replications within Treatments	24	2281.4		0.1094	
Positions	39	6615.5	2.54 ^{***}	0.27797	99.094 ^{***}
Whorls	4	7670.6	2.94 ^{**}	0.6352	5.8074 ^{***}
Linear Response (2W ₃ + W ₄ - W ₂ - 2W ₁)	1	1342.7	0.51 ⁻	0.0075	0.0686 ⁻
Quadratic response (2W ₁ - W ₂ - 2W ₃ - W ₄ + 2W ₅)	1	2812.5	10.78 ^{**}	1.1530	10.5193 ^{**}
Remainder	2	608.6	0.23 ⁻	0.6971	6.3080 ^{**}
Orientation	7	17981.6	6.80 ^{***}	0.3565	3.2591 ^{**}
Upper vs Lower (O _{2,3,8} vs O _{1,4,6})	1	108761.0	41.62 ^{***}	1.0046	9.1828 ^{**}
Flank vs Upper & Lower (O _{3,7} vs O _{1,2,4,5,6,8})	1	2448.7	0.94 ⁻	0.0101	0.0923 ⁻
Within Upper: Linear (O ₈ - O ₂)	1	9769.7	3.74 ⁺	0.0158	0.1444 ⁻
Within Upper: Non-linear (2 O ₂ - O ₂ - O ₈)	1	1865.0	0.71 ⁻	0.3259	2.9789 ⁺
Remainder	3	1009.0	0.39 ⁻	0.3796	3.4698 ⁺
Whorls x Orientations	28	3623.2	1.39 ⁻	0.2073	1.8953 ⁺
Treatments x Positions	117	3705.6		0.13557	
Treatments x Whorls	12	8447.1	3.23 ^{**}	0.1054	0.9634 ⁻
T ₁ vs T ₄ x Linear response	1	81462.2	31.22 ^{***}	0.1215	1.1106 ⁻
x Quadratic response	1	599.9	0.23 ⁻	0.6519	5.0589 ⁺
T ₂ vs T ₃ x Linear response	1	4989.5	1.91 ⁻	0.0262	0.2395 ⁻
x Quadratic response	1	2020.2	0.77 ⁻	0.0328	2.2998 ⁻
T ₁ & T ₄ vs T ₂ & T ₃ x Linear response	1	714.6	0.27 ⁻	0.0019	0.0174 ⁻
x Quadratic response	1	40.3	0.02 ⁻	0.0195	0.1782 ⁻
Remainder	6	1923.1	0.74 ⁻	0.0685	0.6280 ⁻
Treatments x Orientations	21	5180.4	2.06 ⁺	0.2575	2.3544 ^{**}
T ₁ vs T ₄ x Upper vs Lower	1	47120.7	18.06 ^{***}	1.7213	15.7340
x Flank vs Upper & Lower	1	612.5	2.42 ⁻	0.0078	0.0713 ⁻
x Within Upper: Linear	1	2.2	0.01 ⁻	0.0003	0.0027 ⁻
x Within Upper: Non-linear	1	2222.2	0.85 ⁻	0.0019	0.0174 ⁻
T ₂ vs T ₃ x Upper vs Lower	1	3570.3	1.37 ⁻	0.6128	5.6015 ⁺
x Flank vs Upper & Lower	1	81.4	0.03 ⁻	0.2526	2.3090 ⁻
x Within Upper: Linear	1	8228.7	3.15 ⁺	0.5524	5.0494 ⁺
x Within Upper: Non-linear	1	2730.0	1.05 ⁻	0.0337	0.3080 ⁻
T ₁ & T ₄ vs T ₂ & T ₃ x Upper vs Lower	1	599.8	0.23 ⁻	0.4203	3.8414 ⁺
x Flank vs Upper & Lower	1	219.8	0.08 ⁻	0.1262	1.1536 ⁻
x Within Upper: Linear	1	6554.2	2.51 ⁻	0.2422	2.2159 ⁻
x Within Upper: Non-linear	1	786.2	0.30 ⁻	0.4302	3.9324 ⁺
Remainder	9	3840.0	1.47 ⁻	0.1118	1.0219 ⁻
Treatments x Whorls x Orientations	84	2609.5		0.1094	
Positions x Replications within Treatments	936	1870.9			

¹ Shoot length in cm, transformed by taking logarithms, 100 Log (L + 1.1). ² Flowering transformed by SQRT (X + 0.5) where X = No. of flowering.
*** = 0.001p ** = 0.01p > 0.001 * = 0.05p > 0.01 + = 0.1p > 0.05 - = p > 0.1

Abbreviations:

- T₁ = Pendulous bending at the dormant period.
- T₂ = Pendulous bending at the petal fall stage.
- T₃ = Pendulous bending at the second cover stage.
- T₄ = Control treatment; normal vertical lateral.
- W₁ = The first whorl.
- W₂ = The second whorl.
- W₃ = The third whorl.
- O = Orientation. Numerical numbers from 1 to 8 indicates the orientation of buds in the whorl.
- vs = Versus.

and flank orientation of shoots were without meaning, i.e., no obvious relationship can be shown graphically (See. Fig. 7-A). Significant interaction components were therefore expected between T_1 and T_4 , particularly with respect to differences between upper and lower shoot length and between the upper shoots at different positions within the whorl in the laterals. The small almost 'significant (p 0.1)' interaction component involving T_1 and T_4 and comparison of flank shoots with the average of upper and lower shoots suggested that the growth of flank shoots in the laterals bent during the dormant period was less than the average of upper and lower shoots. Therefore, as shown graphically in Fig. 7-A, average shoot length at the lower, flank, and upper side on the horizontal dormant period bending lateral (T_1) is seen as a steep slope of linear effect.

The remaining significant components of the treatment by orientation interaction indicated that the upper-lower effect was greater in the laterals bent at petal fall (T_2) than at second cover (T_3) and that the response to later bending treatments was less than the mid-point between the response in the earliest bending treatment (T_1) and the 'response' in the control treatment (T_4). The horizontal bending during the growing season (T_2 and T_3), therefore, could not induce the shoot growth from the upper side of horizontal laterals as much as that of the horizontal bending during the dormant period (T_1).

4.1.2. Experiment 2 (Red Delicious with 45 positions of laterals, pendulous bending)

The means of total shoot growth from 45 positions among the different seasonal pendulous bending treatments did not show any differences. The results of a detailed analysis of variance of shoot growth of buds in five sub-apical whorls measured in August are shown in Table 3.

4.1.2.1. Effect of treatments

Average shoot length taking positions from five sub-apical whorls showed no significant effects of treatment nor did the length of laterals included in the apical whorl.

4.1.2.2. Effect of positions

The effect of whorl group on shoot growth taken over all treatments was in the form of a quadratic response, with the intermediate whorl group means lower than the first and fifth sub-apical whorl group means. In fact the fifth sub-apical whorl in the pendulous

A. Dormant period pendulous bending (August)

(Apple variety - Red Delicious)(45)

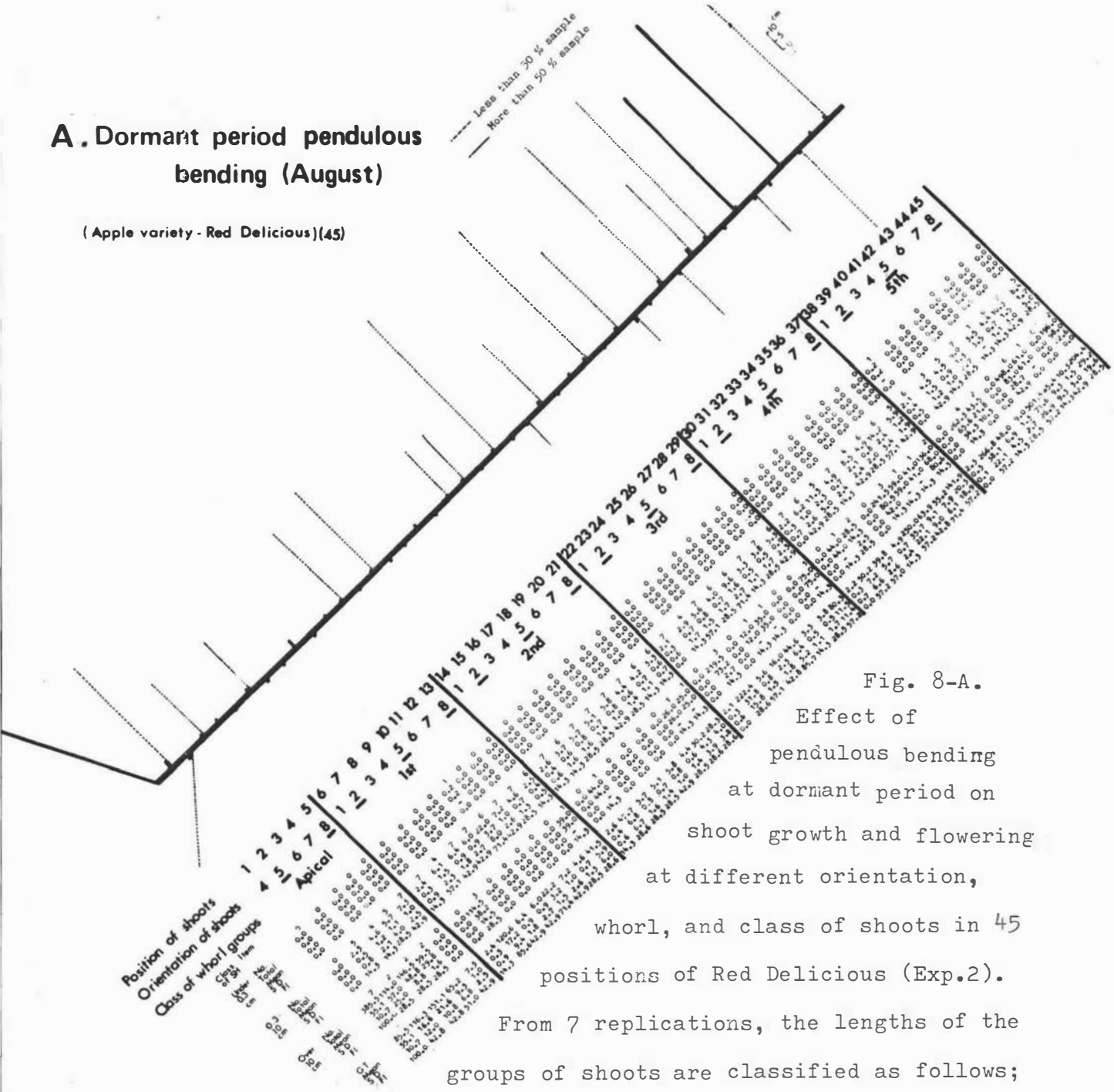


Fig. 8-A.
Effect of
pendulous bending
at dormant period on
shoot growth and flowering
at different orientation,
whorl, and class of shoots in 45
positions of Red Delicious (Exp.2).

From 7 replications, the lengths of the
groups of shoots are classified as follows;

< 0.3 cm, 0.3 - 10 cm, and > 10 cm.

45 positions were examined on the 150 cm lateral.

B. Petal fall pendulous bending (October)

(Apple variety - Red Delicious)(45)

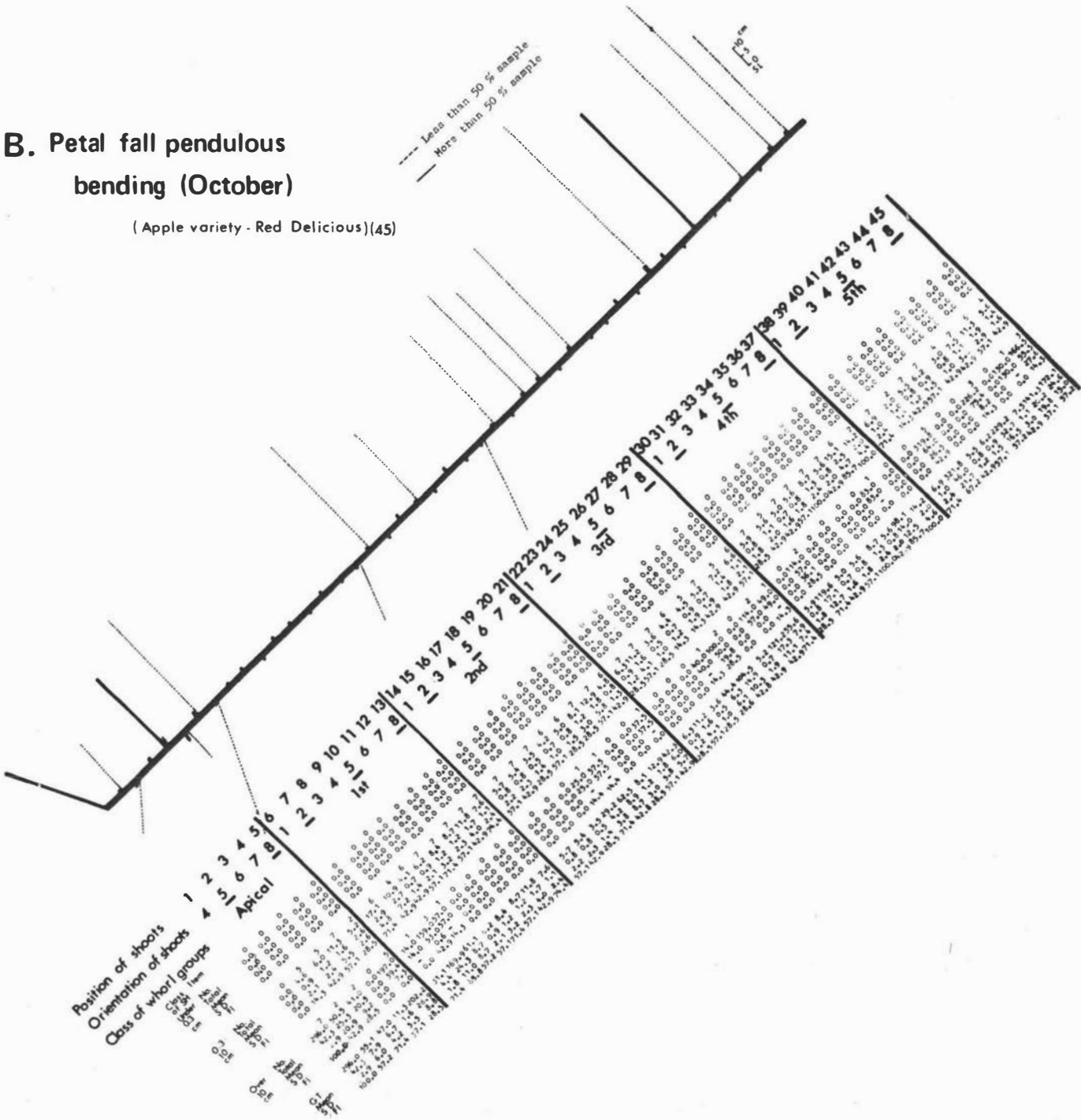


Fig. 8-B. Effect of pendulous bending at petal fall stage on shoot growth and flowering at different orientation, whorl, and class of shoots in 45 positions of Red Delicious (Exp.2).

C. Second cover pendulous bending (November)

(Apple variety - Red Delicious)(45)

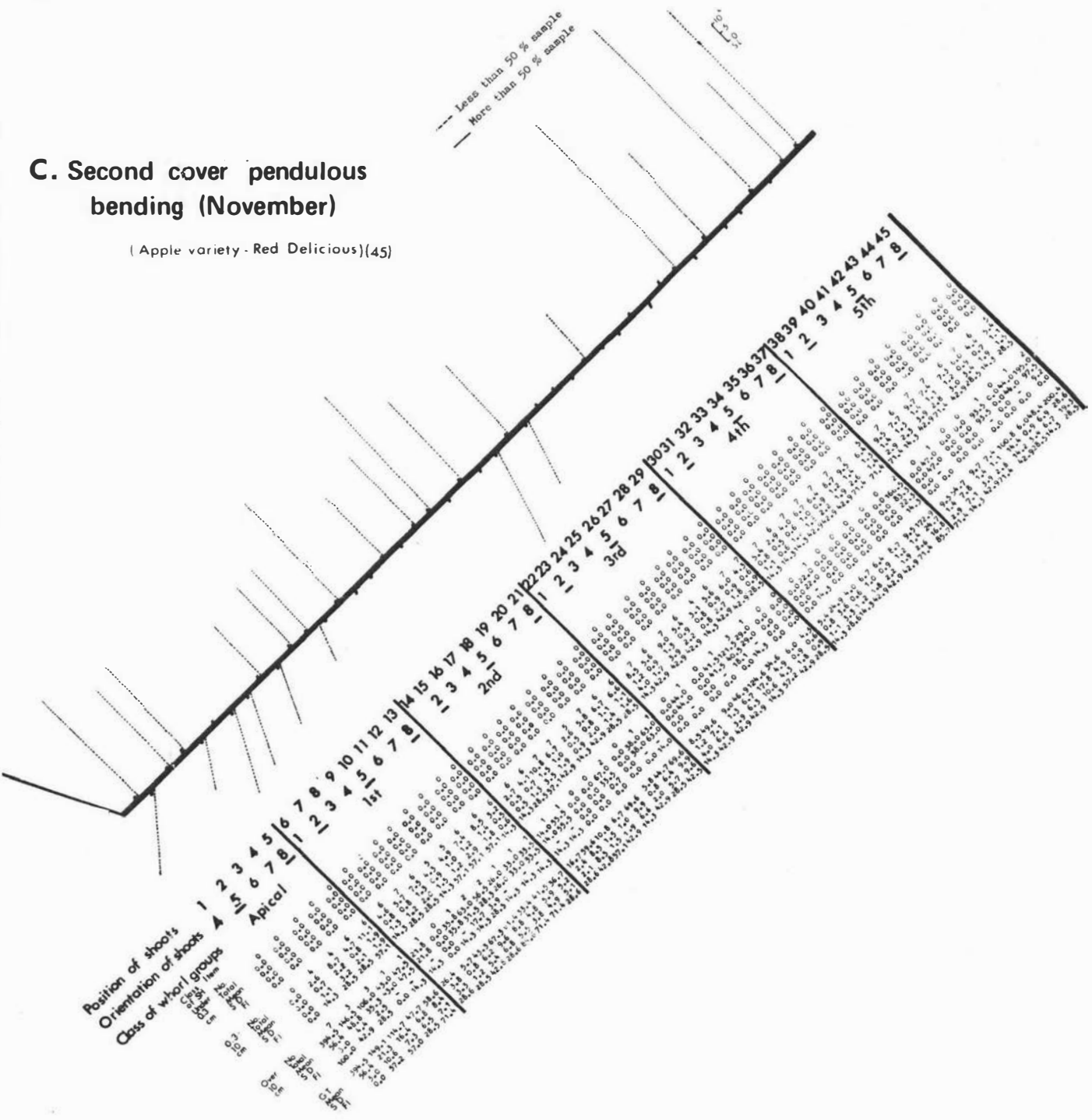


Fig.8-C. Effect of pendulous bending at second cover stage on shoot growth and flowering at different orientation, whorl, and class of shoots in 45 positions of Red Delicious (Exp.2).

D. Vertical treatment (Control)

(Apple variety - Red Delicious)(45)

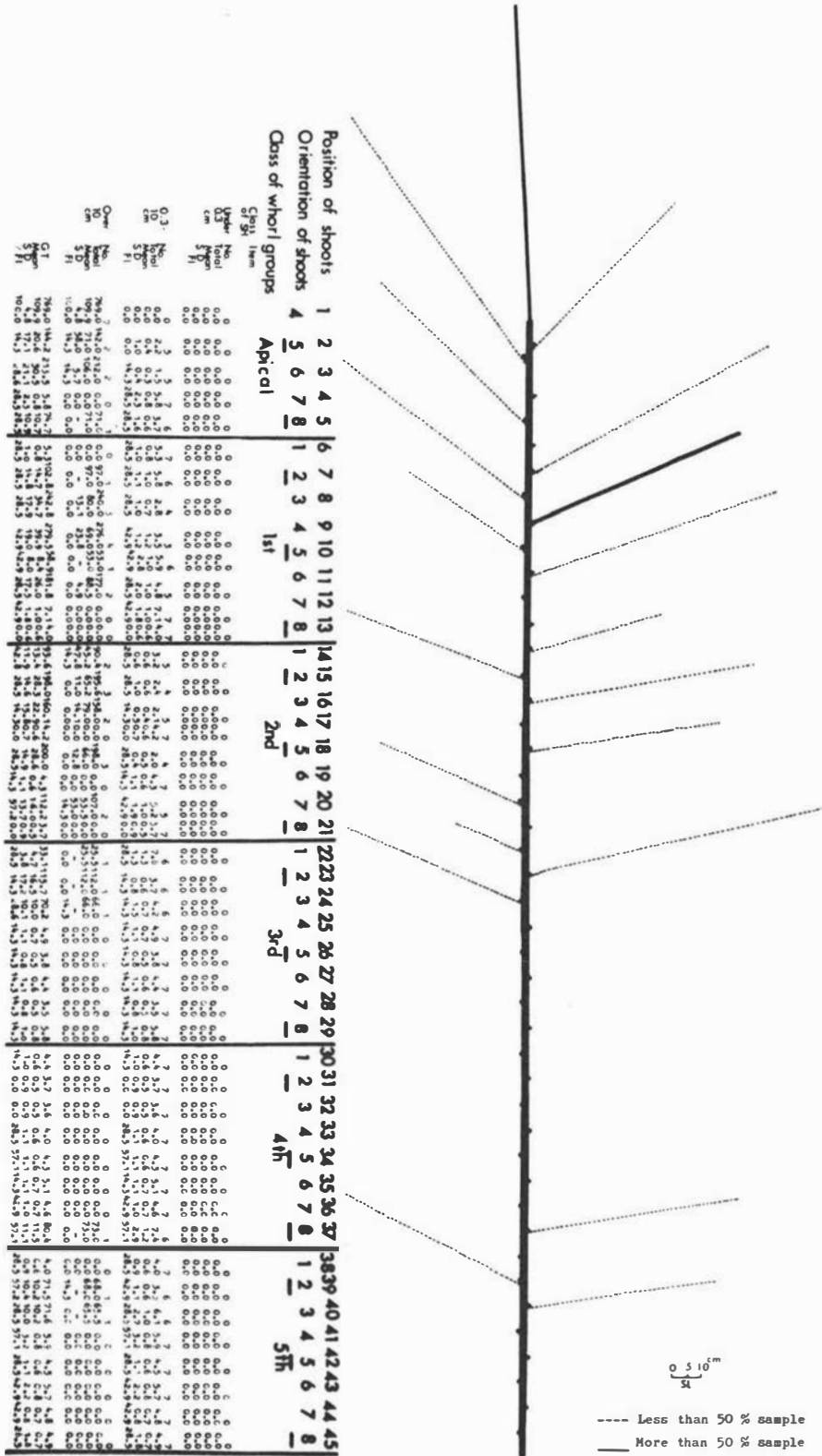


Fig. 8-D. Effect of vertical control on shoot growth and flowering at different orientation, whorl, and class of shoots in 45 positions of Red Delicious (Exp.2).

bending treatments is positioned at the arched part of laterals so that the buds on the upper side of laterals are likely to induce vigorous shoots (Gourmand) as shown graphically in Fig.8-A and B. There were significant differences between individual treatments in the whorl effect and T_2 and T_3 did not differ significantly from each other or collectively from the average of T_1 and the control (T_4). The effect of whorl group was different in the two extreme treatments (i.e., T_1 and T_4). Both had a similar quadratic component but whereas bending to a pendulous position during the dormant period generated a positive linear component in the whorl response, a negative linear component was seen in the control treatment. The earliest bending treatment increased shoot growth in the lower sub-apical whorl, relative to shoot growth in the higher sub-apical whorls (See Fig.8-A,B,C,& D).

The effect of orientation was essentially similar to that observed in Experiment 1. Over all treatments, shoots on the upper side of the laterals (as a result of the bending treatment) were significantly longer than those on the lower side (Fig.7-B; Shoot growth, orientation). The growth of shoots from the flank of the laterals was intermediate. Differences within the shoots from the upper side of the laterals were less obvious and not significantly different for all treatments. The significant components detected in the subdivision of the treatments by orientation interaction were consistent with greater promotion of the shoot growth from the upper side of laterals by earlier pendulous bending. Other effects within the shoots growing from the upper side of the laterals were inconsistent.

4.1.3. Experiment 3 (Granny Smith, horizontal bending)

The patterns of shoot growth in Granny Smith are shown graphically in Fig.7-C and Fig.8-A,B, and C. Granny Smith characteristically has a number of longer shoots spread sporadically from the middle to the apical region of the vertical control laterals. Furthermore, the production of a number of longer shoots in the horizontal laterals resulting from the dormant period bending, shifted from the middle centre in Red Delicious (Exp.1, Fig.6-A) to the middle to apical regions in Granny Smith. The 'two-centre pattern' of shoot growth (Mullins, 1964) is seen more clearly in the dormant period bending horizontal lateral of Red Delicious than that of Granny Smith (Exp.1) so that the main shoot growth is likely to be at the apical parts of the lateral in Granny Smith.

The results of detailed analysis of variance of shoot growth in the three sub-apical whorls measured in August are shown in Table 4.

4.1.3.1. Effect of treatments

The average shoot length on laterals subjected to horizontal bending was significantly greater than in laterals left in the vertical (control) position. There was no significant difference between dormant period (T_1) and petal fall (T_2) bending.

4.1.3.2. Effect of positions

The growth of shoots decreased from the first to the third sub-apical whorl in all treatments. The effect of whorl over all treatments was essentially linear. The two bending treatments differed ($0.1 > p > 0.05$) i.e., in T_2 the whorl effect appeared to have a positive quadratic component similar to that detected for all bending treatments in Experiment 1, whereas in T_1 a negative quadratic component was present.

The upper-lower effect of shoot orientation seen in both Experiments 1 and 2 was also obtained in this experiment where the effect was greatest on laterals bent horizontally in the dormant period, intermediate on laterals bent at petal fall and absent in vertical (control) laterals (T_4). Differences among the shoots on the upper side of the laterals were present only on laterals bent during the dormant period. In contrast with Experiment 1, bending at petal fall did not produce the decrease from orientation 2, through orientation 5 to orientation 8.

4.2. Flowering section

The flowering response of individual shoots was assessed simply as flowering or non-flowering. No account was taken of number of flowers per shoot. All detailed data on flowering, to arrive at a suitable technique to assess the conditions which should be considered as factors for the different classes of shoots are shown in Fig.6-A, B,C,& D for Exp.1, 8-A,B,C,&D for Exp.2, and Fig.9-A,B,&C for Exp.3.

4.2.1. Experiment 1 (Red Delicious 90 cm lateral)

4.2.1.1. Effect of treatments

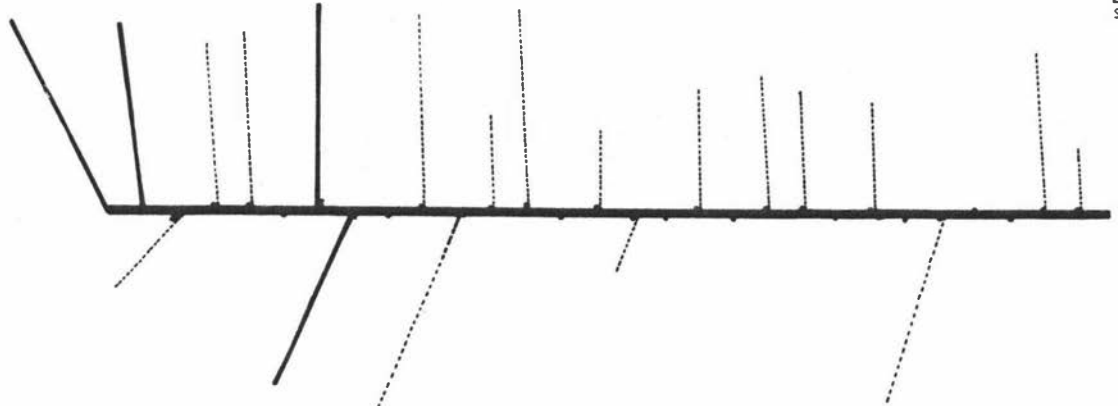
In the horizontal laterals, whether bent during the dormant period or during the growing season, nearly 50% flowering was measured on the horizontal laterals, whereas 25% flowering was recorded in the vertical controls, including flowering in the apical whorl. Horizontal bending during the dormant period (T_1) increased flowering (over all 24 non-apical positions) relative to the control (T_4). The effect of bending at the later stages of petal fall (T_2) and second cover stage (T_3) was to significantly increase flowering above the average flowering

A. Dormant period horizontal bending (August)

(Apple variety - Granny Smith)(29)

----- Less than 50 % sample
 _____ More than 50 % sample

10
5
0
5
10
cm



Position of shoots		1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29				
Orientation of shoots		4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8	1	2	3	4	5	6	7	8				
Class of whorl groups		Apical					1st								2nd								3rd											
Shoot growth cm	Class of SH																																	
	Under 0.3 cm	No.	Total	Mean	S D	% Fl																												
0.3- 10 cm	No.	Total	Mean	S D	% Fl																													
Over 10 cm	No.	Total	Mean	S D	% Fl																													
	GT																																	
	Mean																																	
	S D																																	
	% Fl																																	
	0.3	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0			
	0.3-10	0	4	7	5	6	8	3	4	8	5	7	6	7	8	6	7	8	6	8	6	7	8	6	8	7	8	8	7	7				
	Over	8	4	1	3	2	0	5	4	0	3	1	2	1	0	2	1	0	2	0	2	1	0	2	1	0	0	0	1	1				
	GT	453.0	189.0	25.0	127.0	91.0	0.0	263.5	200.5	0.0	150.0	85.0	9.0	52.0	0.0	41.5	15.0	0.0	92.5	0.0	69.2	31.0	0.0	56.0	0.0	50.0	0.0	0.0	41.5	16.0				
	Mean	56.6	47.4	25.0	42.3	45.3	0.0	52.7	50.1	0.0	50.0	85.0	24.5	52.0	0.0	20.8	15.0	0.0	31.2	0.0	34.6	31.0	0.0	28.0	0.0	50.0	0.0	0.0	41.5	16.0				
	S D	3.7	16.7	-	18.2	48.0	0.0	6.7	17.2	0.0	11.8	-	14.5	-	0.0	8.8	-	0.0	20.9	0.0	50.5	-	0.0	21.9	0.0	-	0.0	0.0	-	-				
	% Fl	100.0	50.0	12.5	37.5	12.5	0.0	37.5	25.0	0.0	12.5	25.0	12.5	50.0	0.0	12.5	25.0	12.5	12.5	25.0	12.5	25.0	12.5	25.0	12.5	25.0	12.5	25.0	12.5	25.0				
	Mean	55.0	207.5	59.2	130.1	93.8	2.7	268.6	204.7	3.6	153.2	90.3	52.1	59.5	3.2	46.1	22.5	4.8	60.4	4.5	71.8	38.2	2.4	58.2	7.3	52.1	2.9	3.8	43.6	21.6				
	S D	56.6	25.9	4.9	16.3	11.7	0.3	33.6	25.6	0.5	19.2	11.3	6.5	7.4	0.4	5.8	2.8	0.6	8.3	0.6	9.0	4.8	0.3	7.3	0.9	6.5	0.4	0.5	5.5	2.7				
	% Fl	3.7	11.2	3.2	9.7	10.5	0.7	10.7	12.4	1.2	10.2	11.3	4.7	8.8	1.8	3.7	1.9	2.9	0.1	1.2	7.4	4.0	0.7	5.8	3.7	6.6	0.9	1.1	5.5	2.1				
	% Fl	100.0	100.0	50.0	50.0	25.0	0.0	100.0	75.0	0.0	50.0	37.5	27.5	62.5	0.0	37.5	37.5	12.5	12.5	5.0	25.0	37.5	0.0	12.5	25.0	12.5	0.0	12.5	12.5	10.0				

Fig.9-A. Effect of horizontal bending at dormant period on shoot growth and flowering at different orientation, whorl, and class of shoots in 29 positions of Granny Smith (Exp.3).

From 8 replications, the lengths of the groups of shoots are classified as follows; < 0.3 cm, 0.3 and 10 cm, and > 10 cm. 29 positions were examined on the 90 cm lateral.

B. Petal fall horizontal bending (October)

(Apple variety - Granny Smith)(29)

--- Less than 50 % sample
 — More than 50 % sample

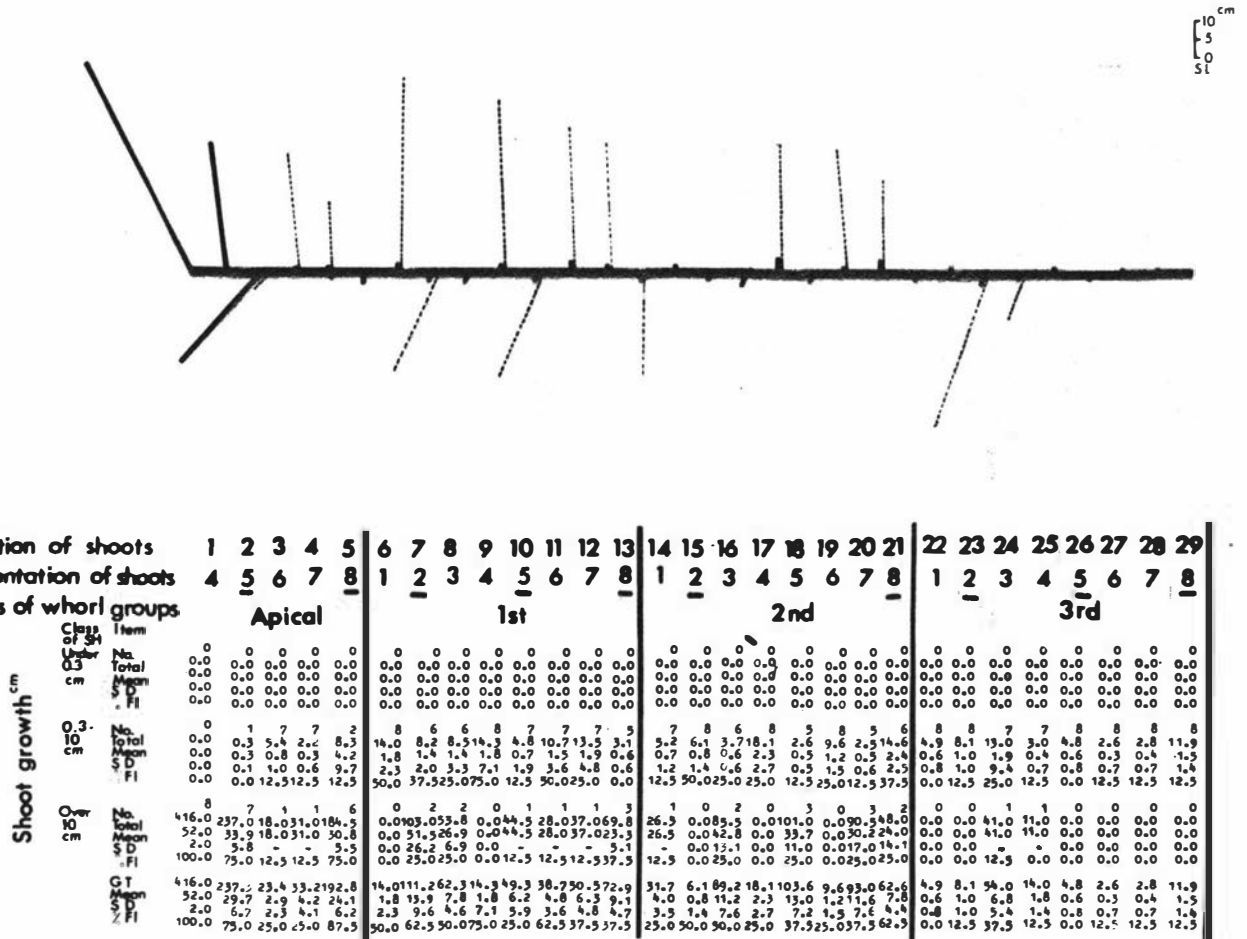


Fig. 9-B. Effect of horizontal bending at petal fall stage on shoot growth and flowering at different orientation, whorl, and class of shoots in 29 positions of Granny Smith (Exp.3).

C. Vertical treatment (Control)

(Apple variety - Granny Smith)(29)

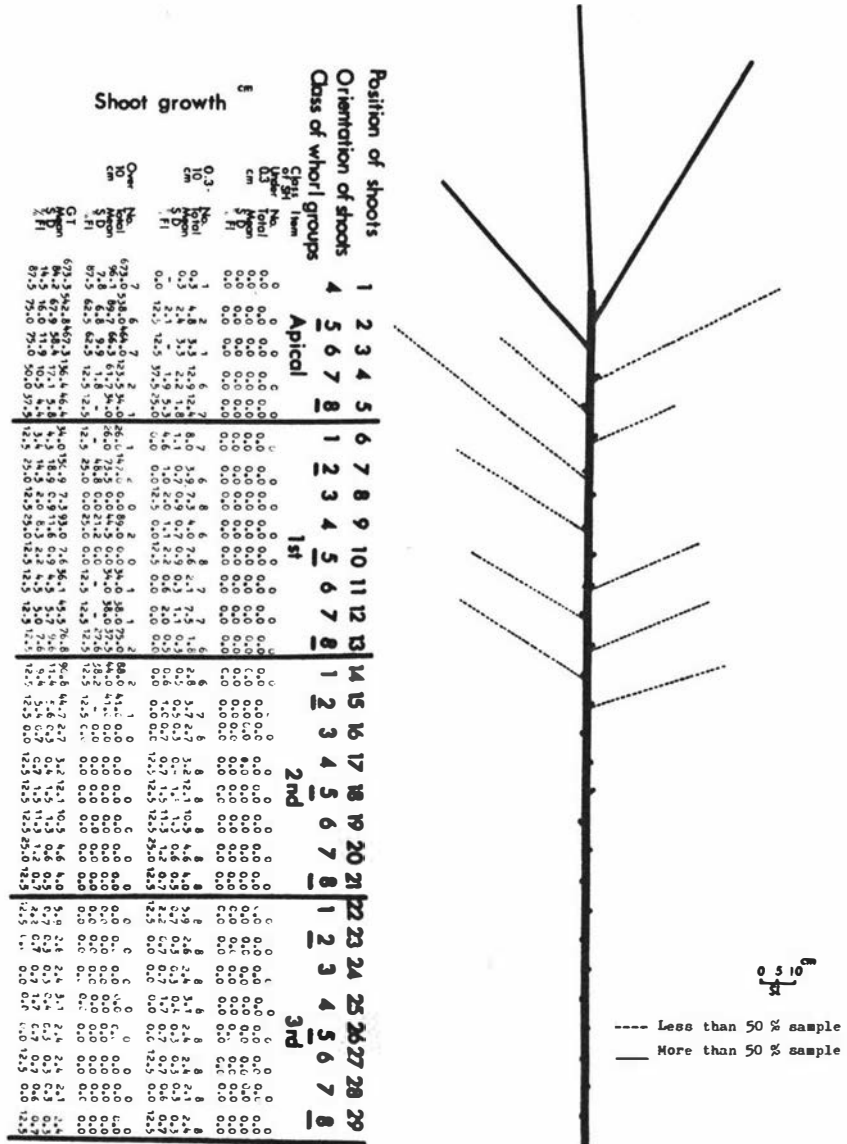


Fig. 9-C. Effect of vertical control on shoot growth and flowering at different orientation, whorl, and class of shoots in 29 positions of Granny Smith (Exp.3).

of the dormant period bending and the controls. Petal fall bending did not bring better flowering than bending at the second cover stage.

4.2.1.2. Effect of positions

The results of the statistical analysis of flowering in the whorl are given in Table 2. Flowering was significantly greater in the first whorl compared with the third whorl (over all treatments). The flowering was markedly increased in the second whorl as compared with the first and third whorls. The bending treatment during the dormant period (T_1) promoted flowering in the third whorl. Flowering in the third whorl was greatly inhibited in the control (T_4) by apical dominance or apical control which also reduced the number of outgrowths. Comparisons of flowering in the whorl between petal fall (T_2) and second cover stage (T_3) showed that there were significant differences in the linear and non-linear response (T_2). The flowering in the third whorl was greatly inhibited at the second cover (T_3), but not inhibited at the petal fall stage (T_2). The greatest effect of the bending treatment on flowering was demonstrated in the second whorl of laterals bent at the second cover stage (T_3). This was much greater than the average of the first and third whorl at petal fall (T_2). The interaction of the bending treatment and whorl in the (dormant horizontal + control and petal fall + second cover) bending treatments (T_1 & T_4 vs T_2 & T_3 x linear response) was reflected in the poor flowering of the third whorl in the second cover bending treatment (T_3).

The effect of orientation within all treatments was to promote flowering on the upper side but inhibit it on the shoots from the lower side of the laterals (Table 2). The effect was more pronounced in the dormant horizontal bending (T_1) relative to the vertical control (T_4). Promotion of flowering on the shoots from the upper side compared with the lower side of the laterals was greater in the petal fall bending (T_2) than in the second cover bending (T_3), and was less in the average of $T_1 + T_4$ than that of $T_2 + T_3$. Flowering on the shoots from the flank of the laterals was intermediate between that of the upper to lower side. Fig.7-A presents percentage of flowering at the lower, flank, and upper shoots from the laterals for each treatment. Interesting results arise from the very steep slope from the lower to the upper orientation of flowering in the dormant horizontal bending (T_1), compared with the zero slope in the vertical control. However, this kind of slope among the orientations did not hold the petal fall (T_2) and second cover stage (T_3), because flowering was similar on the shoots from the lower, flank, and upper sides. The percentage of

flowering on the shoots from the upper side of the horizontal laterals in the second cover stage (T_3) was slightly less than that of the petal fall (T_2) Fig. 7-A), although the difference was not significant.

Within the orientations over all treatments such as 2, 5, and 8 no significant difference was found. However, flowering from the 5 orientation was increased by the dormant horizontal bending treatment.

4.2.2. Experiment 2

4.2.2.1. Effect of treatments

The results of detailed analysis of variance of flowering in five sub-apical whorls are shown in Table 3.

A small 'almost significant' ($p > 0.1$) effect was detected on flowering in the dormant period bending in comparison with the control, excluding the apical whorl. Within the later growing-stage-bending treatments, the petal fall bending promoted significantly more flowering ($p > 0.01$) than in the second cover stage. The average of flowering in the bending treatments during the growing season (T_2 and T_3) was greater than that of either the dormant period bending (T_1) or the control (T_4).

4.2.2.2. Effect of positions

The average percentages of flowering at each whorl for the different treatments are shown in Fig. 7-B. The effect of whorl groups on flowering taken over all treatments was in the form of a quadratic response, with the means of the intermediate whorl group being lower than the first and fifth sub-apical-whorl-group means.

In the interaction component of bending treatments with the whorl groups, a significant quadratic response was detected only between the dormant period bending and the control (Table 3). The effect of whorl on flowering was different in the two extreme treatments. The average flowering at the first and 5th whorl in the vertical treatment exceeded the average flowering at the same whorls in the pendulous bending during the dormant period (T_1). The negative response of flowering in the 5th whorl was associated with the acceleration of shoot growth in the dormant bending treatment. These phenomena are shown diagrammatically in Fig. 8-A in which many gourmand shoots are shown as solid lines at the 5th whorl. However, the over all promotion of flowering by bending was indicated in the dormant pendulous bending (T_1) ($p > 0.1$) compared with the vertical control (T_4). Both had a similar quadratic component but whereas the pendulous bending in the dormant period generated a positive linear component in

TABLE 4. Effect of horizontal bending at various times on shoot length¹ and flowering² measured at the end of the growing season and at blossom: Experiment 3 Grassy Smith
Analysis of variance for all whorls other than the apical whorl

Source of variation	Degrees of Freedom	Shoot		Flowering	
		Mean Square	F Ratio	Mean Square	F Ratio
Treatments	2	10763.6		2,5759	28,1416***
Control vs Tented (T_1 & T_2 vs T_3)	1	21527.1	6.73*	4,9348	53,9333***
Dormant vs Petal fall bending (T_1 vs T_2)	1	0.1	<0.01 ⁻	0.2170	2,3716 ⁻
Replications within Treatments	21	3200.6		0.0915	
Positions	23	6153.6		1,0761	11,2607***
Whorls	2	38727.3	4.34*	2,9455	32,1794***
Linear response ($W_3 - W_1$)	1	77435.4		5,6208	61,4295***
Non-linear response ($2W_2 - W_1 - W_3$)	1	18.3		0.2702	2,9530*
Orientations	7	4521.5	3.19*	0.4426	4,8553**
Upper vs Lower ($O_{2,5,8}$ vs $O_{1,4,6}$)	1	22625.6		15,96**	1,0046
Flank vs Upper & Lower ($O_{3,7}$ vs $O_{1,2,4,5,6,8}$)	1	1947.4		1.37 ⁻	0.0101
Within Upper: Linear ($O_3 - O_7$)	1	3334.2		2.35 ⁻	0.0138
Within Upper: Non-linear ($2O_5 - O_3 - O_7$)	1	2708.0		1.91 ⁻	0.3259
Remainder	3	345.1		0.24 ⁻	0.5806
Whorls x Orientations	14	2316.3	1.63 ⁻	0.1063	1,1614 ⁻
Treatments x Positions	46	2084.7		0.09678	
Treatments x Whorls	4	1635.6	1.15 ⁻	0.1004	1,0967 ⁻
Treated vs Control x Linear response	1	79.0		0.06 ⁻	0.1216
x Non-linear response	1	379.1		0.27 ⁻	0.0087
Dormant vs Petal fall bending x Linear response	1	336.9		0.24 ⁻	0.0824
x Non-linear response	1	5747.2		4.05*	0.1889
Treatments x Orientations	14	3546.6	2.50*	0.2843	3,1058**
Treated vs Control x Upper vs Lower	1	10374.1		7.32*	0.6479
x Flank vs Upper & Lower	1	132.9		0.09 ⁻	0.1912
x Within Upper: Linear	1	3144.7		2.22 ⁻	0.0052
x Within Upper: Non-linear	1	153.3		0.11 ⁻	0.2707
Dormant vs Petal fall bending x Upper vs Lower	1	7318.6		5.16*	1.1860
x Flank vs Upper & Lower	1	284.1		0.20 ⁻	0.0014
x Within Upper: Linear	1	11862.7		8.37**	0.0040
x Within Upper: Non-linear	1	819.5		0.58 ⁻	0.0067
Remainder	6	2594.1		1.83 ⁻	0.2611
Treatments x Whorls x Orientations	28	1418.0		0.0915	
Positions x Replications within Treatments	483	465.0			

¹Shoot length in cm. transformed by taking logarithms: $100 \log(L - 1.1)$. ²Flowering transformed by $\sqrt{X + 0.5}$ where X = No. of flowering.
*** = $P < 0.001$, ** = $0.01 > P > 0.001$, * = $0.05 > P > 0.01$, + = $0.1 > P > 0.05$, - = $P > 0.1$

Abbreviations:

T_1 = Horizontal bending of the lateral at the dormant period.
 T_2 = Horizontal bending of the lateral at the petal fall stage.
 T_3 = Control treatment: normal vertical lateral.
 W_1 = The first whorl.
 W_2 = The second whorl.
 W_3 = The third whorl.
 O = Orientation. Numerical numbers from 1 to 8 indicate the orientation of buds in the whorl.
vs = Versus.

the 2nd, 3rd, and 4th whorls, a negative linear component was seen in the 2nd, and 3rd whorl of the control (T_4), indicating an interaction between the pendulous treatment and whorl. The interaction suggests that poor potential bud qualities were overcome to some extent by the pendulous treatment.

The effect of orientation on flowering in Exp. 2 was essentially similar to that observed in Experiment 1. In over all treatments flowering on the shoots from the upper side of the pendulous laterals was promoted significantly more than those on the lower side. Flowering on the flank shoots was intermediate. Flowering on the shoots from the lower, flank, and upper side of laterals for each treatment is shown in Fig. 7-B. In the dormant pendulous bending treatment (T_1) there was a strong independent relationship of flowering on orientation, in which flowering was insensitive to orientation as indicated in Exp. 1 and 2. Flowering was greatly decreased in the upper side of laterals bent horizontally at the second cover (T_3), but was greatly increased in the petal fall (T_2) bending treatment.

Flowering was promoted in the 5 orientation relative to the mean of the 8 + 2 orientations, and there was no difference between the 8 and 2 orientations. The significant components detected in the subdivision of the treatments (T_1 vs T_4 ; T_2 vs T_3 ; T_1 & T_4 vs T_2 & T_3) by orientation were consistent with greater promotion of flowering on the shoots from the upper side of the laterals, i.e., earlier pendulous bending increased flowering.

4.2.3. Experiment 3

4.2.3.1. Effect of treatments

The analysis of variance in Table 4 exhibits the significant effect of horizontal bending on flowering. Experiment 3 on Granny Smith was carried out in a similar way to Experiment 1 on Red Delicious except the second cover bending treatment was omitted.

The horizontal bending treatment either at the dormant (T_1) or petal fall stage (T_2) significantly increased flowering relative to the vertical control (T_3). There was no significant difference between the dormant period (T_1) and petal fall bending (T_2).

4.2.3.2. Effect of positions

The average percentages of flowering at each whorl for the different treatments are shown in Fig. 7-C. The flowering in the whorl decreased from the first to the third sub-apical whorl in all treatments, as shown in the shoot growth. The effect of whorl over all treatments in flowering was essentially linear. However, the minor effect of whorl on

flowering showed a quadratic response. There was probably less flower in the first whorl of the control. In Granny Smith there was not a clearly detectable interaction between bending treatment and whorl. Therefore, the pattern of flowering is different from Red Delicious in which the flowering at the third whorl was promoted by the horizontal bending treatment.

The consistent effect of the upper-lower shoot orientation on flowering found in both experiment 1 and 2, was also obtained in this experiment. The effect was the greatest in the dormant period, intermediate at the petal fall, and absent in the vertical (Control) laterals (T_3). The flank shoot orientation showed no significant difference in flowering relative to the average of upper-lower orientations and indicated that flowering on the shoots from the flank of the horizontal laterals was due to an intermediate effect of the bending. Flowering at the lower, flank, and upper sides of the laterals for each treatment is shown in Fig. 7-C. The steep slope of the linear regression line in T_1 compared with the zero slope in the control (T_3) was also found in Experiment 1 and 2. Flowering on the upper side of the laterals bent horizontally during the dormant period was increased. Flowering was inhibited on the lower side. There was no variation in numbers of flower in the control (T_3) because of no orientation such as the upper, flank, and lower of the laterals. The interaction between the treatments and orientation indicated significantly increased flowering on the upper side of laterals in T_1 vs T_3 (control) and T_1 vs T_2 . In T_1 and T_2 , the inhibition of flowering in the lower shoots on the laterals during the dormant period bending (T_1) and the promotion of flowering on the lower shoots in the petal fall bending (T_2) are interacting with time of bending to bring about an effect of bending on flowering.

Flowering was promoted at the 5 orientation but there were no differences between 8 and 2 orientation. A similar result is reported above for Experiment 2.

4.3. Relative growth relationship (between leaf area and shoot growth)

4.3.1. Leaf area

The analyses of variance for means of total leaf area and number of leaves are shown in Table 1 and 5. There were no significant differences among treatments for mean total leaf area per shoot and leaf number (Table 1). However, total leaf area among the treatments differed significantly (Table 5). This effect on total leaf area was probably due to many dormant buds on the lower side which had no leaves. Especially the mean of total leaf area was greatly reduced, but total leaf area was the greatest in the dormant period horizontal bending.

Table 5. Studies on shoot growth in relation to leaf area in the vertical and horizontal treatments^a

Items	Horizontal bending			
	Dormant period	Petal fall	Second cover	Control
Initial volume of laterals (cm ³)	<u>83.69</u>	<u>70.51</u>	<u>75.36</u>	80.19
Increment of old lateral growth (cm ³)	<u>117.79</u>	<u>92.61</u>	<u>99.02</u>	236.40
New shoot growth (cm ³)	101.78	<u>62.80</u>	<u>55.04</u>	156.27

Total increment laterals and new shoots (cm ³) (TISV)	219.57	<u>155.41</u>	<u>154.06</u>	392.67

Total leaf area (cm ²) (TLA)	<u>4226.64</u>	<u>3269.07</u>	<u>3518.65</u>	<u>4079.67</u>

Ratio (TLA/TISV)	19.80	22.82	24.40	10.83

^a

Means within a solid underline are not significantly different at the 5% level, and within a dotted underline, at the 1% level by Duncan's multiple range test.

The Log transformed data were used in the case of the mean of total leaf area for the analyses of variance. Fig.10-A,B,C, and D exhibit that there were 25.5% of buds or dards which had no leaves in the dormant period horizontal bending, 13.5% in the petal fall bending, 17.6% in the second cover bending, and 16.9% in the vertical treatment. In the vertical treatment, 40% of total leaf area originated from the terminal shoot and 30% from the second shoot below the terminal one, 20% from the third shoot below the terminal, and the remainder from the rest of the shoots. In the case of the dormant period bending, 45% of total leaf area originated from the first to the third terminals, and the rest of the leaf area originated from 26 positions of laterals. Horizontal bending at the second cover produced a similar leaf distribution. It is interesting to observe that the major percentage of total leaf area originated from the apical whorl in all the treatments. The distribution of leaf area in the laterals at each shoot position is shown graphically in Fig.10-A,B,C, and D.

Thus from the conditions mentioned above, the largest total leaf area was obtained from the horizontal bending during the dormant period, compared with the horizontal bending treatment during the growing season. Bending during the dormant period did not produce significantly more leaves than the vertical treatment. The total leaf area in the vertical treatment was significantly greater than at the petal fall and second cover. The effect was not highly significant at the second cover. There was no highly significant difference between the petal fall and second cover. The total leaf area was highly correlated with the shoot length in each treatment as indicated in Fig.11.

4.3.2. Studies of ratios of total leaf area to total increment of shoot volume (R)

There were no differences in the initial volume of laterals among the treatments at the beginning of experiments (Table 5). The increment of shoot growth was significant at the 5% level (Duncan's multiple range test) between the dormant horizontal bending and the late season bendings but not at the 1% level. The dormant horizontal bending could not obtain as much new increment as that of the vertical. Horizontal bending after shoot growth started did not produce a difference in the volume of new shoot growth. Consequently the total increment of laterals and new shoots was greater in the vertical treatment ($p < 0.01$), next in the dormant horizontal bending, with no difference between the petal fall and second cover bending.

The ratios of total leaf area to total increment of shoot volume were calculated (Table 5). This ratio was greatly reduced

A. Dormant period horizontal bending (August)

(Apple variety - Red Delicious)(29)

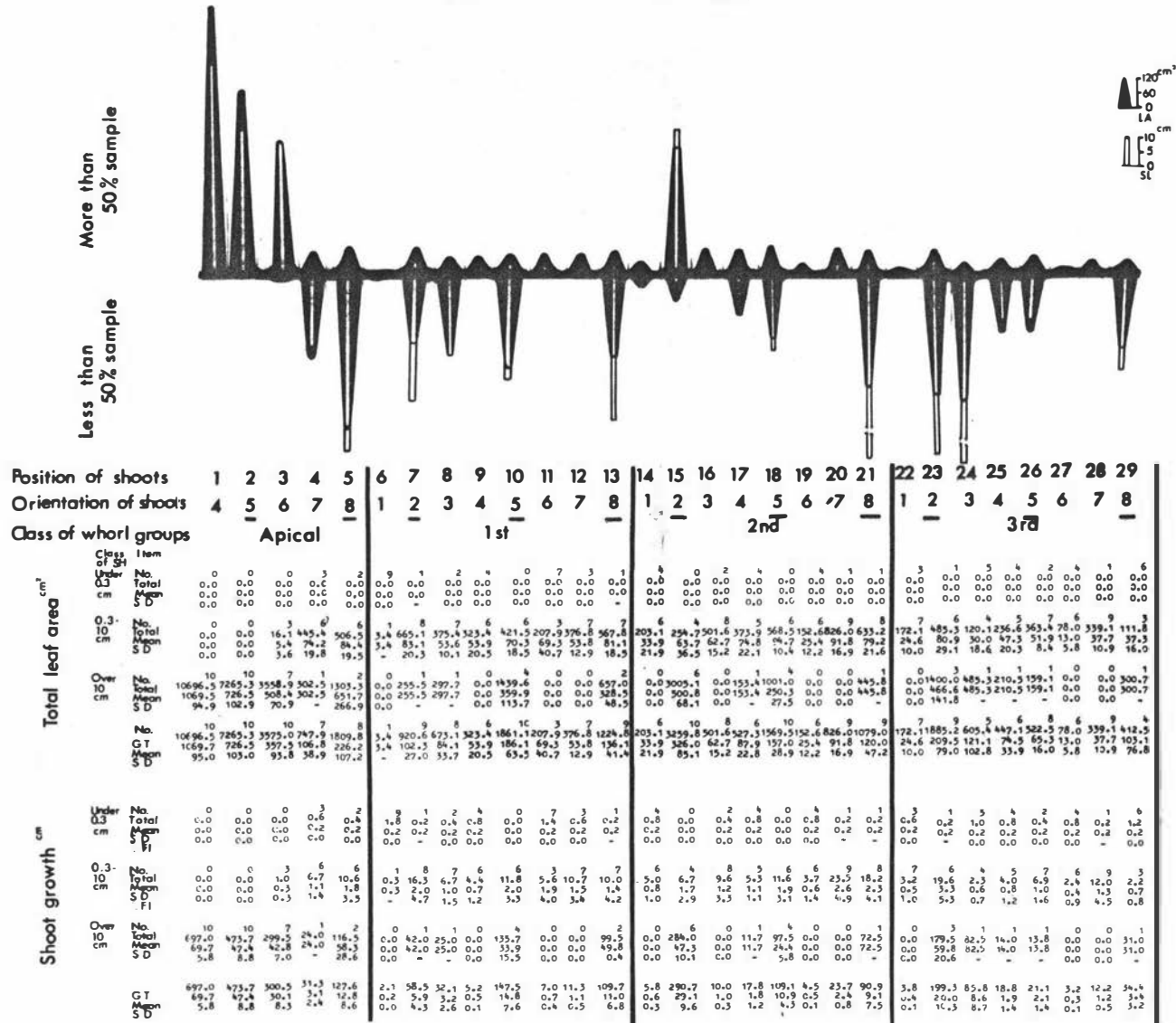


Fig.10-A. Effect of horizontal bending in dormant period on leaf area at different orientation, whorl, and class of shoots in 29 positions of Red Delicious (Exp.1).

From 10 replications, the leaf areas are derived from the groups of shoots as follows: <0.3 cm, 0.3-10 cm, and >10 cm. Leaf areas were only measured in mid-summer. The black areas of peaks correspond to the height of diagram and indicate a mean of leaf area at the position of the shoots. The lengths are represented by a white bar at each position at that time.

B. Petal fall horizontal bending (October)

(Apple variety - Red Delicious)(29)

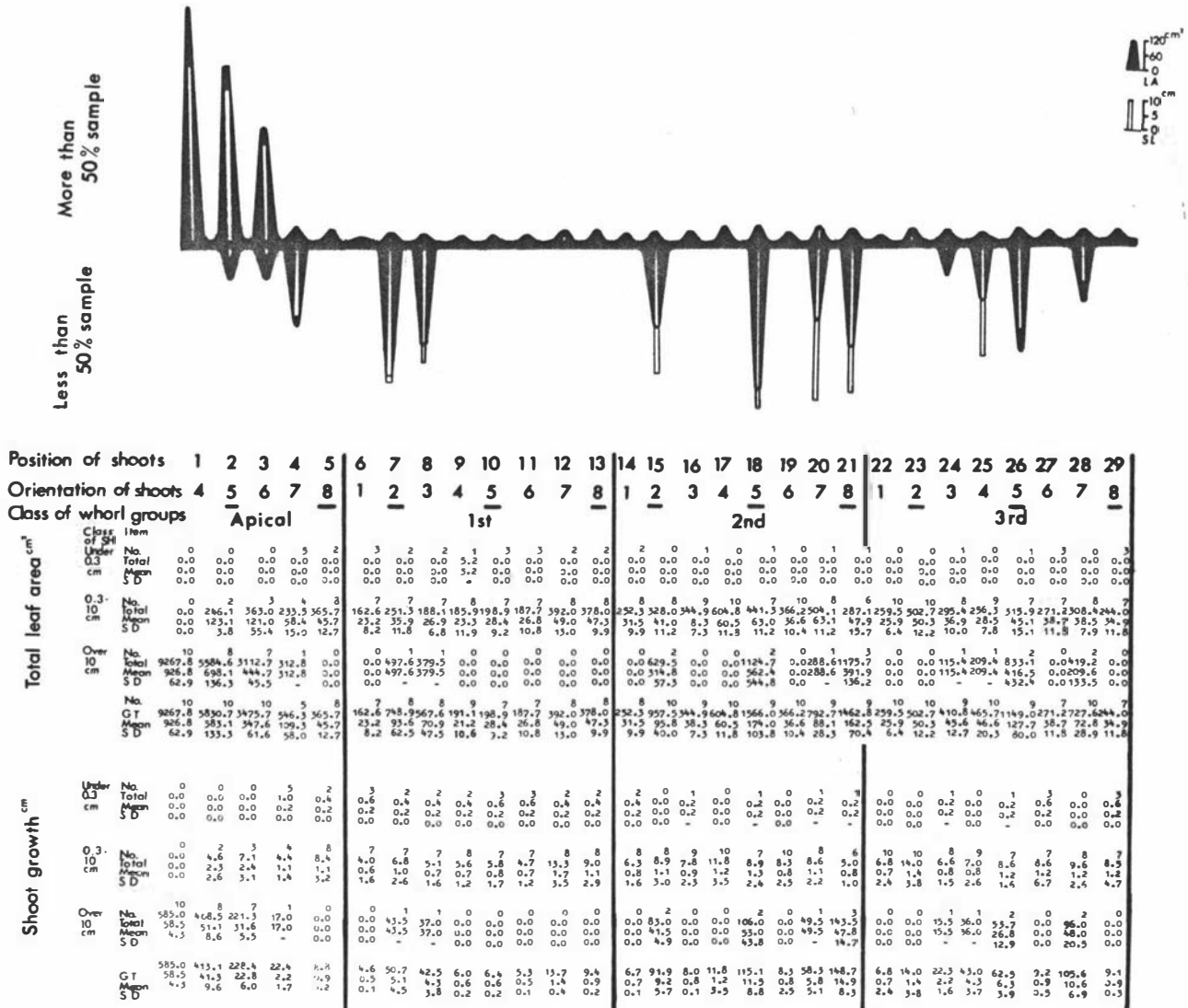


Fig.10-B. Effect of horizontal bending at petal fall period on leaf area at different orientation, whorl, and class of shoots in 29 positions of Red Delicious (Exp.1).

C. Second cover horizontal bending (November)

(Apple variety - Red Delicious)(29)

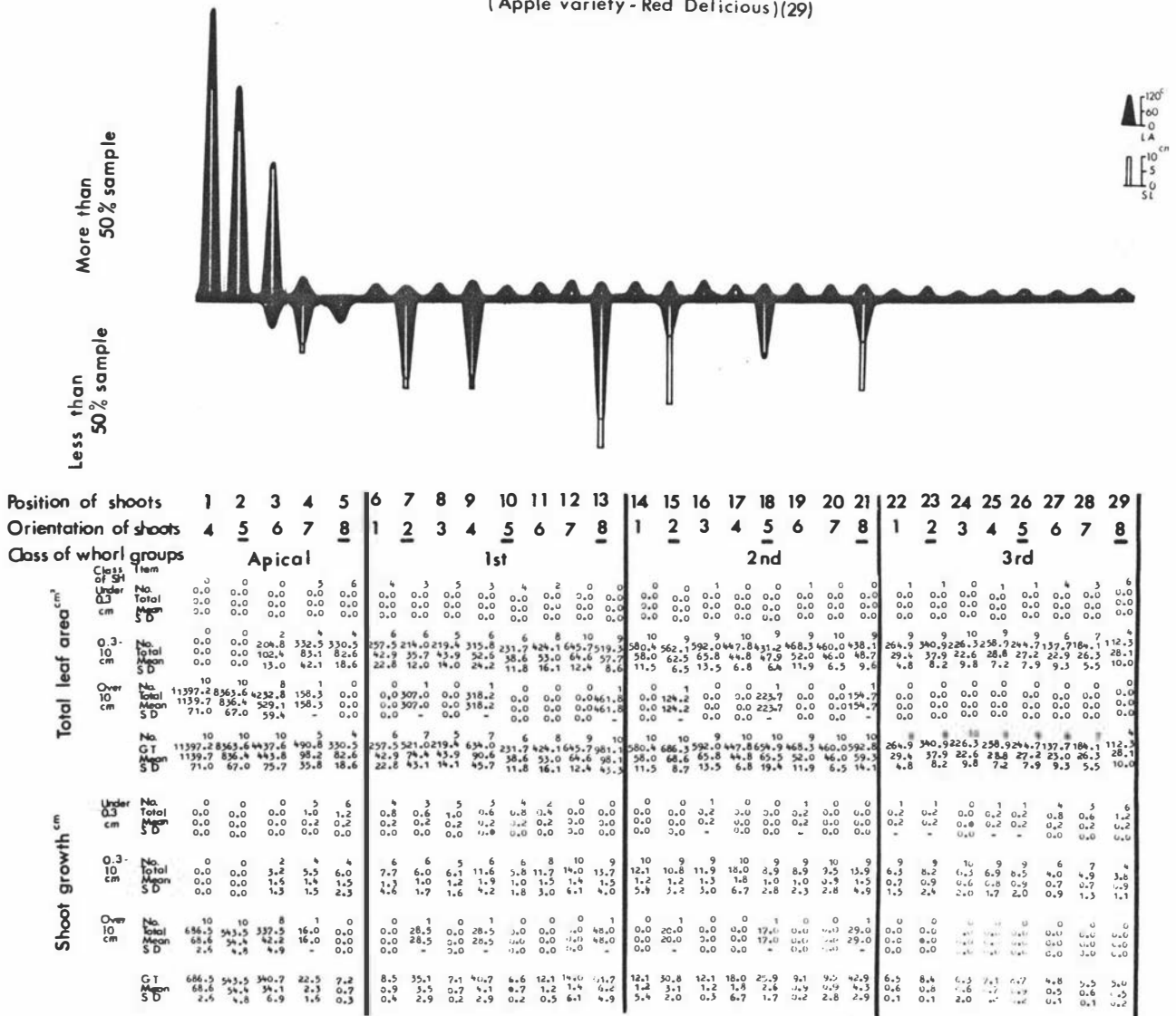


Fig.10-C. Effect of horizontal bending at second cover stage on leaf area at different orientation, whorl, and class of shoots in 29 positions of Red Delicious (Exp.1).

D. Vertical treatment (Control) (Apple variety - Red Delicious)(29)

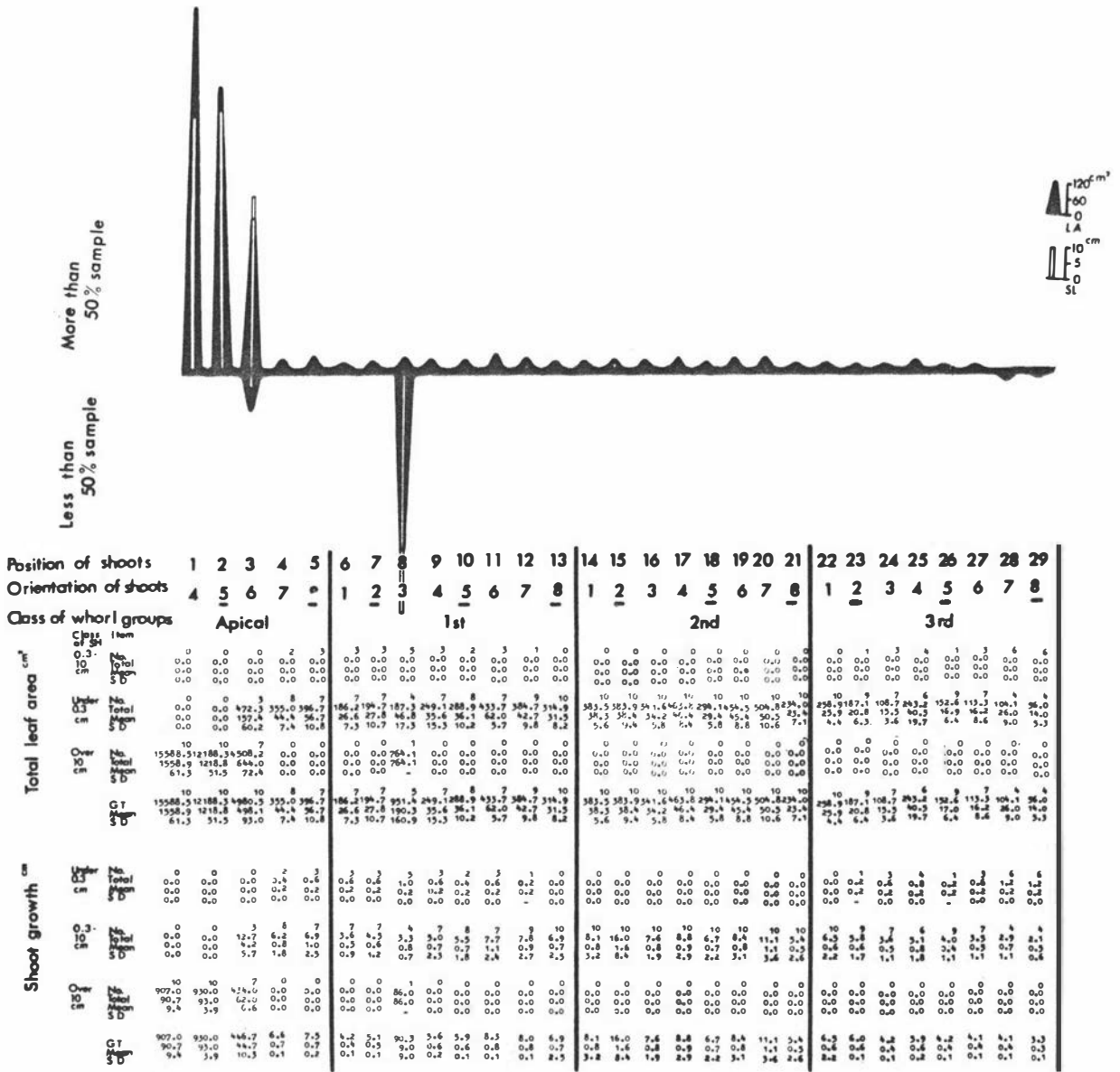


Fig. 10-D. Effect of vertical control on leaf area at different orientation, whorl, and class of shoots in 29 positions of Red Delicious (Exp.1).

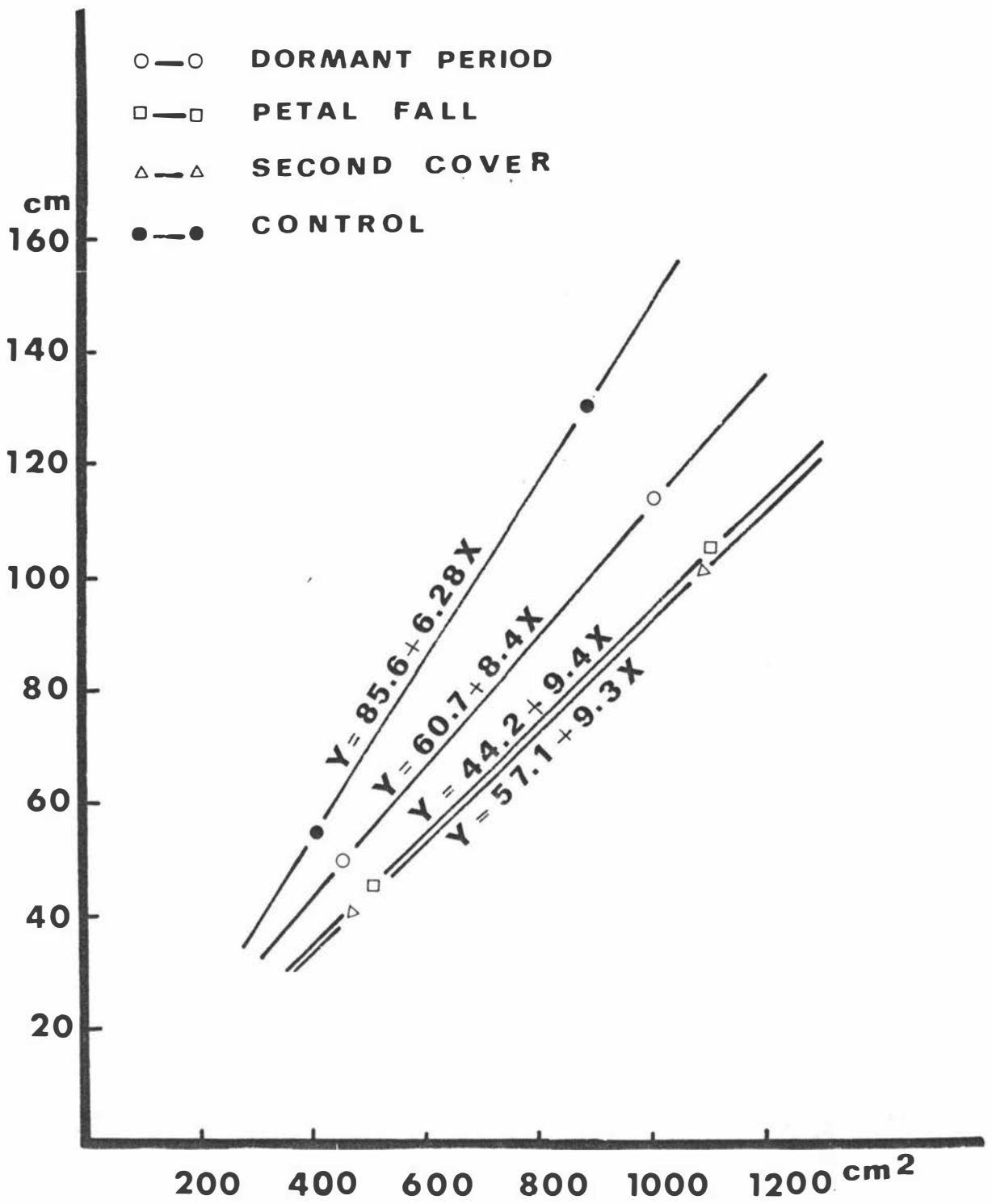


Fig.11. Relationship of the seasonal horizontal treatments on the shoot length and leaf area.

in the vertical lateral, although there were no significant differences among the horizontal bending treatments, compared with the petal fall and second cover (T_2 & T_3) bending treatments.

CHAPTER 5

Discussion

5.1. Shoot growth

5.1.1. Total shoot length

The relative shoot growth at each position on the laterals is illustrated by the empirical polynomial equation in Fig.5 and diagrams of shoot growth on the laterals (Fig.6,8,and 9). The shoots on each lateral were classified according to length, but there were too few samples for appropriate statistical analysis.

The average shoot length in Experiment 1 and 3 using 90 cm laterals (29 positions) was greater in the dormant horizontal bending treatment than in the vertical control. On the other hand in Experiment 2 using the longer 150 cm laterals (45 positions), there were no significant differences in average shoot growth between all the treatments. The results obtained in Experiment 2 are the only ones similar to those of Mullins (1964) who experimented on one year old whole trees grown horizontally, vertically, and inverted in pots, and Mika (1969 a) who experimented with one year old laterals of two-year-old apple trees bent horizontally, vertically, and invertedly under field conditions.

The discrepancy between Experiments 1 and 2 in the average shoot length on the horizontal bending treatment at the different seasons is probably due to the use of 90 and 150 cm laterals. The 150 cm lateral in Experiment 2 did have buds with their potential ability to grow out from the 90 to 150 cm region. There are more shoots over 10 cm long from the half length of the 150 cm laterals in Exp.2, than in Exp.1 in the vertical controls (Champagnat, 1954 b and Maggs, 1963 a). The longer shoots are grown for a longer period from the middle part of the laterals to the terminal region enabling better buds to be sprouted for the next season.

5.1.2. Orientation effect

The effects of orientation and seasonal bending on shoot growth of the lower, flank, and upper side of laterals are shown in Fig.7-A,B,& C.

The greatest increase in shoot growth from the lower to the upper side was after the dormant period bending, intermediate after the petal fall bending, least at the second cover bending, and absent in the control. This pattern of shoot growth suggests that the interaction of shoot growth and apical dorminance, or apical control and

orientation is influenced by the seasonal bending.

The interaction component in the shoot growth results is as follow: the first component is the bud orientation such as lower, flank, and upper buds in the horizontal laterals due to new orientation. The second component is the time of bending. In the laterals bent horizontally at the dormant period, cytokinins from the root could be supplied mainly to the buds on the upper side (Wareing, 1970) rather than the buds on the lower side when the root actively commences in the early spring (Luckwill and Whyte, 1968). In addition, perhaps another reason could be considered i.e., that the accumulation of inhibitors, either auxins or ABA, are counteracted by cytokinins produced from roots so that the buds on the lower side could not have available cytokinins during the dormant period bending treatment. Consequently the horizontal lateral could bear more terminal buds as a result of bending from the vertical to the horizontal position, whereas the vertical lateral has only a few terminal buds. The newly orientated upper buds become the physiologically terminal buds (Champagnat, 1954) on the horizontal lateral. Thus shoot outgrowth from the upper side of horizontal depends on the inherent properties (Champagnat, 1954 b).

Severe winter pruning induces longer and strong leader shoots from the remaining highest bud on the pruned vertical (Maggs, 1963; Krivko, Grigorov, Lepesheva, 1972). Synthetic cytokinins induce outgrowth of buds by releasing them from winter dormancy and a correlative inhibition (Pieniazek, 1964; Pieniazek and Jankiewicz, 1965, 1966; Poll, 1968 a; Williams and Stahly, 1968; Pieniazek and Saniewski, 1970). The dormant period horizontal treatment might have a similar effect to winter pruning by inducing the vigorous shoots to supply cytokinins (Jones 1965, 1973; Luckwill and Whyte, 1968) to either the terminal buds on the vertical lateral or the upper side of buds on the horizontal lateral. This has led to have the sporadic distribution of gourmand shoots along the upper side of the horizontal (Fig.6-A,B,& C, 8-A,B,& C and 9-A & B). This explains why bud conditions which obtain positively inherent properties for shoot outgrowth in the previous year can be stimulated by additional horizontal bending in the current year. The higher percentage of gourmand shoots produced at the 15th upper bud orientation position is indicated with a solid line in Fig.6-A.

The great decrease in shoot length within the 1st whorl group (Fig. 7-A) and the slight increase in shoot length within the 2nd whorl group in 90 cm laterals of Red Delicious agreed with the

two centre pattern of shoot growth established by Mullins (1964). However, the degree of inhibition of shoot growth on the lower side of the laterals bent during the growing season, is not the same as the dormant period bending, because the bud outgrowth along these laterals occurred after bud-burst on the vertical laterals, following the phenomena of apical control (Champagnat, 1954 b; Brown, McAlpine, and Kormanik, 1967).

The decrease of slope in the linear component from the dormant period bending to the second cover stage occurs because there was number of shoots over 10 cm in length on the upper side of buds on the horizontal laterals. This could explain why the inhibitors produced from the expanding terminal leaves and from expanded leaves of spurs are counteracted by cytokinins either synthesised in the buds (Lee, Kesser, and Thimann, 1974) or transported from roots together with gibberellins, consequently reducing the secondary flush of terminated buds. However, the striking phenomenon of the second cover stage bending is the induction of gourmand shoots, although a few gourmand shoots were present on the upper side of buds **after** bending. Those shoots grow continuously until the later in the season (Mullins, 1964). However, the flank shoots are not induced by the second flush of growth after bending in the growing season, probably because they are supplied with less cytokinins from the roots relative to the shoots on the upper side of the horizontal. The shoot growth from the flank buds is always intermediate at the dormant period bending.

The angle of orientation in the shoot growth appears to have some physiological consequences. The angle of apical meristem to the horizontal appears to affect the inhibition of shoot growth. Therefore, to discuss the gravitational effects on shoot growth both the original and final orientation of shoots and buds must be considered. The difference between the orientation of the apical meristem of the shoot and that of the shoot itself must be considered. The growth habit of a shoot trained in the horizontal direction at 90° to the current shoot which developed from the buds on the vertical laterals reduces shoot extension compared with the shoot trained vertically (McKenzie, 1956; Wareing and Nasr, 1961; Koltunov, 1971; Park, 1971 in unpublished data). In the present studies, the upper, flank, and lower side of bud orientation could be considered as 0° , 90° , and 180° to the apical meristems at bud burst time.

The present experiments suggest that the linear relationship in the shoot length from 0° ; 90° and 180° could be explained by the sine rule on gravity effect (Onaka, 1949; Larsen, 1962; Lundegårdh, 1962;

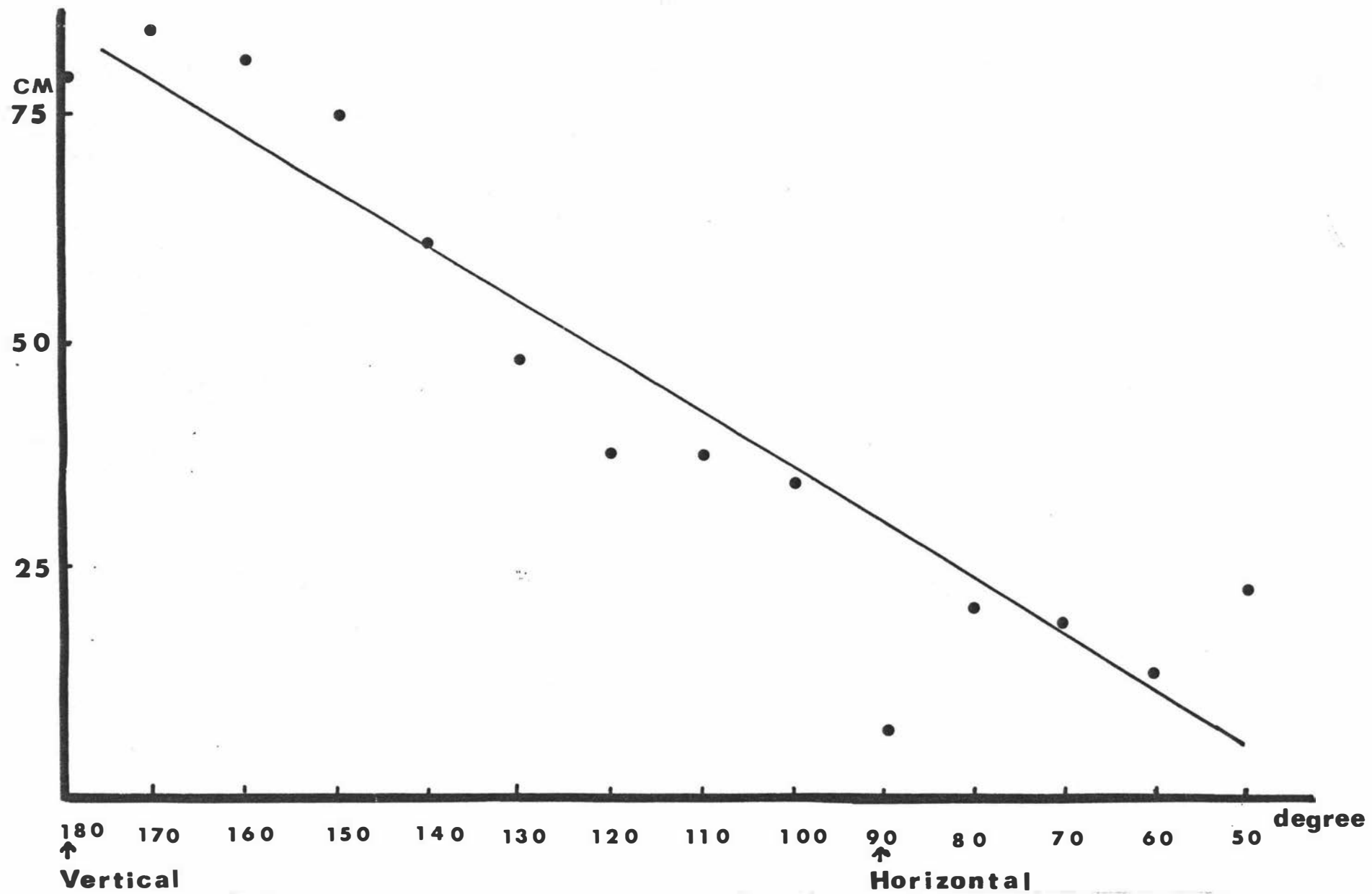


Fig.12. Shoot growth at the different angle (After Edminster, 1917).

Wardrop, 1964; Robards, 1966). Data of Smith and Wareing (1964) indicate that the percentages of bud-break on the lower side buds either from the concave and convex lateral of the arched willow or from the 0° to 180° of the inclined cuttings of willows, were generally proportional to the orientation angle (an angle of bud insetion to the horizontal) and this was evident in the short period of 12 days. When the apple shoot growth from the vertical to the invertical direction (from 180° to 0°) is plotted (See Fig. 12), the shoot length indicates a similar linear relation to the angle as the sine rule (Edminster, 1917). Bud-break also exhibits a similar result in apple shoots (Edminster, 1917).

The mechanisms which cause shoot length to be greater in laterals with vertical than invertical apical meristems is far from clear. The angle of the meristems to gravity (Polarity of cell division to gravity) may help either to determine the direction of shoot growth or to regulate the rate of cell division, by mediating the metabolism via balance of plant hormones in the apical meristem.

The association of apical meristem orientation with the habit of plagiotropic and crthotropic growth must be influenced by the apical meristems. A profound effect of bud position on growth habit after propagation is shown in Araucaria excelasa (Vöchting, 1904; Robbins, 1964; Hartmann and Kester, 1968), Sequoia sempervirens (Robbins, 1964), Hydrocarpus spp., Coffea arabica (Hartmann and Kester, 1968), and in Taxus baccata.

The application of synthetic gibberellic acids can cause diageotropic or plagiotropic branches to become erect in several species (Rufelt, 1961; Wilkins, 1966). On the other hand, a growth retardant, counteracting gibberellin biosynthesis, induced the reduction of negative geotropic growth of shoots (Cathey, 1964). The runner of the peanut plant, sprayed with GA assumes an erect habit of growth from the diageotropic form, whereas the growth retardants such as Alar and reversed growth habit modified by the spray of GA (Halevy, Ashri, Ben-Tal, 1969). The content of endogenous gibberellins varied little between the erect and runner type of peanuts, but antagonism between the native gibberelline and inhibition is substantially higher in runner plants and the Rf of one of these inhibitions corresponds to that of abscisic acid (Halevy, Ashri, Ben-Tal, 1969). Therefore, Halevy, Ashri and Ben-Tal (1969) suggest that there is a possibility the geotropic growth of shoots can be regulated by antagonistic effects arising between endogenous gibberellins and endogenous inhibitors as controlled by genes and plasmatic factors. Woolley and Wareing (1972 a)

showed that the ratio of plant hormones at the bud insertion is primarily important in influencing the habit of shoot growth; i.e., a high ratio of gibberellins to cytokinins induced a diageotropic habit with stolons and a low ratio an orthotropic habit with leafy shoots.

The habits of branch growth, which may be correlated with the shoot growth at the different bud positions as well as the orientation angle of the shoot is known to be controlled by plant hormones. A wide crotch angle can be induced following the application on decapitation with a paste containing 1% IAA, 0.15% 2,4-D (Jankiewicz, 1970), a spray of TIBA at 7.5- 12.5 cm long (Bukovac, 1968; Poll, 1968 b), a spray of Maleic hydrazide (MH) at 23-29 cm long (Jankiewicz, 1960), and the treatment with cytokinins BA and BAP with GA_4 and GA_7 of the dormant buds (Williams and Billingsley, 1970). All treatments with the above growth substances controlled the balance of endogenous plant hormones in the plant tissues, thereby inducing a wide branch crotch angle. Although the direction of shoot growth did not alter permanently, some shoots tended to be of negative geotropic growth during the season. Therefore, the angle of the apical meristem at the tip of shoots appears to be affected by the production of plant hormones. In general, the polarity of apical meristems seems to be governed by a gravitational effect.

5.1.3. Whorl effect

The well developed shoot growth revealed at the second whorl regardless of the treatments is embodied in the quadratic relationship (Table 3) illustrated for Red Delicious in Exp.1. At the first and third whorl in Exp.1 the shoot growth was poorer even at the dormant horizontal bending. This pattern of shoot growth is more or less similar to the 'two centre pattern' of shoot growth as described by Mullins (1964), although the vertical treatment as control did not show it, it did show an extreme acrotony type of shoot growth (Champagnat, 1954 b). The poor shoot growth at the fourth whorl of Red Delicious in Exp.2 coincides with that of the first whorl in Exp.1. Developmentally the positions of the fourth whorl in Exp.2 are morphologically and chronologically the same as the first whorl in Exp.1. The induction of shoots over 10 cm in length on the laterals is shown in the vertical 150 cm length of the control in Exp.2 (Fig.8-D). Shoot growth among three treatments of vertical laterals Exp.1,2 and 3 (Fig 6-D, 8-D and 9-C) differs. Many shoots over 10 cm are sprouted from both the middle and apical parts of the laterals in Exp.2 and 3 but only three shoots from the apical part in Exp.1 (Barlow and Hancock,

1962; Maggs, 1963; Mullins, 1964). This pattern of shoot growth appears to follow the concept of 'inherent' properties (Proprietes fixeas, Champagnat, 1954 b).

Champagnat (1954 b) states that apical dominance in correlative growth is different in woody plants from that of herbaceous plants in the following ways: (1) the quality of buds with 'inherent' properties on the one-year-old shoot or lateral, these properties having been acquired during the phase of shoot development preconditioning phenomenon, and (2) the inhibition mechanism by which some plant hormones in the tissues prevent bud growth. These substances are transported either in an acropetal or basipetal direction, resulting from the action of the apical groups on the organs of the basal parts or in a more general way, or as a combined influence of these parts.

These 'inherent' properties could be considered as the 'potential bud qualities' acquired during the process of the developmental phase, and preconditioned by a gradient of several plant hormones. Thus growth and development processes in the sequence from seed germination to a seedling (at the juvenile phase) or from a bud on the scion wood to a nursery tree (at the clonal juvenile phase) (Park, 1969) are analogous to shoot growth from bud burst on the laterals during the current growing season. This analogy covers the gradient phenomenon in development or a process in the developmental phase. These issues interacting to acquire certain physiological, biochemical, and morphological properties (Gregory and Veale, 1957) in the buds by a gradient of several plant hormones at that particular bud position. These properties are determined by the proportions of IAA, Gibberellins, and Abscisic acid, to cytokinins; or a combination of each other at the stage of differentiation of the leaf primordia. For example a high ratio of IAA/CK + GA/CK or GA/CK, IAA/CK, or ABA/CK may induce abnormal differentiation which induces a juvenile leaf in the case of a high ratio of IAA/CK + GA/CK and an arrested leaf bud with a high ratio of IAA/CK or ABA/CK (Fig.2). Such a bud may require a higher content of cytokinins when the bud is commencing to burst and grow subsequently. In fact, these potential bud qualities at the different positions recall a problem of phase changes in the meristematic cells and within the terminology of tophophysis (Molish, 1915), Cyclophysis (Seelinger, 1924), and periphysis (Busengen and Much, 1919) (Briefly reviewed in Juvenility by Schaffalitzky de Muckadell, 1959 and Park, 1969).

The potential bud qualities at the different positions on the one year old shoot, or on the current growing shoots, are

documented for the flower bud initiation at the middle part of the current apple shoots (Zeller, 1960 a; Feucht, 1963) in plum (Hassib, 1967), in the morphological and structural differences in the three developmental phases (slow, rapid and slow) in the long apple shoots (Mazurowa, 1971), in the centres of the first flush growth zone at the nodes 10-11 and 13-14 and in the second flush centre at the nodes 21-32 (Crabbe, 1972), and 'Two centre pattern' of shoot growth (Mullins, 1964). Veale (1955) found a changing gradient of bud growth potential along the stems from the basal to the apical part, when isolated buds of flax (Linum usitatissimum) are cultured. Cytokinins especially can change the morphogenetic qualities in terms of both topophysis and cyclophysis, providing evidence that the flower bud formation occurred in Bryophyllum verticillatum at the marginals on the upper leaves and the vegetative shoots on the basal leaves after the same treatment of BA (Dostal, 1970). Barlow and Hancock (1960) demonstrated an experimental modification of branch form in the defoliation and decapitation of current growing apple shoots; i.e., the number of induced axillary buds growing in the current apple shoots is more or less proportional to the number of leaves removed, indicating a quadratic relationship in shoot growth at the centre of the branch. This gave an expression to the bud qualities in the developmental stages of the axillary buds (Barlow and Hancock, 1960, 1962). Maggs (1959 a) also suggested that the potential of the individual buds may vary according to position in the developmental stages, although he did not obtain any difference in shoot growth from the different bud positions in the graftstick on the rootstock M XVI. Barlow and Hancock (1960) suggested that the outgrowth of axillary buds in current shoots under apical dominance may be brought about by the development of buds in the meristematic cell, giving evidence for scale formation by the presence of the subtending leaf and crescent shaped leaf production by its absence.

5.2. Flowering

5.2.1. Total flowering

Explanations of the effect of gravity on flowering are contradictory in the literature. In present studies, in the 90 cm length laterals bent horizontally either at the dormant period or during the growing season in both Red Delicious and Granny Smith, Flowering was almost doubled compared with the vertical control. Between the horizontal bending treatments in different seasons there was no variation in the number of flowering buds. On the contrary, the 150 cm length laterals in Exp.2 had almost the same degree of

flowering between the pendulous bending during the dormant period and the vertical control, whereas the pendulous bending treatments in the growing season increased flowering as compared with the vertical control. Similar results were obtained by Mullins (1964) and Mika (1969 a). This may arise because the increased flowering on the upper side is offset by decreased flowering on the lower side of the pendulous laterals. In addition gourmand shoots are produced on the upper side of pendulous laterals in the dormant period bending. The increased flowering on the vertical laterals is due to the production of reasonable shoot growth in regions from the apex to the 3rd whorls (Zeller, 1960 a; Feucht, 1963; Mullins, 1964; Hassib, 1967; Mazurowa, 1971; Crabbe, 1972).

The flowering of a shoot can also be interpreted in terms of the 'inherent' properties of the shoot Champagnat (1954 b). The effects of the petal fall bending of pendulous laterals on flowering may be balanced by the stimulated induction of shoot growth from along the 150 cm laterals and the cessation of shoot growth from the lower to upper side of bending laterals. On the other hand, generally the poor flower bud formation occurred in the 90 cm lengths of Exp.1 and 3. Because the better buds from the middle to the terminal end of shoots were eliminated by reducing the laterals to a 90 cm length at the beginning of the experiments.

5.2.2. Orientation effect

The orientation of shoots on the horizontal and pendulous bending laterals during the dormant period is a critical factor in inducing flowering. The upper side of a shoot had greatly increased flowering, the lower side of shoots almost no flowering, and the flank had intermediate flowering. In these laterals, apical dominance no longer exists, but new orientations such as the upper, flank, and lower side of buds on the horizontal or pendulous laterals play a role in governing the outgrowth of shoots and subsequently the inhibition of flower bud formation by an imbalance of auxins and cytokinins between the upper and lower side of the bending laterals. This emphasizes that the buds which are impeded at the early burst stage by the high auxin production from expanding leaves (Wardlaw, 1965) and apical meristems (Luckwill, 1964) on the upper and flank sides of shoots may require a higher amount of cytokinins to burst. Therefore, such buds and dards which remain at a dormant stage throughout the growing season are no longer able to initiate flower buds, unless the required minimum number of leaf primordia in the buds is achieved

after bud burst, by a reduction in the plastochrone (Fulford, 1965, 1966 a,b,c). The inhibitory effect of shoot outgrowth and consequent restriction of flower bud formation on the buds and the dards of the lower side of the horizontal and pendulous laterals is similar to the third whorl development of the vertical control. There are no leaves in the dormant buds and few primary small leaves in the dards on the lower side of the horizontal laterals.

The promotion of flowering on the lower side of shoots and spurs of the shoots bent during the growing season may be due to conditions established before bending. Some of the shoots which were continuously growing in the vertical position can slow down shoot growth, but after bending are situated on the lower orientation of the horizontal or pendulous laterals so that flowering can be initiated. Some of the shoots, spurs or buds which are now on the upper side of laterals can be stimulated to renewed growth by supplying cytokinins from the roots or by receiving an optimum level of cytokinins sufficient to induce flowering without encouraging the second growth.

It has been long observed that there is a close relationship between the cessation of shoot growth, spur leaf expansion (Fulford, 1962) and fruit bud formation in apple trees (Swarbrick, 1929; Davis, 1957). These phenomena are now associated with morphological changes in bud development in relation to plastochrone and bud-scale formation (Fulford, 1965, 1966 a,b,c; Abbott, 1970). The relationship between the time of cessation of shoot growth and renew of shoot growth is changed by orientation either in the dormant period or the growing season, and is closely associated with the induction of positive and negative flowering. The pattern of interrupting plant hormones in the growing season by the change of orientation may have a physiological significance. Tromp (1968) obtained an increased flowering on the laterals bearing fruits by changing the orientation of either vertical or horizontal laterals which were kept up to the cessation of shoot growth, whereas he could not increase flowering on the horizontal laterals bearing fruit which were kept horizontal for the whole growing season. On the other hand, Mullins (1964) did not obtain increased flowering when he tied down the branches at different times in the season from both eight-year-old and 15-year-old apple trees, because the second flush of shoot growth induced gourmand shoots. Furthermore he found that summer pruning induced a greater number of flushed shoots on the bending laterals, thereby reducing the number of fruit buds in the bending branches. Lalatta (1958) obtained fewer

flower buds on the laterals bent pendulously during July (Northern Hemisphere). Fulford (1962) also reported that defoliation reduces flower bud formation by allowing a new flush of leaves. The inhibition of flowering occurred by the flushing of the terminated shoot (Ogaki et al, 1963). The flower initiation from the shoots which were flushed from the terminated spurs and dards by the defoliation, defruiting, and horizontal or pendulous bending during the growing season is dependent on the subsequent growth conditions of shoots or spurs. If there is no change of plastochrone (Fulford, 1960, 1965, 1966 a,b,c) after flushing of leaves from the terminated buds, there were no inhibition of flower bud formation. If the plastochrone is delayed 18 days a bud will not convert into a flower bud. In fact, the plastochrone is consistently 7 days in those buds which are initiated into a flower bud, whether the buds are terminated in the early period of the growing season after being released from dormancy, or are flushed from the spurs which are already terminated in the growing season by defruiting and defoliation. Fulford (1966 c) obtained flower bud formation after the flush of bud growth from the bourse by defruiting at the end part of June (Northern Hemisphere) before flower bud initiation. This defruiting effect might be due to the elimination of the auxins and the gibberellin inhibitory effects (Fulford, 1966 c; Luckwill, 1970) from the fruits to the bourse buds and subsequently the supplied cytokinins from the roots can interact with the inhibitory effects from the expanded foliage so that the buds can recommence growth for a certain period to have a terminated bud at the 7 days of plastochrone, at which stage the flower bud is formed (Fulford, 1965, 1966 a,b,c). Therefore, if the horizontal bending treatments induce the spurs, terminal, or axillary buds of the new current shoots on the horizontal lateral by the 7-day plastochrone there will be a flower bud formed even after flushing the leaves from the terminated buds by bending in the growing season. In gourmand shoots if the time at which growth of the flushed buds ceases does not allow the development of a terminal bud by the 7-day plastochrone at the late autumn, a flower bud will not be borne on them. Some gourmand shoots could bear flower buds on axillary buds if the plant growth substances are balanced to induce the production of bud primordia, as the 7-day plastochrone in the terminal buds. The consistent decrease in flowering of the upper side of laterals bent horizontally at the stage of second cover in Red Delicious of Exp. 1 and 2, is brought about by the second flush of growth, although some of the shoots which were delayed during the rest period by the

induction of horizontal laterals can be expected to have an initiation of flowering in Autumn (Zatylso, 1970).

The second growth flush brings slender and thin spurs but seldom develops flower primordia. On the other hand, the consistently increased flowering, in all experiments, on the upper side of laterals bent during the dormant period suggests that there is an association with shoot extension. It is not concluded that shoot extension and flower bud formation are correlated in the present data, because most of the long shoots bore the flower primordia in the axillary buds (Zeller, 1960). Pieniazek, Mika and Soczek (1967) also demonstrated that flower bud formation on the different types of shoots, such as long shoots or spurs, depends on the varietal characteristics. The climatic conditions are also involved as factors inducing flower bud formation. The terminal buds of long shoots and even those produced from bourse buds can easily produce flowering in Granny Smith, whereas these can be easily produced on the short shoots or spurs in Red Delicious. In the present studies, flowering occurred on the long shoots on the upper side of the horizontal lateral, bent during the dormant period, both in Red Delicious and Granny Smith.

There was an increased flowering in the terminal buds of the short shoots and spurs on the upper side of the horizontal laterals. The reason might well be due to the level of cytokinins being inadequate to induce the second flush of growth, but sufficient to induce an activity of cell mitosis or production of primordia in the bud and subsequently flowering was induced in the buds. Summer application of nitrogen after cessation of shoot growth stimulates flower bud formation markedly (Williams and Rennison, 1963; Williams, 1965), probably due to the increased cytokinins from the roots. Apical dominance which can be released by high nitrogen application in flax plants (Gregory and Veale, 1957; McIntyre, 1968) is probably due to the increase of cytokinins produced from the roots.

5.2.3. Whorl effect

The effects of whorl on flowering among the bending treatments are remarkable as is shown in the physiological significance of the potential bud qualities in shoot growth. The most abundant flowering occurs in the apical whorls in all treatments, because of the vigorous extension shoot growth in the current year (Rudloff and Feucht, 1959; Zeller, 1960). The extent of flowering on the current shoots has been mentioned already in the discussion of shoot growth. Increased flowering from the middle part of the current shoots from

the horizontal bending laterals were observed by Mullins (1964, 1966 b). The effect of gravity on flower bud formation and shoot growth are evident in terms of the potential bud qualities of the different whorls. The first whorl had small buds on the laterals in the previous year so that the growth of buds and shoot were also affected (Fig.7-A). The buds on the first whorl in Red Delicious had been severely influenced by a drought in the previous year, but Granny Smith was less affected. The potential bud qualities in the second whorl demonstrate a better flower bud formation which was increased by the seasonal horizontal bending in Exp.1 of Red Delicious. Poorer flower bud formation occurred in the third whorl compared with the vertical control. The poorer flower bud formation in the third whorl can be overcome by the horizontal bending treatments with Red Delicious but not in Granny Smith. Champagnat (1954 b) emphasized that there are different responses between species to shoot outgrowth from the upper side of a bud by the arching treatment at the different times. This fact is seen in the third whorl in Granny Smith, where flower bud formation was not stimulated by the bending. All phenomena are correlated with 'inherent' properties (Champagnat, 1954 b). Here again the potential bud qualities in the flower bud formation of the vertical control (150 cm length of lateral) in Exp.2 are positive so that there was only a small effect of dormant period horizontal bending on flowering. The increased flowering in the 3rd and 4th whorl was compensated by the decreased flowering in the 5th whorl from which gourmand shoots had been produced. This was the arched part of the pendulous bending laterals.

The reason why there are contradictory data on flowering and shoot growth with the regard to the effect of gravity, is possibly due to the variation in potential bud qualities in the laterals used for the experiments as well as the degree of vigour in the laterals and trees used by the different research workers. Potential bud qualities with positive response will induce an outgrowth of buds so that flower bud formation will be increased in that lateral. In this case the effectiveness of bending may reduce it by bringing more vigorous shoots, whereas the potential bud qualities which have a negative response may induce an outgrowth of buds, thereby inducing a proper shoot growth so that flower bud formation will be increased by the bending. Therefore effectiveness of gravity varies with the 'inherent' properties of buds.

5.3. Relative growth relationship

5.3.1. Leaf area

It is interesting to note that 90% of total leaf area is derived from the three uppermost terminals in the vertical lateral in contrast with 45% of total leaf area in the dormant period bending horizontal lateral. Total leaf area was not affected by horizontal bending during the dormant period, because of the gourmand shoots produced from the upper side of shoots on the horizontal laterals. However, Vassev and Mitov (1968) found that total leaf area was greatly reduced by arching laterals during winter pruning compared with the vertical laterals. Alexander (1969) also found that leaf weights in the vertical laterals of peach and orange trees were greater than that of 45° and horizontal laterals. In the present studies, total leaf area was greatly reduced in the horizontal laterals bent during the growing season. These were reduced by a number of gourmand shoots and terminal shoot growth.

Total leaf area is closely correlated with shoot length, although each treatment has a different regression coefficient as shown in Fig. 11. Vassev and Mitov (1968) found that average leaf size was greater in the vertical laterals than in the arched ones. There was a tendency for the largest leaves to be observed on the vertical laterals (Maggs, 1960). Blinovskij (1971) reported that horizontal bending increased the leaf area per unit of shoot length. However, leaf areas on the three upper most terminals were similar in all the treatments in the present studies (Fig. 10-A,B,C,D). There is some variation in leaf area among the different shoot positions on the horizontal and vertical lateral, because of the orientation of shoots in the horizontal laterals and the vigorousness of shoot growth.

5.3.2. Ratio of total leaf area to total increment of shoot volume

Shoot growth has been discussed already in terms of the shoot extension among the horizontal and vertical treatments. Quantitative growth using the volumetric method has been measured in order to find a simple physiological index of vegetative and reproductive growth. In the present studies the terminology of growth analysis in terms of relative growth rate (RGR), unit leaf ratio (ULR), and leaf area ratio (LAR) (Evans, 1972) could not be used. The formula of

$$R = \frac{\text{Total leaf area}}{W_2 - W_1} \quad \text{which is analogous to leaf area ratio in}$$

growth analysis was used in the present studies. $W_2 - W_1$ is only the increment of shoot volume of new and old shoots during the whole growing season. Leaf weight, therefore, is not included in the increment factor of growth. The weight of roots could not be measured in these studies.

When shoot extension was compared between the dormant period horizontal bending and vertical treatments, the average shoot length was greater in the horizontal laterals. On the other hand, the volume of shoot growth was significantly greater in the vertical treatment. These results suggest that the number of shoots which were sprouted from the upper side of horizontal laterals did not increase its volume. In the growth index R in the present studies, the smaller ratio found in the vertical treatment was due to the markedly increased increment of new laterals and not total leaf area changes. On the other hand, the higher ratio was found in all the horizontal bending treatments. The smaller ratio in the vertical laterals can be due to an increase in the vegetative shoot growth. Therefore, it could be used as an index of promotive reproductive growth. The smaller ratio may also be a measure of leaf efficiency to produce the increment of shoot growth. The leaf efficiency of the growing area of the vertical laterals may be increased, because the fixation of CO_2 could be greater in the expanding leaves than in the extension of shoots (Hansen, 1967 a & b, 1969). The better shoot growth occurs in long duration growth (Cook, 1941). There must be a longer growing period in the vertical laterals. There was no significance in the extra period of growth of the vertical laterals (Table 2) probably because there were only two or three shoots which could grow for the longer period from the 29 positions of shoots. This continuous growth from the upper and nearby shoots in the vertical laterals was observed and could contribute to the increased thickness of new laterals in the vertical treatment. The cambial activity throughout the growing season is dependent on the auxin and gibberellin production from the expanding terminal growing area in the laterals (Wareing, Hanney, and Digby, 1964; Digby and Wareing, 1966; Kozlowski, 1972). The increased cambial activity in the laterals and a newly established growing area in the terminal parts of shoots in the vertical treatment act as sinks for nutrients and photosynthates transported from other parts of the trees and utilized for vegetative growth. In this way, a sink system in the vertical treatment may also contribute to an increase in efficiency of leaves as fruits establish to increase leaf efficiency (Chandler & Heinicke, 1925, 1934; Mochizuki, 1962; Maggs,

1963; Hansen, 1967 a; Lenz, 1967). On the other hand, the photosynthates in the leaves of the horizontal laterals could be transported to other branches and roots so that the growth increment of the horizontal laterals is less than that of the vertical laterals.

The pattern of distribution of assimilates from the various leaf positions is different between the horizontal and vertical laterals. The assimilates of CO_2 from the young leaves in the fast growing vertical shoots are mainly transported to the actively growing areas, and not to other regions (Quinlan, 1964; Mika, 1971; Hansen, 1967 b). Assimilates from leaves at the intermediate region of vertical laterals were distributed in both directions, i.e., the growing and other parts of the tree tops and to the roots (Mika, 1971). The ^{14}C -assimilates in the basal leaves in the vertical shoots were transported to another part of the tree (Hansen, 1967 b). Alexander (1969) found that the inclination of peach and orange trees to the horizontal increased the dry matter of the roots relative to that of the vertical trees.

The distribution of growth increment among the various parts of apple trees is nearly constant (Maggs, 1959 b). According to the equation below which describes the response to pruning established by Maggs (1959 b): i.e.,

$$\frac{\text{weight of stem of new shoots}}{\text{increase in weight of old stem}} = 0.6 \times \frac{\text{old stem length before pruning}}{\text{old stem length after pruning}} - 0.3$$

the more severe pruning, the greater increment occurring in new shoots. In the present studies ratio of increment of new shoots to the increment of old laterals in volumes was calculated by the equation of Maggs (1959 b). The ratio was 1.16, 1.48, 1.80, and 1.52 (T_1, T_2, T_3, T_4) which is similar to the range 1.4-2.4 calculated from the data of Maggs (1960). In fact the ratio of new stem to old stem varies according to variety (Maggs, 1960). A lower ratio in the dormant period horizontal bending indicates a greater response to bring a vigorous long shoot from the upper side of the horizontal laterals as the dormant pruning induced the vigorous longer shoots, as compared with the summer pruning (Maggs, 1965). Therefore, the dormant horizontal bending and dormant pruning responses suggest that those treatments during the dormant period can produce a bud where cytokinins can be accumulated and stimulate an activity of bud growth to obtain a dominant factor, and subsequently a vigorous growth induction. On the other hand, summer pruning stimulates a

secondary flush of growth for a number of buds, but the growing period was reduced to 30-50% of the growing season (Maggs, 1965). Therefore, growth activity may be greatly reduced by the horizontal bending treatments during the growing season which were found in the present studies.

5.4. Summary of discussion

The shoot growth from one year old laterals in woody plants is a different phenomenon from the axillary bud growth of a herbaceous plant (Champagnat, 1954 b), although the phenomenon of apical dominance is the same in the current shoot of the woody plants (Champagnat, 1954 b; Phillips, 1969). Therefore, the buds on the one year old laterals have been preconditioned during the previous growing period by internal factors such as apical dominance and external environmental factors. Those buds acquired properties during the previous year which are 'inherent' properties (Champagnat, 1954 b), e.g., potential bud quality.

In the present studies, the one year old lateral at which 90 cm length was headed back could have about 38 internodes. The positions of buds from the terminal to the basal buds were numbered from 1 to 38. However, 1 to 29 positions were examined in Exp.1 and 3, and 1 to 45 positions in Exp.2. Those basal scars and buds (e.g., from 38, 37, 36 positions etc.) which originated from an embryonic state were not seen or were very small. The larger buds were present around the middle part of laterals and towards the apical part buds were gradually smaller in the long shoots. The larger buds had a well developed vascular connections to the axillary buds in the current season. This was probably the season that the 'inherent' properties could be revealed in the coming season. Although bud size was not measured, the distribution of bud sizes along the laterals followed a normal distribution pattern. This kind of bud size pattern can be changed by environmental conditions such as drought which increase ABA levels, thereby both inhibiting bud formation and inducing smaller size of buds as observed in the first whorl of Exp.1 and third whorl of Exp.2. The arrested buds were hardly able to induce an outgrowth by the dormant horizontal bending because of 'inherent' properties controlled by correlative inhibition and environmental conditions may be due to the inception of axillary bud formation which is controlled by a quantitative distribution of cytokinins and other root hormones (Luckwill and Whyte, 1968) and auxins, gibberellins, and ABA from the apices and leaves.

During the release from dormancy in woody plants all buds are likely to receive equal supplies of plant hormones synthesized in the roots as well as nutrients from roots. However, some buds are not ready for bud break and may require greater quantities of cytokinins to be neutralized with inhibitors. By observation all except the severely arrested buds were simultaneously ready to start bud burst and to remove the bud scales in Red Delicious, but some buds in Granny Smith were not ready to separate the bud scales, so that those scales were kept for the new growing season. There seems to be a varietal difference in the time at which bud scales are removed. As soon as the buds move and the flow of inhibitors under the gravitational effect has already been established, correlative inhibition occurs between the upper and lower side of the horizontal.

The development of dominant buds may vary according to the potential bud qualities and treatments. The following factors have to be considered: (1) 'inherent' properties;

- (a) Position of the bud on the shoot during the previous year's growth.
- (b) Bud size; development of the vascular connection and number of primordia.
- (c) Distance from the proximal to the distal position

(2) Treatments

- (a) New orientation of buds; e.g., upper, flank, and lower position
- (b) Time of bending

(3) The rate of growth and development

- (a) Ratio of cytokinins to IAA and GA, quantities of ABA and IAA supplied from the adjacent tissues such as bud scale, and young leaves, and the ratio of GA to IAA.
- (b) Supply of nutrients required to synthesize plant hormones and the constituents of plant cells in the developing tissues.

These three factors are interacting during development and growth. In the present studies, although the dominant shoots appeared as the 'two centre pattern' (Mullins, 1964) at the apical whorl and 15th position in the dormant period bending of Exp.1, the gourmand shoots were spread sporadically along the upper side of the laterals. This means that any bud on the upper side of the horizontal and pendulous laterals which meet the essential factors mentioned above can grow as a dominant shoot on the terminal of a vertical lateral, apical whorls of the bending laterals, and upper side of bending laterals. Cytokinins and nutrients are limited and inadequate to

supply to 29 or 45 buds in the laterals. Therefore, the dominant buds, which are conditioned for vigour, can exhibit compensatory growth (Barlow, 1970)

The buds newly oriented to the upper side of horizontal and bent during the dormant period can receive much more cytokinin hence burst into outgrowths as soon as root activity begins. Therefore, the upper buds on the horizontal are equivalent in effect to a physiological apical meristem (Champagnat, 1954). The upper buds dominate the lower and flank buds by a mechanism similar to apical dominance or lateral bud dominance (Cutter and Chiu, 1972), being due to a gradient of inhibitors under the gravitational effect. On the other hand in the late growing season, the horizontal bending could not induce shoot outgrowth from the upper side as much as the horizontal bending during the dormant season. Thus the decreased effect of horizontal bending on shoot outgrowth from the upper side increased as the season advanced. The effect of inducing bud outgrowth on the upper side by bending during the growing season depends on antagonism between cytokinins, both supplied from roots and those synthesized by the bud itself, and inhibitors produced in the expanding, mature leaves, and bud themselves especially bud scales. In a similar way, inhibition of axillary buds is overcome by defoliation, defruiting, removal of bud scales and summer pruning, which eliminates the source of inhibitory plant hormones.

The inhibitory effect present during different developmental stages on the outgrowth of buds, spurs, and bourse buds is shown by the progressive sequence of developing organs on the different parts of the tree. For example in apricot (Ramsay, Martin, and Brown, 1970), when the terminal and young leaves are still growing and expanding, decapitation alone can induce a release of inhibited axillary buds, but when the spurs are terminated and leaves expanded, decapitation and defoliation are necessary for inducing axillary bud growth. These effects vary between species (Champagnat, 1965).

There is a number of similar phenomena in the whole tree in the correlative inhibition brought about by defruiting and defoliation. In apple, defruiting by late June (Northern Hemisphere) induces the outgrowth of bourse buds without defoliation of spurs (Fulford, 1966 c). This could be due to an axillary correlative inhibition at the early stage of development produced by the apex and expanding leaves. Further inhibition is due probably to the mature leaves (Eagles and Wareing, 1964), and in seeds concomitantly a small

quantity of IAA could maintain its inhibitory effect together with ABA. The function of ABA at this stage could be to have a major role compared with IAA, leading gradually to a dormant state of buds. The bud scales may also serve such a function. Therefore bending treatments during the later growing season interact with the preventing hormonal balance to induce the outgrowth of buds. This gradient of inhibitors which produces activities in different organs and tissues establishes a steep linear response of shoot growth and flowering from the lower, flank, and upper shoot orientations. This is associated with the apical meristem orientation in the dormant period bending and no steep linear response in the late season bendings.

The greatest difference between the dormant period bending and growing season bending occurs on bud outgrowth on the lower side of shoots in the horizontal and pendulous laterals. Bud outgrowth is almost completely inhibited at the bud burst stage by the dormant period bending.

Thus the lower buds on the dormant bending horizontal laterals have shown the greatest inhibition of flowering. There is no doubt that the buds in which outgrowth is completely arrested could not induce flowering because of the high content of inhibitors. However, flowering on the lower and flank shoots of the horizontal and pendulous laterals bent during the late season gave an intermediate response without the inhibition of flowering which occurred with dormant period bending. The shoots or spurs which had already grown in the vertical laterals before bending had formed vascular connections (Gregory and Veale, 1957; Sorokin and Thimann, 1964; Panigrahi and Audus, 1966). Thus the newly obtained shoots or spurs oriented in the lower side after bending could not inhibit flowering. Flowering was promoted on the extending shoots where growth was slowed down by the new orientation. The same physiological phenomena occurred in the flank orientation of the bending laterals. Therefore, flower bud formation was the same as for the lower side of the laterals bent during the late season.

The newly oriented shoots or spurs on the upper side of the horizontal shoot can achieve a flush of growth if the essential conditions are not; (i) a vigorous growing condition, possibly receiving cytokinins beyond a bud which can be neutralized with the inhibitors, (ii) a degree of correlative inhibition such as summer dormancy, (iii) before the onset of a winter dormancy. Therefore the flush of growth does not occur to all the shoots or spurs on the upper side of laterals by the late season bending. The number of shoots outgrown from the upper side of the horizontal gradually decreased as the

season advanced. If the flush of terminated buds does not induce the formation of a minimum primordia (a critical value such as 18 internodes in the flower bud, Fulford, 1960) flower bud formation may not occur. The bud must have sufficient time to produce a certain number of primordia before initiation of flower buds. Therefore flushing in summer by defoliation, pruning, and bending induced no flower induction (Fulford, 1960; Mullins, 1964). Summer application of nitrogen (Williams and Rennison, 1963; Williams, 1965) and changes of orientation of laterals after cessation of shoot growth (Tromp, 1968) increased flower bud formation. These practices could increase the cytokinin content of the flush growth and possibly increase the number of primordia to more than a critical value (Fulford, 1960) in the flower buds (Zatyko, 1970). In analogy, the shoots or spurs of the upper side on the bending laterals may have an accumulation of cytokinin without flushing so that the number of primordia could be increased in the buds leading to flower induction.

Flowering is closely related to shoot extension and terminated spurs. The upper shoots on the horizontal lateral may have a positive flowering response and a proper vegetative growth in several individual shoots which gave a reproductive growth index as 19.80 to 24.40 of R in Table 5. The excessive vegetative growth which produces a negative flowering response occurs when R is 10.83 as a vegetative growth index. The spurs on the vertical control may have a higher content of IAA and GA levels produced by the young levels and apex associated with the intensive increase of activity in xylem and phloem production under the feedback system. Flower bud formation therefore was greatly reduced on shoots or spurs in the vertical controls in Exp. 1 and 3. Leaf area was not related to the flower bud formation in the vertical control (Fulford, 1970). Flower bud formation was not inhibited in the vertical controls in Exp. 2 nor in the apical whorls in all the experiments. Axillary buds in the long current shoots were induced to flower in the buds in the vertical and on the upper side of the dormant period bending, depending on the growth condition (Coombe, 1969; Luckwill, 1970). In connection with the vegetative growth condition, the shoots which are formed during the juvenile tissue conditions in terms of high contents of IAA and GA in the developmental stage cannot be reproductive. The presence of reproductive tissues might be determined by a high ratio of bark to xylem (Mosse, 1952; McKenzie, 1956). GA sprays at bloom inhibit flowering. This is probably due to the formation of axillary buds on the tissues at

the early stage of growth where the spur buds or bourse buds are affected at the high level of IAA and GA, and give conditions similar to juvenile tissues, consequently no flower bud formation occurs. On the other hand, Alar promotes flower bud formation when sprayed at 2-4 weeks after bloom, because shoot growth is reduced probably by inhibition of IAA synthesis (Reed, Moor, and Anderson, 1965).

There is a close relationship between the cessation of shoot growth and flower bud formation (Swarbrick, 1929; Davis, 1957). Terminal bud formation in the growing apex begins with the development of bud scales (Fulford, 1966 a). Abbott (1970) suggested that the formation is due to a deficiency of cytokinin. Wareing explained that ABA induces the inhibition of development of the lamina so that in blackcurrant a bud scale was formed (Abbott, 1970). Drought induced rapid increases in ABA so that there is no synthesis of cytokinins. The relationship between ABA and CK may be a cause-effect relationship and vice versa, as indicated in a 'stop-and-go' growth system between cytokinins and ABA in Lemna minor (van Overveek, 1968). In the terminated buds there are still influences on production of primordia by which vegetative and reproductive buds will be determined by a ratio of plant hormones. If primordia can continue to produce a critical value as in 18 internodes (Fulford, 1960) the bud will be a flower bud. During the production of primordia there are established certain rates of production (Plastochrone) (Fulford, 1965, 1966 a, b, c). For flower buds there is no change in a 7 day plastochrone, but for a non-flower bud a 7 day plastochrone keeps until about 4 weeks after full bloom, and thereafter an 18 day plastochrone occurs in the bourse bud because of fruitlet influence. Therefore if any factors lengthen primordia production from a 7 day plastochrone to an 18 day plastochrone, the bud will not establish as a flower bud.

The developing apple fruitlet greatly inhibits flower bud formation due to the presence of seeds in the fruit (Chan and Cain, 1967; Griggs, Martin, and Iwakiri, 1970). The auxin contents in the bourse bud and GA contents in the seed were peaked during 4-9 weeks and 5-10 weeks after full bloom respectively (Luckwill and Whyte, 1968). The physiological phenomena of inhibiting flower bud formation in the bourse bud is analogous with a correlative inhibition as shown in apical dominance. That is the first factor is an auxin-like hormone which initiates an effect on the inhibition of the bourse bud and so lengthens the plastochrone. The second factor is a gibberellin-like substance. From seeds, hormones are produced which encourage leaf primordia to continue development into leaves. Although GA acts by

shortening of the plastochrone in the growing apex to a 2-day plastochrone (Fulford, Quinlan, and Barlow, 1968), the bud inhibited with IAA by correlative inhibition may not become active with GA, unless the buds are at mitotic condition (Ali and Fletcher, 1970). On the other hand, if sufficient cytokinins are present to antagonize IAA and GA the bourse bud can be released from a correlative inhibition arising from the developing fruitlet so that flower bud formation occurs. This phenomenon occurred in blackcurrant in which the axillary buds induced flowering after being released from a correlative inhibition (Nasr and Wareing, 1961). In these cases the spur tissues are renewed by passing through juvenile conditions (Schwabe and Al-door, 1973).

Plant hormones and chemicals which can promote flower bud initiation link directly or indirectly with the inhibition of biosynthesis, translocation, and degradation of anti-flowering IAA and GA (Lang, 1961 and 1965) which stimulate vegetative growth. In most cases where sprays of growth regulators reduced shoot growth, e.g., Alar, inhibition of synthesis of IAA (Reed, Moore, and Anderson, 1965), inhibition of effective gibberellin but not as anti-gibberellin (Greenhalgh, 1967); CCC, inhibition of biosynthesis of gibberellins (Cross, 1968), an increased level of CK in xylem sap (Skene, 1968), TIBA, inhibition of transportation of IAA and GA (Bukovac, 1963); NAA, induction of producing phlodizin which induces the inhibition of biosynthesis for IAA (Grochowska, 1963, 1964); NAA, induction of ethylene (van Overbeek, 1945; Burg and Burg, 1966).

In several cases, ABA, CK, and ethylene induce flower bud formation (El-antably and Wareing, 1966; Michniewicz and Kanienska, 1964, 1965; Maheshwari and Venkataraman, 1966; Nitsch, 1968; Gupta and Maheshwari, 1969).

In conclusion, there must be a delicate balance of plant hormones required to proceed the shoot growth and the induction of flowering. In shoot growth, bud burst appeared to be controlled by cytokinin-like substances with which IAA (a correlative inhibitor) and ABA (a bud scale inhibitor) are antagonistic. After bud burst the extension of shoot growth is influenced by the interaction of four factors, IAA, GA, CK and ABA. However, IAA and GA are dominant factors together with CK for the shoot growth to retain a juvenile condition whereas CK and ABA moderate the action of IAA and GA to give an adult condition (Fig. 1 and 2). These phenomena can reveal an interaction between bending at the time of a particular physiological condition of laterals and buds and shoot orientation. During the formation of buds in the

shoot, a bud scale formation and continuous primordia production occurs. In this process a vegetative or reproductive phase is terminated by an optimum gradient of CK, ABA, IAA, and GA. In flower induction the two major plant hormones may be cytokinins and ABA. At the period of induction of flowering levels of IAA and GA are greatly reduced because of no active producing loci such as exist in expanding leaves and apices. The amount of IAA and GA transported from the other parts of organs may be antagonized by the existence of bud scales. The bud scales and primordia themselves also may have an antagonistic function especially in relation to cytokinins produced in the roots. Therefore ABA from the bud scale and mature leaves plays a very important role along with CK and other plant hormones. If an excess of cytokinin is available for primordia the bud will burst. When cytokinins are well balanced with ABA, a minimum quantity can encourage new primordia to develop so that a production of floral-RNA leads to the initiation of floral primordia leading to a possible supply of IAA and GB via the self-regulating systems in primordia production.

If there is a florigen it must be regulated by ABA and CK. One can draw a model of a biochemical switch from a vegetative and reproductive bud in terms of a qualitative method (Thornley, 1972).

PART II
CHAPTER 6
Introduction

Plant hormones such as auxins, gibberellins, cytokinins, and abscisic acid together with the gaseous hormone ethylene and possibly as yet unclassified plant hormones are vital factors in controlling the overall physiological and morphological phenomena in the growth and development of a plant, serving as agents in the integration and co-ordination of growth and differentiation (Wareing and Phillips, 1971). The integration of growth probably depends on a delicate ratio of these plant hormones interacting with basic metabolic pathways and linked with the supply of nutrients, as indicated in Fig.1. In this model the feedback system of cause-effect phenomena between plant hormones and nutrients is influenced at specific times and places by both internal and external conditions. The delicate balance of plant hormones may then affect plant growth and development by interacting directly or indirectly with over-all metabolism. It should be possible to represent this balance in terms of concentration of plant hormones as determined by plant hormone analysis. However, analysis of plant hormones has given many problems to investigators, because of the extremely low concentration of labile plant hormones present in plant tissue.

Many analysts have met problems in extracting plant hormones. Powell (1972) described the difficulties of plant hormone analysis as follows: "The analysis of plant extracts or diffusates for hormones has always bordered on the mysterious, and has been fraught with pitfalls for even determined investigators".

The investigations during the past 40 years of, not only the analysis of endogenous plant hormones, but also the application of authentic plant hormones to a tissue or an entire plant, could embrace a concept of hormonal balance theory for control of growth and development in a plant. Accordingly to fully investigate the hormonal basis of growth and development it is necessary to analyse most or all groups of hormones but this is of course very difficult and time consuming; only recently have attempts been made to achieve this goal. For example, Isenberg, Thomas, Pendergrass, and Abdel-Rahman in 1974 reported differences in four hormonal groups in normal and maleic hydrazide treated onions stored over winter while Shindy and Smith (1975) investigated the hormonal balance in cotton ovules by combined gas chromatography-mass spectrometry (GC-MS) of a single extract. At the beginning

of this investigation, various extraction and bioassays techniques published were tried and modified for use with woody tissue and leaves (see, Table 6), as the research progressed.

During the studies of extraction procedures the loss of plant hormones at each step were tested. In particular, ^{14}C -IAA and ^3H -zeatin were used to assess the loss of these compounds during various purification methods. It was found that the greatest loss of IAA occurred at pH 8.0 in the aqueous phase. Therefore, the literature review has attempted to focus on how to develop the purification procedures at different pH's after removal of methanol from the extract. In addition to this a few new techniques reported recently are summarized.

Various bioassays have been tested, and modified to obtain better results using the modified purification procedures.

When the laboratory facilities were available the technique of gas liquid chromatography was tested but the technique needed considerable effort to establish optimum condition of use. Therefore in the present studies of plant hormones, analysis by GLC was used only to a limited extent.

The extraction procedures and bioassays outlined in Table 6, were used with apple leaf samples to analyse auxins, gibberellins, cytokinins and abscisic acid, thereby estimating a ratio between free plant hormones. However caution needs to be exercised in interpretation of the results since firstly, the hormones may have been released from a bound form during the procedures used and secondly, no estimate was made of the bounds forms.

CHAPTER 7

Review of literature

In the last 40 years endogeneous plant hormones have been widely isolated and determined both quantitatively and qualitatively. The principle extraction procedure used was to homogenize the plant material in ether, aqueous alcohol or acetone. Initial purification was then carried out by solvent partitioning, the separation achieved depending on the hydrophilic or hydrophobic nature of the extracted compounds. The extraction and purification procedures have been empirically investigated by many research workers. For the purpose of the following experiments, it is necessary to give an outline of the vast volume of literature published on the determination of auxins, gibberellins, cytokinins and inhibitors.

7.1. Auxin-like substances

7.1.1. The historical development of extraction procedures for acidic, neutral, and basic fraction in the determination of auxin-like substances

"
Kogel and his co-workers in 1934-1935 isolated IAA from human urine and yeast plasmolyzate by fractionation of extracts between ether and water at different pH values (Larson, 1955). Thimann in 1935 also isolated IAA from Rhizopus suinus cultures. One of the earlier purification procedures for IAA was to use sodium bicarbonate to give an alkaline pH and then partition between ether and water, that is the ionized acidic compounds are separating into the aqueous phase from the ether while the basic compounds remained in the ether phase (Boysen Jensen, 1941). The method used by Boysen Jensen was that ether extracts from plant materials were partitioned three times with 8 % NaHCO_3 in water, and the ether phase then was discarded. The sodium bicarbonate solution was then acidified and extracted three times with fresh ether into which the acidic ether soluble compounds partitioned. The combined ether fractions were then dried and bioassayed. In the early period of auxin research, the ether phase only was studied. After the development of paper chromatography, however, the finding of a neutral hormone such as indolealdehyde in cabbage (Larsen, 1944, 1951) and indole-3-acetonitrile in cabbage (Link, Eggers, and Moulton, 1941; Linser, 1940; Avery, Berger, and White, 1945), stimulated an investigation of further detailed extraction procedures (Bentley and

Housley, 1952). Finally IAN was isolated from cabbage from a neutral fraction in the extraction procedure (Jones, Henbest, Smith, and Bentley, 1952). In the extraction procedure the aqueous phase after evaporation of ethyl alcohol was acidified to pH 3.0 and was then extracted four times with ether. The ether fractions were back washed with distilled water and then were filtered over anhydrous magnesium sulphate. The residue after evaporation of the ether was redissolved in fresh ether and was extracted with 1 % sodium bicarbonate to separate acidic and neutral fractions, that is the neutral compounds remained in the ether phase after the partitioning. The ether phase was reduced to the residue after back washing with distilled water and IAN isolated by paper chromatography.

In 1955 a, Kefford enumerated a number of papers which dealt with acidic growth substances and reported a highly active promotion and inhibition area in the paper chromatogram, named accelerator α and inhibitor β in the acidic fraction. He did not investigate neutral and basic and certain phenolic substances.

In 1956, Housley and Bentley investigated auxin components in all possible fractions, classified into acidic ether and neutral ether, and basic ether fraction of an extract of cabbage leaves. They found no IAA and IAN in the acidic fraction, but IAN and a growth promoter at Rf 0.0-0.1 in the neutral fraction and a neutral inhibitor in the aqueous fraction.

In 1959, Kefford investigated all fractions such as acidic, basic, and neutral ether soluble substances in tobacco extracts. He found only IAA-like substances at the Rf of IAA and no auxin-like substances in the neutral and basic fractions. On the other hand, Paleg and Muir (1959) found changes of neutral and acidic auxins in developing tobacco fruit. Lahiri and Audus in 1960 found auxin-like substances from all fractions in the roots of Vicia faba.

7.1.2. The acidic auxin-like substances at pH 2.5-3.0 and pH 8.0-8.5

During the purification of a plant extract a process of acidification and alkalization may bring several problems such as hydrolyzation in weak acidic and alkaline solutions (Gordon, 1954), conversion of tryptophane to IAA with the presence of phenols at alkaline pH (Gordon and Paleg, 1961), and the possibility of IAA formation from labile IAA conjugate (Kefeli and Turetskaya, 1968). For example, simple transfer of indoles from ethereal solution to aqueous solution induced IAA (Terpstra, 1953) and indole acetaldehyde without enzymatic conversion (Zenk, 1962). Mild alkalinity can cause

the formation of IAN from a precursor, possibly an auxin complex (Housley and Bentley, 1956) while enzymatic inactivation of IAA occurred at pH optimum 6.2-6.7 (Tang and Bonner, 1947). Vlitos and Meudt (1954) examined the stability of IAA in phthalate buffer (1.5-9.0) at different times and found that 70 per cent disappeared at pH 3.0 after 72 hrs of incubation.

In the conventional partitions, because the use of sodium bicarbonate partition from ether extracts in the early period of auxin analysis (Boysen Jensen, 1941; Larsen, 1955, Heide, 1967; Moe 1971; Rudich, Halevy, and Kedar, 1972), the aqueous phase could be adjusted to alkaline solution at the beginning of partition after evaporation of solvent extract. Dannenburg and Liverman (1957) tested the fractionation of several indole ring compounds such as IAA, IAN, IAAP, IpyA, TTP, TNH_2 , and TOH at pH 8.1 with Na_2CO_3 in the extraction procedure. There is a number of papers in which partitions begin at pH 8.0-8.5 in the aqueous phase after evaporation of solvent extracts (Tafari, 1966; Dullaart, 1967; Klingström, 1967, 1969; Avery and Lacey, 1968; Eliasson, 1969; Alden, 1971; Igoshi, Yamaguchi, Takahashi, and Hirose, 1971; Aung, 1972).

Steen (1972) avoided to use pH 8.2 in the extraction procedures of Eliasson. Because the reason given to use pH 2.8 instead of pH 8.2 in the aqueous phase was that IAA might be formed from tryptophan by enzymatic activity in alkaline conditions (Gordon and Paleg, 1961; Whitmore and Zahner, 1964). Since the extraction procedure of Kefford (1955 a & b, 1959) has been widely adapted to study auxin-like substances in plant materials, the partitions have begun with the aqueous phase at pH 2.5 - 3.0 (Thurman and Street, 1960; Phillips, 1964; Wheeler, 1966; Wodzicki, 1968; Ramsay and Martin, 1970 a; Hoad, et al., 1971; Steen, 1972).

7.1.3. A bound or conjugate-auxin-like substance

The presence of unidentified ether-soluble auxin and related compounds, unknown water-soluble auxins, and non-indolic auxins in plant extracts has been published in many papers (reviewed by Bentley, 1958). The free and bound auxins and auxin precursors have been reviewed by Bentley (1958), Fawcett (1961) and Thimann (1969). At the present time the study of bound auxins is of great interest because of their physiological significance. In part I (in the section 1.6.2; IAA conjugating system with aspartate) the physiological significance of IAA-conjugates, for example, indolylacetylaspatic acid has been briefly reviewed. Bound auxins which exist as IAA-protein or tryptophan-

protein, tryptophan, and ascorbigen can readily be converted to IAA (Fawcett, 1961). In addition there exists low molecular weight auxin precursors composed of IAA linked to various sugar groups (Thimann, 1969). One of these groups is identified as indole-3-acetyl-2-O-meso-inositol. These indole, or non-indole compounds have been determined by enzymolysis, hydrolysis, or autolysis after removed of free auxins from the plant extracts. The citrus auxin was found in lemon seeds and other citrus fruits by column chromatography (Khalifah, Lewis, Coggins, 1966), although Goldschmidt, Monselise, and Goren (1971) suggested that the native auxins of citrus plant were identical with IAA.

7.1.4. Column chromatography

7.1.4.1. Adsorption column

7.1.4.1.1. Alumina column

Earlier investigators have used alumina column adsorption chromatography for the purification of indole compounds (Linser, 1951; Blommaert, 1954; Luckwill, 1957).

7.1.4.1.2. Silica gel-celite column

A clear separation between IAA and citrus auxin in the fraction was revealed when a silica gel-celite adsorption column was employed with a gradient elution system using chloroform and ethyl acetate (Khalifah, Lewis, and Coggins, 1966). The existence of citrus auxin was demonstrated in citrus (Khalifah, Lewis, and Coggins, 1966, Khalifah, Lewis, Coggins, and Padlick, 1965) as well as other genera (Lewis, Khalifah, and Coggins, 1965) by solvent partition and paper chromatography. Adsorption column chromatography with silicic acid, similar to the method of Hirsch and Ahrens (1958), was used by Rayle and Purves (1967) in isolation and identification of indole-3-ethanol from cucumber seedlings. The solvent system used was a stepwise gradient using increasing concentration of ethyl ether in petroleum ether. The brief extraction procedures are as follows: From the ether extract, the residue after evaporation of ether was passed through charcoal for adsorption of pigments and then through DEAE-cellulose. The residue was purified again with a magnesium silicate M-1 (Bio-Rad) column packed with carbon tetrachloride and eluted in a stepwise pattern using increasing concentrations (0 to 50 %) of chloroform in carbon tetrachloride. Further purification was made with a Sephadex LH-20 column (2.5 x 70 cm) eluted with MeOH. Two peaks of biological activity were found with an absorption maximum at 280 nm. After thin layer chromatography the active fractions from the Sephadex LH-20 were rechromatographed on Sephadex LH-20. From 93 Kg of cucumber tissues, 2.5 mg of a light yellow

compound was obtained.

7.1.4.2. Ion exchange column

Anion and cation ion exchange column chromatography using cellulose phosphate and DEAE-cellulose has been intensively used to purify the root tissues of 12 day old Vicia faba plants (Burnett, Audus, and Zinsmeister, 1965). The ether soluble fraction partitioned from the aqueous phase at pH 3.0 was loaded on the top of a charged DEAE-cellulose (Anionic) column. Elution with distilled water eliminated neutral compounds, and then was eluted with 0.05 M Na₂SO₄. Without using a 5 % sodium bicarbonate step, DEAE-cellulose was found to give a good separation of IAA, representing 85-97 per cent recovery of IAA. Water soluble compounds were also passed through cellulose, cellulose phosphate, and DEAE-cellulose columns to separate the various active components (Burnett, Audus, and Zinsmeister, 1965). However those active compounds were not identified. The cellulose phosphate cationic column passed some phenolic materials running at Rf 0.12-0.5 in the standard solvent, but there were some phenolic compounds retained by anionic DEAE-cellulose.

7.1.4.3. Partition column

7.1.4.3.1. Silica gel column

Powell (1960, 1963) reported that the silica gel partition column was successfully employed to separate a number of synthetic indole derivatives and naturally occurring plant hormones, eluting with n-butyl alcohol saturated with 0.5 M formic acid in n-hexane by stepwise techniques. Further investigations showed the techniques included a number of other useful solvent mixtures, such as methylene chloride, chloroform and ethyl acetate in n-hexane. Most of the solvent systems could separate the indole compounds tested in the same order. Powell and Pratt (1964) analyzed plant hormones in the developing fruit of peach by those techniques eluted by n-butyl alcohol saturated with 0.5 M formic acid to petroleum ether by a stepwise method. They found several growth promoting substances determined qualitatively and quantitatively for seasonal trends at each growth phase.

Hamilton, Bandurski, and Grigsby (1961) choose the silica gel partition column employing a phosphate buffer aqueous phase (pH 6.5) and using ether as the mobile phase, recovering 77 % from 25 µg applied authentic IAA to the column.

7.1.4.3.2. Gel chromatography

The separations of the active substances from some of the interfering substances for bioassay have met with many difficulties when the conventional solvent partition chromatography with paper and thin layer chromatography only was employed. Recently gel chromatography has been developed as a means of further purification after solvent partition chromatography. Gel chromatographic separation depends primarily on the differences of molecular weight and is particularly effective for structurally analogous oligomers. Among many different grades of gel, Sephadex G-10 as a stationary phase can be eluted by a buffer solution. Anderson in 1968 found that the order of elution from the fermentation products of Claviceps purpurea PRL 1980 was indole acetic acid, tryptophan, chanoclavine, 5-hydroxyindoleacetic acid, unknown b and c, and agroclavine, lysergic acid, unknown a, d, and e. Successful purification and fractionation of 50 % aqueous acetone extraction by Sephadex G-10 eluted with distilled water or 50 % acetone was demonstrated for a quantitative estimation of alkali-labile indole-3-acetic acid in dormant and germinating maize kernels (Ueda and Bandurski, 1969). Without using solvent partition chromatography Ellicot and Stowe (1970) isolated and identified a new indole glucosinolate as 1-sulpho-3-indolyl methyl glucosinolate from Isatis tinctoria by using Sephadex G-10 column eluted with distilled water followed by paper chromatography.

Sephadex LH-20 which can be eluted by organic solvents has been employed to remove various inhibitory components in pine tree extracts (Klingström, 1967, 1969). The improvement of qualitative and quantitative estimation of IAA from Populus tremula (Eliasson, 1969), Picea abies Karst (Steen and Eliasson, 1969), and Pinus silvestris (Alden and Eliasson, 1970) was achieved with a Sephadex LH-20 column eluted with 96 % ethanol containing 0.001 M HCl for the acidic fraction or elution from paper chromatogram. A certainty of occurrence of IAA in conifers, therefore, is clearly demonstrated by using Sephadex LH-20 (Steen, 1972). Ellicott and Stowe (1970) used Sephadex LH-20 for isolation and identification of indole-3-ethanol (tryptophanol) from cucumber seedlings. Sephadex LH-20 could reduce to 3.5 mg of residue, 33 mg of residue collected from a magnesium silicate column. A column of 'modified Sephadex LH-20' has been developed for analysis of plant terpenoids with benzene and alcohol solvent systems by Keates and Brooks (1969).

7.2. Abscisic acid and other inhibitors

The physiology and chemistry of abscisic acid has been reviewed by Addicott and Lyon (1969), Kefeli and Kadyrov (1971) and Milborrow (1974). They mentioned briefly the natural occurrence and methods of determination of ABA. Milborrow (1974) described the chemical properties of ABA such as the structure of (+)-(S)-abscisic acid and some derivatives, e.g., phaseic acid, and analogs, and also the structural requirement for activity. He listed several methods for quantitative determination of ABA as follows: Racemate dilution method, use of labelled material, adding a known amount of the 2-trans-isomer, and determination by spectrofluorimetry, spectropolarimetry, gas liquid chromatography, immunological assays, and bioassay of the inhibitory effect of ABA. For the analysis of ABA, some preliminary purification by solvent partitioning and paper chromatography must be carried out. Further purification using TLC, CC(count current), electrophoresis, and gas-liquid chromatography are often needed.

In this section, a brief historical outline of the isolation of ABA is given, and the methods and techniques used for the quantitation of ABA are summarized.

7.2.1. Historical progress in the isolation of ABA

Since the study of auxin activity in extracts of plant materials, the existence of inhibitory substances were known (See Hemberg, 1960). As soon as a paper chromatography technique using the solvent system isopropanol or n-butanol aqueous ammonium mixture to study plant extracts was introduced, inhibitory zone were observed at Rf 0.5-0.7 (Bennet-Clark, Tambiah, and Kefford, 1952; Luckwill, 1952). Bennet-Clark and Kefford (1953) called the inhibitory zone 'inhibitor- β '. The inhibitor- β was derived from the acidic fraction of an alcoholic extract of etiolated leaves of broad bean and contained many different phenolic compounds. The presence of inhibitor- β in different plant organs was reviewed by Bently (1958) and Hemberg (1960).

Since Hemberg (1947, 1949) proposed a significant role for inhibitors in the dormancy of potato buds and buds of Fraxinus, a number of research workers investigated specific inhibitors in the extracts. There were three groups who intensively attempted to isolate from the β -inhibitor a pure compound in crystalline form which would account for most or all of the inhibitory activity.

Phillips and Wareing (1958 a & b, 1959) found that a quantitative change of inhibitory substances in Acer pseudoplatanus throughout the annual cycle was closely correlated with the growth and

dormancy of the shoots. In the investigation of 'inhibitor- β ' eluted from Rf 0.65-0.95 using extracts of birch leaves (Betula pubescens), Eagle and Wareing (1963) applied the inhibitor- β solution back to the birch seedlings under long day conditions and induced the dormancy of those seedlings. Therefore they proposed the term 'dormin' for substances which appear to function as endogenous dormancy-inducers. Robinson, Wareing, and Thomas (1963) isolated the inhibitor- β from the leaves of Acer pseudoplatanus, and found -OH, -CO-, -CH₂- and /or -CH₃ groups were present but did not obtain evidence of aromatic substances being present. Robinson and Wareing (1964) isolated an inhibitor from the inhibitor- β zone with Acer pseudoplatanus, using paper chromatography and two different solvent systems, that is, the inhibitor- β at Rf 0.55-0.8 in paper developed by isopropanol/ammonia/water was eluted and the residue of the eluate was chromatographed in n-butanol/ammonia to eliminate phenolic compounds. Finally Conforth, Milborrow, Ryback, and Wareing (1965, 1966) isolated identified and confirmed by synthesis as the major inhibitory component in the leaves of Acer pseudoplatanus.

During the investigation of hormonal substances in cotton, led by Carns and Addicott (Addicott and Lyon, 1969), Ohkuma, Lyon, Addicott, and Smith (1963) first isolated a compound 'abscisin II, from young cotton fruit.

In the third group, van Steveninck (1959) eluted the inhibitor- β zone from paper chromatography, using the ether extract from rapidly growing fruit of lupins (Lupinus luteus). He found that the inhibitor- β counteracted IAA wheat coleoptile section growth and also accelerated abscission of lupin fruit. Therefore he called the inhibitor- β an abscission-accelerator. Rothwell and Wain (1964) furthered the initial investigation of van Steveninck, and isolated a fruit abscission substance in lupins.

It became clear that the three inhibitory compounds thus isolated were the same and the trivial name abscisic acid was agreed upon (Addicott and Lyon, 1969).

7.2.2. Solvent partition at different pH's with different organic solvents

ABA is an acidic compound and the solvent partitioning procedure is similar to IAA. The fractionation of ABA in the solvent partitioning can be referred to in the sections as IAA and gibberellins. The special characteristics of ABA during purification will be covered in this section. Partitioning from water into diethyl ether or ethyl

acetate has been commonly used for both IAA and ABA by many research workers. However, chloroform gave a good separation from the aqueous phase in an acidic condition (Mayak and Halevy, 1972; Mayak, Halevy, and Katz, 1972), while Rudich, Halevy, and Kedar (1972) preferred using methylene chloride to fractionate ABA in the initial solvent partition after removal of MeOH from MeOH extract; the aqueous solution at pH 8.3 was extracted with methylene chloride and then the alkaline solution was adjusted to pH 3.0 and extracted five times with methylene chloride into which the ABA partitioned.

The behaviour of ABA in solvent partitioning has been tested at different pH values with a different solvent system in studies on hormonal balance (especially auxins, gibberellins, and ABA) (Monselise, Goren, and Costo, 1967). Diethyl ether could remove ABA and IAA from the aqueous phase at pH 7.0, although the optimum pH range for ether partitioning of IAA was reported as being between pH 5.5 and 2.5 by Larson (1955) quoted in Goldschmidt and Monselise (1968). Goren and Goldschmidt (1970) modified the extraction procedure to use pH 6.0 in the aqueous phase, obtaining 88 % of IAA-2-¹⁴C in the diethyl ether. They also found that di-isopropyl ether can remove ABA at pH 6.0, but only 7 % IAA (See Goldschmidt et al., 1973). On the other hand this solvent could not transfer IAA and ABA-like substances at pH 7.5 nor gibberellin-like substances at pH 6.0 (Goren and Goldschmidt, 1970; Zucconi and Goren unpublished data from Goldschmidt, et al., 1973).

On the basis of these results Goren and Goldschmidt (1970) studied ABA, IAA and gibberellins in orange fruit tissues, as follows: The concentrated aqueous phase of the 80 % MeOH extract was adjusted to pH 6.0 and extracted with di-isopropyl ether for ABA and subsequently extracted with diethyl ether for auxin-like substances. The aqueous solution then was adjusted to pH 3.0 and extracted with diethyl ether for gibberellins (Goren, Goldschmidt, and Monselise, 1971). Furthermore a neutral inhibitor fraction could be separated by di-isopropyl ether at pH 7.2 after removal of MeOH from the aqueous phase (Goldschmidt, Goren, Even-Chen, and Bittner, 1973).

In order to improve removal of mildly acidic components in the organic phase which was extracted from the aqueous phase at pH 2.5-3.0, extraction was alternated 3 x between 1/4 v of .5% sodium bicarbonate and 1/4 v of distilled water (Badr, Martin, and Hartmann, 1971; Firn, Burden, and Taylor, 1972).

7.2.3. A neutral inhibitor in the neutral fraction

Recently a number of discrete neutral inhibitors in the neutral fraction have been isolated. Xanthoxin was isolated and identified from dwarf bean (Phaseolus vulgaris) and wheat (Triticum vulgare) by Taylor and Burden in 1970. Since then, the presence of xanthoxin in many kinds of plants has been detected, although not in some plant tissues such as tomato shoots, maple seed, rose hips, potato peelings, pea and bean roots, liverwort (Marchantia polymorpha) and a brown seaweed (Fucus serratus) (Firn, Burden, and Taylor, 1972). Cis, trans-xanthoxin is now reported as an endogeneous growth inhibitor which exhibits structural similarities and equivalent activities in a range of physiological tests to ABA. The method by which xanthoxin was extracted by Firn, Burden, and Taylor (1972) is to take only the neutral fraction. That is, the ether phase after it was extracted from 5 % sodium bicarbonate solution was reduced in vacuo and the residue partitioned between 80 % MeOH and an equal volume of light petroleum (60-80°C). The 80 % MeOH phase was made up to 50 % MeOH and then extracted again with petroleum ether. Xanthoxin remained in the MeOH phase, the MeOH was removed in vacuo and the residue suspended in 5 % sodium sulphate solution and partitioned with ether. The ether phase was used for further purification by thin layer chromatography.

In the neutral fraction, capric acid is also isolated in iris bulb (Ando and Tsukamoto, 1974) and batatasine, from yam bulbils (Hasegawa and Hashimoto, 1973). The former can inhibit both seed germination and elongation of lateral shoots, while the latter seems to be causally involved in the dormancy of yam bulbils.

A neutral inhibitor identified as 1-acetoxy-2,4-dihydroxy-n-heptadeca-16-ene, a fatty acid, was also isolated from a basic aqueous phase at pH 9.0 with petroleum ether from avocado mesocarp (Bittner, Gazit, and Blumenfeld, 1971). This new compound inhibited not only soybean callus growth but also wheat coleoptile sections. The inhibition was not due to toxic material and the growth could resume when the medium containing the inhibitor was removed. When a methanolic extract of avocado mesocarp was chromatographed on paper in isopropanol/ammonia/water (10:1:1 v/v), the inhibitory activity at Rf 0.8-0.1 was well separated from the inhibitor- β zone (probably ABA) at Rf 0.6-0.75.

7.2.4. A conjugate of abscisic acid

Since Koshimizu, Inui, Fukui, and Mitsui (1968) isolated and identified a conjugate of ABA as abscisyl- β -D-glucopyranoside from yellow lupin (Lupinus luteus), there has been a number of reports which

indicate that the conjugate exists in various plants, e.g., in developing and ripe strawberries (Rudnicki and Pieniazek, 1971) in tomato plants (Milborrow, 1970), in citrus fruit peel (Goldschmidt, Goren, Even-Chen, and Bittner, 1973), in blackcurrant (Ribes nigrum) buds and beech buds (Fagus sylvatica) (Wright, 1975).

A common technique used to determine conjugates of ABA is to hydrolyze and then extract as for free ABA, i.e., the aqueous phase after ether extraction at pH 3.0 is adjusted to pH 11.0 and held for 30 min. at 60°C which hydrolyses the conjugate and free ABA is then extracted at pH 3.0 with an organic solvent.

7.2.5. Column chromatography for purification of ABA

Column chromatography is often used to purify ABA extracted from large quantities of plant material. For example, Ohkuma, Lyon, Addicott, and Smith (1963) extracted 225 kg of fresh cotton fruits with 80 % acetone and 147 gram of crude acidic fraction was obtained after solvent partitioning with ether at pH 3.0; a celite/charcoal column (2 : 1) reduced this to 4.15 g of oily material, when eluted with increasing acetone in water (10 to 100 per cent). Goodwin and Gordon (1972) used 5 per cent steps in the same solvent and column system. Walton, Dorn, and Fey (1973) changed the ratio of charcoal : celite to 3 : 2, and MacMillan and Pryce (1969) isolated phaseic acid from fraction 40, eluted with 47 % acetone in water from a celite-charcoal (2 : 1) column.

In the purification of ABA in the plant extracts, one of the commonly used columns was of celite-silicic acid (2 : 1), using a stepwise elution technique as mentioned for gibberellin purification (MacMillan, Seaton, and Suter, 1960; Cavell, MacMillan, Pryce, and Sheppard, 1967). Gabr and Guttridge (1968) used twice the celite-silicic acid column for purification after first using a B.D.H. granular animal charcoal column. The first celite-silicic acid column eluted with ethyl acetate in chloroform starting with 5 %, then 10 % and after that in 10 % steps ending with pure ethyl acetate, exhibited an inhibitory activity in the fractions of 5, 10, and 20 % ethyl acetate. For further purification of the inhibitory substances the combined fractions were applied on the second celite-silicic acid column and eluted with increasing concentrations of ethyl acetate in chloroform in 10 % steps, inhibitory activity being confined to the 10 % ethyl acetate in chloroform fraction. Most (1971) employed a different

solvent system such as benzene containing 0, 20, 40, 60, 80, and 100 % of ethyl acetate or 10, 20, 25, 30, 40, 45, and 50 %, with the final fraction being pure methanol. Most (1971) modified this solvent system by grading 100 % ethyl acetate to 100 % methanol in 20 % steps followed by Bio-gel P-2 column (40 % ethanol or water) (Most, Gaskin, and MacMillan, 1970)

Mayak and Halevy (1972) employed a 30 g silica gel (0.05 - 0.2 mm) column for the purification of rose petal extract using the solvent benzene: ethyl acetate: acetic acid (50 : 5 : 2 v/v/v). In this column ABA was detected in a 132 ml volumn from fraction 27 to 35 and was then analysed by GLC. In silica gel partition column chromatography, Glenn, Kuo, Durley, and Pharis (1972) could not resolve well ABA from GA₄ and GA₇ using the gradient elution techniques of Powell and Tautvydas, using ethyl acetate in n-hexane (saturated with 0.5 M formic acid).

In Sephadex G-10 and G-25 column chromatography ABA has a nearly identical retention volumn to GA₃ (Glenn, Kuo, Durley, and Pharis, 1972) and also on G-15 (Reynolds, 1970).

Klingström (1969) used Sephadex LH-20 to remove a number of inhibitory substances from pine extracts. A successful purification of inhibitors was achieved by Sephadex LH-20 eluted with 96 % of ethanol with 0.001 M HCl (Eliasson, 1969; Alden and Eliasson, 1970, Steen and Eliasson, 1969), ABA and IAA are well resolved on Sephadex LH-20 eluted with 96 % ethanol. ABA in the column was eluted from about 110 to 140 ml where the greatest inhibition of wheat coleoptile sections was found in Picea abies (Eliasson, 1969; Alden and Eliasson, 1970).

One of the greatest difficulties in plant hormone analysis is the presence of interfering inhibitory substances in the plant extracts. One of the most promising techniques is to use Polyclar AT, which is an insoluble form of the polymer, poly-N-vinylpyrrolidone (PVP). Polyclar AT can retain most phenolic compounds in plant tissue extracts by hydrogen bond formation. A number of research workers have used PVP either as a slurry which is filtered or column chromatography (Lenton, Perry, and Saunders, 1971; Eady and Eaton, 1972; Hewett and Wareing, 1973 a; Ivy, 1974; Tustin, 1975) and have obtained greatly improved results. Glenn, Kuo, Durley, and Pharis (1972) demonstrated that ABA, IAA, GAs, and zeatin could be well resolved by different pH buffer solutions. The data of Glenn et al., (1972) indicated that ABA was eluted more or less within the same range as GA₁, GA₇, GA₄, GA₅, GA₂, GA₈, GA₉, and GA₁₃, but was completely resolved from IAA and zeatin at

pH 8.0, and GA₁₃ at pH 5.0. After the initial purification of plant material through the PVP column, the residue was subjected to the silica gel-celite column for further purification (Glen, et al., 1972).

7.3. Gibberellin-like substances

7.3.1. Historical progress of isolation for gibberellins

The evidence of existence for a growth promoting substance produced by a rice disease fungus, Gibberella fujikuroi by Kurosawa in 1920's led in 1939 to the isolation of the crude gibberellin, a crystalline material with plant growth promoting properties, by a group of Japanese scientists in Tokyo (Yabuta and Hayashi, 1939). Immediately on spreading information about the gibberellins from East to West after 1945, almost simultaneously two groups, Stodola in 1951 at the Northern regional Research Laboratory of U.S.A. and Curtis in 1954 at Imperial Chemical Industries in Britain isolated gibberellin A and gibberellin X and gibberellic acid respectively. The name proposed by Cross in 1954 was gibberellic acid and this was agreed to by the above three groups (See Weaver, 1972).

7.3.2. Gibberellin-like substances in different fractions

7.3.2.1. An acidic fraction

7.3.2.1.1. Extraction of gibberellins at pH 2.5-3.0

The extraction methods used by Curtis and Cross (1954) and Stodola, Raper, Fennell, Conway, Sohns, Langford, and Jackson (1955) originated from Japanese workers. However a slight difference in the methods between Curtis et al. and Stodola et al. was that the ethyl acetate phase extracted from aqueous solution adjusted to pH 4.0 or less was partitioned with phosphate buffer at pH 6.3 by Curtis and Cross; whereas Stodola et al. (1955) partitioned the acidic aqueous solution with ethyl acetate. The extraction methods for gibberellins used by Curtis and Cross (1954) and Stodola et al. (1955) are more or less the same as outlined by Bentley and Houseley (1952) and Kefford (1955) for IAA, except that ethyl acetate was used instead of ether. That is the aqueous phases which were acidified to pH 2.5-3.0 before partition were either extracted directly from plant materials or eluted from activated charcoal. Endogenous gibberellin-like substances were partitioned by this method at pH 2.5-3.0 by many research workers (Radley, 1958; Radley and Dear, 1958; MacMillan, Seaton, and Suter, 1960; Corcoran and Phinney, 1962; Dennis and Nitsch, 1966; Ogawa, 1966; Murofushi, Takahashi, Yokota, Kato, Shiotani and Tamura, 1969; Badr, Martin, and Hartmann, 1971; Jones, Stoddart, MacMillan and Cloke, 1971;

Naito, Inoue, and Bukovac, 1972).

7.3.2.1.2. Extraction of gibberellins at pH 8.0-8.5

West and Phinney (1959) were probably the first who modified the extraction procedure to use the aqueous phase at pH 7.0. At this pH ethyl acetate was particularly efficient at removing inhibitors. Petroleum ether and chloroform at pH 5.0 were also used for the same purpose (Kohler and Lang, 1963). There are a number of research workers who have used the ethyl acetate method, although the pH used varies from 6.2-9.0 (Hayashi, Blumethal-Goldschmidt and Rappaport 1962; Hayashi and Rappaport, 1962, 1965; Hashimoto and Rappaport, 1966 a & b; Reid and Carr, 1967; Crozier, Aoki, and Pharis, 1969; Durley, MacMillan, and Pryce, 1971; Pieterse, Bhalla and Sabharwal, 1971; Ross and Bradbeer, 1971 a and b; Zeevaart, 1971; Eady and Eaton, 1972; Yamamura and Naito, 1973).

7.3.2.2. A neutral fraction

Since Wierzchowski and Wierzchowska (1961) found two gibberellin-like substances in the neutral fraction from Gibberella fujikuroi (Saw) Wr. cultures, Hayashi and Rappaport (1962, 1966) attempted to extract a gibberellin-like substance in the neutral fraction as well as acidic fraction. In their extraction procedures MeOH extract was evaporated to the water phase and the water phase, adjusted to pH 7.5, was extracted three times with ethyl acetate. The ethyl acetate phase then was partitioned with 1 per cent sulphuric acid, leaving only neutral substances. The concentrated ethyl acetate fraction was chromatographed on paper with isopropyl alcohol : ammonium hydroxide : water (10 : 1 : 1 v/v/v). The Rf 0.3-0.4 eluate showed activity in the dwarf maize d_1 or d_2 , bioassay and was called potato factor I. After standing for two weeks in contact with the air at room temperature potato factor I was rechromatographed with the same solvent system mentioned above. The new activity of dwarf pea growth was found at Rf 0.5-0.6, whereas the previous growth activities at Rf 0.3-0.4 disappeared (Hayashi and Rappaport, 1965), suggesting that the neutral gibberellin had been changed into an acidic gibberellin.

With the same technique as mentioned above, Hashimoto and Rappaport (1966 a & b) found a neutral gibberellin-like substance similar to that of potato factor I (Hayashi and Rappaport, 1962). They concluded that the neutral gibberellin-like substance in the neutral fraction could be a reserve form of gibberellin. They demonstrated that the contents of neutral gibberellin remained constant in immature

seed (Phaseolus vulgaris L.) but markedly increased in mature seeds. Skene (1962) also demonstrated the presence of a non-acidic gibberellin-like substance in green bean seeds.

7.3.2.3. Bound or conjugate gibberellins

McComb (1961) demonstrated the occurrence of 'bound' gibberellin in mature runner bean seeds using a simple technique of paper chromatography with three different solvents; a mixture of acetone and water, phosphate buffer pH 6.2, and phosphate buffer (pH 6.2) after hydrolysis with ficin, a proteolytic enzyme. A number of investigators have obtained similar results using different species as reviewed by Lang (1970). Lang cited the terms 'conjugated GAs' (introduced by Sembdner et al.) and 'bound GAs' terms which have continued to be used for these, as yet unidentified substances. These compounds cannot be extracted with ethyl acetate from the aqueous phase at pH 2.5-3.0 but are extractable with butanol so that they are called water-soluble and butanol-soluble gibberellins. The bound compounds must first be hydrolyzed with acidic, basic, or enzymatic treatments. There was also evidence that $^3\text{H-GA}_1$ applied to young Pharbitis plants was easily converted to two water-soluble compounds (Barendse, 1971). Harada and Yokota (1970) also isolated gibberellin A_8 -glucoside from shoot apices of Althaea rosea by extraction with n-butanol from the aqueous phase which had been previously extracted with ethyl acetate at pH 3.0. Similarly GA_3 -glucoside was isolated from immature seeds of Morning Glory (Tamura, Takahashi, Murofushi, Yokota, and Kato, 1968) and GA_3 glucoside from immature seed of yellow broome (Cytisus scoparius L.) (Yamane, Yamaguchi, Murofushi, and Takahashi, 1971). Glucosyl esters of GA_{38} was isolated from mature seeds of Phaseolus. The glycosyl ester of this gibberellin is one of the butanol-soluble neutral gibberellins.

7.3.3. Partition of gibberellin with different solvent

It is apparant that different gibberellins have markedly different polarities.

The partition coefficients of gibberellins between phosphate buffer and some commonly used organic solvents for 27 gibberellins were reported by Durley and Pharis (1972). At pH 3.5 or less, gibberellins GA_4 and GA_7 or GA_9 and GA_{10} totally partitioned into ethyl acetate. However a number of gibberellins, for example, GA_4 and GA_7 or GA_9 and GA_{10} partitioned significantly into the ethyl acetate at pH 8.0, whereas GA_5 , GA_6 , GA_{16} , GA_{19} , GA_{20} , and GA_{27} did not. GA_1 , GA_2 , GA_3 , GA_8 , GA_{22} ,

GA₂₆, and GA₂₉ did not appreciably partition into ethyl acetate at pH 8.0 or 6.5. GA₂₁ and GA₂₃ could also be included in this group. Most gibberellins entered ethyl acetate at pH 2.5, but di- and tricarboxylic GA₁₂, GA₁₃, GA₁₄, GA₁₇, GA₁₈, GA₂₁, GA₂₃, GA₂₄, GA₂₅, and GA₂₈ (exception GA₁₉) exhibited a rapid increase of partition coefficient with increasing pH, more so than the monocarboxylic acids.

Badr et al. (1972) reported that diethyl ether could remove 50 % of labelled GA₃ from the aqueous phase at pH 2.5. Methylene chloride was used at low pH for gibberellin extraction (Luckwill, Weaver, and MacMillan, 1969). Hayashi, Blumenthal-Goldschmidt, and Rappaport (1962) tested solubility of gibberellin A₁ and A₃ to A₉ in chloroform from water at pH 8.0, 6.0, 5.0, and 2.5 using bioassay with Morse's NO.9 dwarf pea. GA₅ and GA₁ were partially partitioned with chloroform at pH 6.0, 5.0, and 2.5 but not at pH 8.0. Furthermore the degree of solubility of GA₇ was greater at pH 6.0 than that at pH 5.0 and 2.5, whereas GA₅ was the opposite. The other gibberellins were almost negligible in solubility for chloroform.

Coombe and Tate (1972) isolated a polar gibberellin A₃₂ from Prunus armeniaca L.. They macerated frozen apricot seeds with 0.5 M Na₂SO₄, and 1.0 M Na₂S₂O₅, adjusted to pH 2.5 with H₂SO₄ and then blended the mixture with n-butanol, separating the two phases by centrifugation. The aqueous phase was re-extracted with n-butanol and the combined butanol fractions were further partitioned with water at pH 10.0. In fact, a polar gibberellin-like substance partitioned partially with ethyl acetate at pH 2.5 from apricot seeds could be GA₃₂ in view of partitioning characteristic and polarity (Jackson and Coombe, 1966). Yamaguchi, Yokota, Murofushi, Ogawa, and Takahashi (1970) also isolated GA₃₂ from immature seed of Prunus persica in the n-butanol-soluble acidic fraction after partitioning with benzene and ethyl acetate respectively. In Prunus cerasus L. cv Montmorency, Naito, Inoue, and Bukovac (1972) partitioned firstly ethyl acetate soluble-GA-like substance and subsequently a GA-like butanol-soluble substance in the acidic aqueous phase. They suggest that this butanol-soluble gibberellin could be GA₃₂, because it was not a characteristic glycoside but resembled GA₃₂ in chromatographic behaviour and partition characteristics.

7.3.4. Counter current distribution

Counter current distribution has provided an improved purification method for special large scale extractions. This technique was

used at the middle steps of purification after solvent partition (West and Phinney, 1959; Murofushi, Takahashi, Yokota, Kato, Shiotani, and Tamura, 1969), and then a further purification was made by adsorption or partition column chromatography and paper or thin layer chromatography.

7.3.5. Column chromatography

7.3.5.1. Partition column

Gibberellin extracted from the filtrate of fungus culture have been absorbed onto carbon or a mixture of carbon and celite. Further purification was by solvent partition and then column chromatography. Stodola, Nelson, and Spence (1957) developed the use of partition chromatography on a buffered celite column with ether as the initial step for the chromatography of gibberellin-like substance in plant extracts and managed to separate 'gibberellin A' and GA_3 . Grove, Jeffs, and Mulholland (1958) also separated gibberellin A_1 and GA_3 with a similar celite column. They confirmed that 'gibberellin A' was identical to GA_1 . Kende and Lang (1964) modified the celite column to use ethyl acetate instead of ether used in the Stodola's method, by using 0.5 M phosphate buffer (pH 6.4). They found that 1 μ g of GA_5 and GA_1 were well resolved by the column. A number of research workers have detected gibberellin-like substances from various plants with this method (Most and Vlitos, 1964; Crozier and Audus, 1968). Most and Vlitos (1964) had met some difficulties of bioassay with dwarf pea and cucumber when using this method. With the further purification of plant extracts through a buffered celite column they found the existence of free and bound forms of gibberellin-like compounds in stem apices of sugar cane.

After studying indole compounds in the silica gel partition column (Powell, 1960), Powell and Taudvydas (1967) applied the same partition column to the resolution of 7 gibberellins with n-hexane saturated with 0.5 M formic acid and ethyl acetate by stepwise and gradient elution techniques. The results obtained by them were that a stepwise technique was a far better characterization than gradient elution, although the former took 5-6 hours to elute all the gibberellins. This system was applied by Kozel and Tukey (1968) and Ruddat, Pharis, Aoki, and Crozier (1968). Durley, Crozier, Pharis, and McLaughlin (1972) tested the silica gel partition chromatography over three years. They found that the most satisfactory products were Mallinkrodt No. 2847 (100 mesh), Mallinkrodt SilicAR CC4 (100-200 mesh), and Woelm Silica Gel containing 20 % water as stationary phase. However, because of difficulties such as 'dry out problem' with Mallinkrodt

products, they reported that Woelm Silica Gel was a useful and convenient means for the partial analysis of gibberellins in plant extracts, although 33 gibberellins were not completely separated.

7.3.5.3. Gel chromatography

Sephadex gel appears to give a good purification of gibberellins from plant extracts by partition chromatography (Kohler, 1965; Koshimizu, Fukui, Kusaki, Ogawa, and Mitsui, 1968). Crozier, Aoki, and Paris (1969) had compared several purification methods. They found that the methods using counter current distribution, Sephadex G-10, and silicic acid partition improved 25 fold compared with ion exchange resins, basic lead acetate treatment, and a phosphate buffered celite column from 1,000 Phaseolus multiflorus seedlings. Sephadex G-50 has been used with phosphate buffer and a n-butanol-ethyl acetate system to isolate new gibberellins such as PG-I and PG-II which have different solubility in the solvents used by Yamaguchi, Yokota, Murofushi, Ogawa, and Takahashi (1970). Pitel, Vining, and Arsenault (1971) have also improved the separation of closely related gibberellin on a preparative scale with initial fractionation by silicic acid adsorption chromatography. Of the several grades of Sephadex tried, G-15 eluted with water gave the sharpest separation (Reynolds, 1970). Vining (1971) successfully separated gibberellin A₁ and dihydrogibberellin A₁ by argentation partition chromatography on a Sephadex G-25 column.

7.3.5.4. Ion exchange column

Cation and anion exchange resin column chromatography has been used by Radley (1958), Jones (1964) and Crozier and Audus (1968). However, Crozier (personal communication) did not recommend using the resin column for gibberellin separation.

A cellulose column for purification of gibberellins was eluted at the final purification by petroleum, chloroform, butanol, ethyl acetate, and 3 % NH₄OH ethanol succeedingly (Hayashi, Bluemthal-Goldschmidt, and Rappaport, 1962). A cellulose column was also used by Reinhard, Kato, and Lang (1960) and DEAE-Sephadex column (300 g) was used by eluting with a water (2 L) and 2 N acetic acid (2 L) linear gradient by Coombe and Tate (1972).

7.4. Cytokinin-like substances

7.4.1. Discovery of kinetin

In the literature reviewed from 1940-1959 by Steward and Shantz (1959), evidence was given that growth promotive substances extracted

from coconut milk, immature fruits of Zea mays, Aesculus woerlitzensis, Juglans sp., Musa sp., and the female gametophyte of Ginkgo biloba could stimulate cell division. This stimulation seemed similar to that produced by kinetin, 6-furfurylamino purine, which was isolated by Miller, Skoog, van Saltza, and Strong (1955). In a review of kinetin and related compounds in plant growth (Miller, 1961 a), the discovery of kinetin is briefly summarized as follows: A group of scientists at Skoog's laboratory studied the chemical control of differentiation of plant tissue in sterile culture. The continuous cell division of callus tissue required IAA plus some growth factor derived from coconut milk or yeast extract. This growth factor could be extracted with 95 % ethanol from yeast, could be precipitated with silver nitrate under acidic conditions, the material precipitated with silver and extracted into ether. The active component eluted from paper chromatography has an absorption maximum at 268/nm. This evidence suggested that the active component might be a purine and in fact an active component was found in aged DNA but not in RNA. Finally the active component was isolated in crystalline form from autoclaved herring sperm DNA, and identified as 6-furfurylamino purine (kinetin) formed from the molecular re-arrangement of deoxyadenosine released from the DNA.

7.4.2. Historical progress of isolation for zeatin

In the early 1960's there were a number of groups who had attempted to isolate a kinetin-like substance from immature maize or young fruits (Miller, 1961 b; Beauchesne, 1961; Letham, 1963 b; Zwar et al., 1964; Kende, 1964; Stowe et al., 1964; Loeffler and van Overbeek, 1964; Wood, 1964). The methods used for isolating the substitute purine derivative from the immature maize was basically the same as the purification of kinetin from autoclaved DNA, finding a chemical and physical properties of kinetin, that is, (1): amphoteric properties acting as a cation under acidic conditions and as an anion under alkaline conditions and therefore ion exchange column such as Dowex 50 (cation) or Dowex 1 (anion) could be used at pH 2.5 or 10.2, (2): precipitation properties from an acidic solution by silver ions, possibly natural related compounds will be substituted with groups such as sugar which could prevent the precipitation, (3): extractable properties from water solution into diethyl ether at pH 9.3, although natural compounds have slightly or no solubility in ether, (4): stabilities in strong acidic or basic conditions (Miller, 1961 a & b, 1963). Therefore Miller already presented some criteria to purify cytokinin-like substances to be extracted from plant materials. Miller's extraction procedure to isolate a purine derivative

presented kinetin-like activities and was as follows: A 65 per cent ethyl alcohol extract from a creamier maize was prepared at room temperature. The ethanol extract on its aqueous residue was passed through a Dowex 50 W-X 8 (H^+ , 50-100 mesh) column and then was eluted with 6 N NH_4OH after a large volume of water washing. In some cases it was eluted with 1.5 N HCl, and followed by 4 N HCl. The concentrated solution free from NH_4OH could be passed once more through Dowex 50 (H^+) column. Whatman No.1 paper was used for paper chromatography and the solvent system was a mixture of n-butanol, water, and conc NH_4OH . The final yield of active compounds was 17.7 mg from 84 kg of immature corn maize. In fact this compound was confirmed to be identical with zeatin which Letham isolated first as pure crystals in 1963 b independently (See below). Later research by Miller (1965, 1967 a & b) and Miller and Witham (1964) followed his main pattern of extraction procedures, although some have slightly modified his methods. Klambt, Thies, and Skoog (1966) used nine steps to purify crude bacterial extracts, that is, Dowex 50 W-X 4 H^+ column, Ag precipitate, butanol and $CHCl_3$ extracts, and Whatmann 3 mm paper chromatography, identifying the fractions as 6-(α,α -dimethylallylamino)purine, nicotinamide, and 6-methylaminopurine.

It was Letham working for the D.S.I.R. in New Zealand who first isolated a cytokinin-like substance after having found a kinetin-like activity in crude apple extract (Letham and Bollard, 1961). This compound was found to be identical to the active compound isolated from sweet corn extracts and was given the trivial name of Zeatin (Letham, 1963 b). In the development of his extraction procedures he examined the cytokinin activities in the plum fruit extract fractions (Letham, 1963 a), Letham found that ethyl acetate extraction from the natural aqueous phase (pH 3.3), after evaporation of alcohol, had a high content of inhibitors but the butanol phase extracted from the aqueous phase adjusted to pH 7.0 had a significantly higher content of cytokinin activity as measured by proliferation cell division of carrot phloem tissues. He found also that the level of inhibitors in the ethyl acetate phase reduced in order from pH 2.0, 3.3, 4.0, and to 7.1 in the aqueous phase. Probably this scheme of extraction procedure for cytokinins in plant tissues, as the solvent partitions such as ethyl-butanol pattern was first to suggest, separates cytokinins from IAA-like, ABA-like, and gibberellin-like substances. The methods used by Letham have varied to some extent, although the principle extraction procedures were the same. Both Letham's and Miller's methods are based on ion exchange column chromatography and precipitation with silver nitrate. The detailed extrac-

tion procedures that Letham has used are as follows: (1) the initial purification of cytokinin-like substances with ion exchange resin from ethanol extract before or after solvent partitioning with ethyl acetate-n-butanol at different pH's or, in some case the use of ethyl ether (Letham, 1974); (2) pretreatment with charcoal (Letham, 1974) before loading on to the ion-exchange column; (3) precipitate with silver nitrate, and in some case partition chromatography on a cellulose column and paper chromatographic purification (Letham, 1963 a & b, 1964, 1966 a, b, and c, 1973, 1974).

One of other groups who had investigated cytokinin-like substances from apple fruitlets were Zwar, Bottomley, and Kefford in 1963 at C.S.I.R.O. in Canberra, Australia. The scheme of extraction procedures used by Zwar et al. basically originated from the method of Miller. The methods used by them after purification with ethyl acetate were as follows : (1) adsorption on cation-exchange resin (Dowex 50) and displacement with 2 N ammonium hydroxide, (2) adsorption on carbon and elution with pyridine-ethanol ammonium hydroxide, and (3) passage through polyamide resin. They found at least four zones of highly cytokinin-like activity from the aqueous phase after paper chromatography. In 1970 Zwar and Bruce obtained cytokinin activities from extract of young apples and from coconut milk. They, however, suggested that cytokinins in apple fruit extract may not be the same as zeatin and zeatin riboside, as suggested by other work (Letham, 1963 a, Letham and Williams, 1969).

7.4.3. An outline of extraction procedures and column chromatography

Late in the 1960' much literature has reported cytokinin-like substances in various plant tissues, using the above methods. A brief summary for the extraction procedures is as follows. Initial extraction from plant tissues was made using 80 % aqueous ethanol or methanol either at room temperature or at 1-2°C from 1 to 3 days. After alcohol extraction in order to remove some inhibitors from the aqueous phase, solvent partition was employed with petroleum ether (Maheshwari and Gupta, 1967), or diethyl ether or ethyl acetate at the acidic condition (Letham, 1963 a), although petroleum can remove some cytokinin activity, suggesting resemblance to the compound found by Sassa (van Staden and Wareing, 1972). Before subjecting to solvent partition, the aqueous solution can be slurried with polyvinylpyrrolidone (PVP) and then the slurried solution filtered (Hewett and Wareing, 1973 a).

Cytokinins are now known to be 6-substitute purines as free bases and their ribosides and ribotides. The 6-substitute purine and their riboside can be extracted by butanol at pH 7.0-8.5 (See section 7.4.2.1.) while ribotides remaining in the aqueous phase can be hydrolyzed at the alkaline condition by phosphatase as mentioned in section 7.4.2.2.

7.4.3.1. The 6-substitute purines and their ribosides

7.4.3.1.1. Dowex 50 column

The residue of the butanol phase or the water phase before butanol partition, can be purified with ion exchange columns. Particularly Dowex 50, cation exchange resin with which free base and nucleosides can be retained and then eluted with 1.5-6.0 N ammonia has been employed by many workers (Miller, 1961 b; Zwar, et al., 1964; Seth and Wareing, 1965; Maheshwari and Prakash, 1967; Klambt, Thies, and Skoog, 1966; Gupta and Maheshwari, 1970; Tegley and Witham, and Krasnuk, 1971; Woolley, 1971; Hewett, and Wareing, 1973 a; Rybicka and Engelbrecht, 1974).

7.4.3.1.2. Zeo-Karb 225 column

The cation exchange resin Zeo-Karb 225 was used by Letham (1963-1975) and Hewett and Wareing, 1973 b). 'Zeo-Karb 225' as H form was eluted with 4 N HCl (Letham, 1963 b) and 'Zeo-Karb 225' as NH_4 form was eluted with 1.5 N NH_4OH (Letham, 1968). Letham (1968) separated cytokinin activity from sweet corn using firstly a cation exchange column, and secondly an anion exchange column.

7.4.3.1.3. An anion exchange resin column as Dowex 1

Dowex 1 with its strong anion exchange properties has given poor recovery of cytokinin activity (Zwar and Skoog, 1963). However, most of coloured materials in the extract was removed by Dowex 1 (50-100 mesh; formate form) and further purification was made by using DEAE cellulose (Browning, 1973). Dowex 1 x 10 (200 mesh) also was used by Seth and Wareing (1965) and Gupta and Maheshwari (1970), BIO-RAD AG 1 x 8, 200-400 mesh (Cl^-) form by Tse (1974), and De-Acidite FF (Acetate form) by Letham (1968, 1974).

7.4.3.1.4. Other columns

Sephadex LH-20, using 25-30 % ethanol gives good resolution between several cytokinins (Armstrong, Burrows, Evans, and Skoog, 1969; Burrows, Skoog, and Leonard, 1971; Hewett and Wareing, 1973 a,b,& c;

Horgan, Hewett, Purse, and Wareing, 1973 a).

Paper roll column chromatography has been used by Matsubara and Koshimizu (1966). With the plant hormone analysis, much difficulty is caused by the presence of phenolic compounds. To discard phenolic compounds as well as other plant components a number of different columns have been employed such as polyamide powder (Zwar, Bottomley, and Kefford, 1963; Letham and Williams, 1969) and cellulose phosphate in NH_4^+ form (Letham, 1969; Skene, 1972 a; Skene and Antcliff, 1972). One of the best columns for removing phenolic compounds may be insoluble polyvinylpyrrolidone (Glenn, Kuo, Durley, and Pharis, 1972; Thomas, Carroll, Isenberg, Pendergrass, and Howell, 1975).

7.4.3.1.5. Precipitation

Silver nitrate has been used to purify purine unsubstituted in position 9 (Miller, 1961 a; Letham, 1964, 1966 a, and 1969; Maheshwari and Prakash, 1967). Silver precipitation, however, could bring about considerable loss of cytokinin activity when especially low activity solutions were used (Woolley, 1971).

Mercury salts can also be used for nicotinamide-type cytokinins (Wood, 1964; McKenzie and Street, 1972). Of several heavy metal precipitants tested, mercuric acetate was the most effective precipitant for crude extracts of pea seedlings (Zwar and Skoog, 1963).

7.4.3.1.6. Paper and thin-layer chromatography

Paper and thin-layer chromatography have been employed when further purification is necessary, using various solvent systems.

7.4.3.2. Zeatin ribotide and other bound forms of cytokinins

Miller (1965) and Letham (1966 c) also investigated cytokinin-like compound in sweet corn other than Zeatin and finally Miller (1965) and Letham (1966 c) crystallized Zeatin ribotide.

In solvent partition, Zeatin ribotide and other bound form of cytokinins remain in the water phase after butanol extraction at pH 7.0-8.5.

In order to purify nucleotide cytokinins from the water phase after butanol partition or from crude ethanol extracts, the following techniques have been used (1) adsorption with charcoal (Letham, 1966 a, 1974), (2) binding with anion exchange resin, such as De-Acidite FF at pH 11 as mentioned in section 7.4.2.1.2. (Letham, 1968) and anion exchange columns at neutral pH's (Yoshida and Oritani, 1972), (3) precipitation with barium salts (Miller, 1965; Woolley, 1971). After eluting

the columns or releasing precipitant, the residue of nucleotide cytokinins can be resuspended with water and then further purification by solvent partitioning after enzymic conversion, using alkaline phosphatase to form the riboside (Woolley and Wareing, 1972; van Staden, Webb, and Wareing, 1972).

7.4.4. The occurrence of cytokinins in different plants

Zeatin has been widely found either as purified crystal form or by co-chromatographic studies in, Zea mays kernels (Letham, 1963 b, 1964, 1966 b & c; Letham and Miller, 1965), Rhizopogon roseolus (Miller, 1967 a, 1968), apple and plum fruitlet (Letham, 1964, 1966 a, 1969; Letham and William, 1969), immature sunflower fruits (Miller and Witham, 1964), sunflower leaves and root exudate (Klambt, 1968), pumpkin seeds (Gupta and Maheshwari, 1970), shoots of grape (Skene, 1972 a), buds and leaves of Populus tremula, and Acer platanoides (Engelbrecht, 1971), leaves of Populus x Robusta (Hewett and Wareing 1973 b), Zeatin riboside and Zeatin ribotide have been reported from almost all the plant tissues in maize kernels, coconut milk (Letham, 1968, 1974), Rhizopogon roseolus, and Cichorium intybus roots (Bui-Dang-Ha and Nitsch, 1970), and leaves and root exudates of sunflower (Klambt, 1968), some other Zeatin-like substances derivatives have been reported in various parts of the plant kingdom; 6-(3-methylbut-2-enylamino)-purine in Corynebacterium fascians (Helgeson and Leonard (1966), 6-(3-methylbut-2-enylamino)-9- β -D-ribofuranosylpurine in tRNA of E. Coli and yeast or sRNA of several higher plants (Skoog and Armstrong, 1970 ; Gauss, et al., 1971), 6-(4-hydroxy-3-methylbutylamino)-purine (Dihydrozeatin) in immature lupin seed (Koshimizu, et al., 1967), Cis-zeatin riboside in sRNA of several plants (Hall, et al., 1967; Babcock and Morris, 1970; Playtis and Leonard, 1972; Vreman et al., 1972), 6-(3-methylbut-2-enylamino)-2-methylthio-9- β -D-ribofuranosylpurine in S. Epidermidis (Armstrong, et al., 1970), in tRNA of E. coli (Gauss, et al., 1971), 6-(4-hydroxy-3-methylbut-2-enylamin)-2-methylthio-9- β -D-ribofuranosyl-purine in sRNA of wheat germ (Burrows, et al., 1970), in sRNA of tobacco callus (Burrows, et al., 1971), and pea shoots (Vreman et al., 1972), 6-(2-hydroxybenzyl)amino-purine riboside (Horgan, et al., 1973 a; Hewett and Wareing, 1973 c).

7.5. Determination of plant hormones by gas-liquid chromatography (GLC)

In recent years, the technique of gas-liquid chromatography (GLC) has been used to analyse plant hormones. The technique can often resolve the closely related compounds. Furthermore combined gas-liquid chromatography and mass spectrometry (GC-MS) gives conclusive

identification of plant hormones. As gas chromatographic instruments supplementary equipment has improved, the use of GLC and GC-MS has opened a new era in which plant physiologists and biochemists can more clearly identify and quantify plant hormones which are linked with physiological and biochemical phenomena in plants.

7.5.1. Auxins by GLC

Ikegawa, Sumiki, and Takahashi in 1963 first reported the behaviour of GA₁-GA₉ in GLC and in 1964, Stowe and Schilke reported the characteristics of 19 indole compounds in a gas chromatographic separation on 10 % of Versamid 900 (an ethylenediamine linoleic acid polyamide of uncertain structure), polyneopentylglycol succinate, silicon SE-30, SE-52, and QF-1. They found satisfactory techniques for indoleacetic, indolepropionic, and indolebutyric acids by esterification with BF₃, but indole-3-carboxylic, indole-3-glycolic, indole-3-pyruvic, and indole-3-lactic acids were destroyed by the BF₃ catalysis method.

Powell (1964) was probably the first to determine IAA from plant materials such as maize and indoleacetonitrile in cabbage by GLC and spectrophotofluorometry. In his procedures, the residue of the collected fraction from the silica gel column was methylated by diazomethane and further purification was made by GLC using 7 % Versamid stationary phase and an Argon ionization detector (90 Sr) for individual indoles which were then determined by a spectrophotofluorometer.

With the same stationary phases used by Stowe and Schilke (1964) Grunwald, Mendez, and Stowe (1968) found that 3 % Versamid 900 and 3 % HI-EFF 8BP gave better results than 7.3 % silicone SE-30 and 4.8 % silicone SE-52 for 11 methylated indoles.

Davis, Heinz, and Addicott (1968) suggested the feasibility of analysis of plant hormones using the trimethylsilyl (TMS) derivatives of IAA, ABA, and GA₃ from crude acidic plant extract since the derivatives gave (1) no rearrangements, (2) stability, (3) easy separation, and (4) easy regeneration. However they did not determine IAA in developing cotton fruit but only ABA (See ABA GLC).

Using the stationary phase 3 % SE-30 and 3 % QF-1 and an interchangeable hydrogen flame detector the methylated esters of Indole-3-acetic acid, Indole-3-carboxylic acid, Indole-3-propionic acid, and Indole-3-butyric acid in several Nicotiana species were detected by Bayer (1969). The activity of the indole compounds obtained by GLC were checked by bioassay and spectrophotofluorometry.

Ueda, Ehmann, and Bandurski (1970) improved the fractionation of the myoinositol esters of IAA from maize kernels through several

complicated steps and identified IAA myoinositols, IAA myoinositol arabinosides, and IAA myoinositol galactosides by GC-MS analysis, using a hydrogen flame ion detector and columns packed with 3 % OV-1 on Chromosorb W, 2 % OV-1 on Gas-chrom Z, 3.8 % UC-Wp8 on Diatoport, and 3.7 % HiEff-8BP on Gas-chrom Z for GLC.

Alden (1971) confirmed only the presence of IAA in buds of Pinus silvestris using hydrogen flame ionization. Alden could not find any great advantages of GLC over fluorescence spectrophotometry in the determination of indole compounds.

El-Antably (1975) demonstrated the presence of a ratio 1 : 3.8 (137.5 : 52.5 $\mu\text{g/g}$ dry weight) for IAA and 2.1 : 1.0 for ABA (47.5 : 100 $\mu\text{g/g}$ dry weight) from the upper and lower halved horizontal shoots in Ribes nigrum by GLC. The methods used for the purification of extracts was solvent partition with ethyl acetate, thin layer chromatography with silica gel GF-254 developed with ethyl acetate: chloroform : acetate (15 : 5 : 1 v/v/v), and gas chromatography with a flame ionization detector and Epon 1001 on WW DMCS chromosorb W (60-80 mesh).

7.5.2. Abscisic acid by GLC

7.5.2.1. Determination of ABA by a flame ionization detector

Of plant hormone ABA has been the most widely measured in plant extract by GLC using both flame ionization and electron capture detectors.

After Davis, Heinz, and Addicott (1968) had characterized the behaviour of TMS derivatives of IAA, GA_3 , and ABA by BSA silylated reagent (bis(trimethylsilyl)acetamide) in gas liquid chromatography, they examined only ABA in developing cotton fruit in the acid fraction of ethyl acetate solvent partition at pH 2.0 by GLC. In the preliminary test on the TMS derivatives of the residue of the ethyl acetate fraction they found that there was a large peak which would mask ABA. Therefore they tested two different methods. The first method was to purify the TMS derivatives of the acidic fraction using 5 % SE-30 or 5 % QF-1 columns and a thermal conductivity detector and then rechromatograph the collected samples on the flame ionization instrument. This method resulted in a considerable loss of ABA. In the second method tested additional purification was made by a celite-charcoal column using a step wise elution and then injecting TMS derivatives of the sample on columns packed with 5 % SE-30 and 5 % QF-1 stationary phase on 60-80 mesh Chromosorb W. This method gave better recovery and they estimated that the level of ABA was 0.65 mg/kg fresh weight of developing cotton fruit.

Lenton, Bowen, and Saunders (1968) also attempted to determine ABA levels from phloem and xylem sap of willow (*Salix viminalis* L), by a GLC technique using 1.5 % QF-1 on AW-DMCS Chromosorb W. Successful gas chromatographic separation was obtained after purification by silica gel TLC using two different solvent systems. They estimated 10 µg and 1-5 µg of ABA in 100 ml of phloem sap and xylems sap respectively.

In order to confirm the presence of ABA in the plant extracts which had been examined by bioassays, Ramsay and Martin (1970 b) purified the acid ether fraction of apricot bud extracts. Firstly a silica gel TLC, with n-propanol : n-butanol : water : 30 % ammonium (6 : 2 : 2 : 1 v/v/v/v) was used, then the ABA marker position was scraped off and the eluted residue purified by paper chromatography using isopropanol : 30 % NH₄OH : water (8 : 1 : 1 v/v/v). Finally the ABA position on the paper was eluted by MeOH, rechromatographed on silica gel TLC using the solvent system (benzene : acetic acid : water, 8 : 3 : 5 v/v/v) and the eluted samples from TLC were methylated and analyzed by gas chromatography. They found that the apricot bud inhibitor had the same retention time as ABA and also a small peak of trans-ABA.

Most (1971) demonstrated that the abscisic acid in sugar cane leaves subjected to drought stress was greatly increased. The abscisic acid content determined by GLC using 2 % QF-1 on 80-100 mesh Gas-Chrom A or 2 % SE-33 on 80-100 mesh Chromosorb G represented only about ½ the total inhibitory material determined by bioassay.

Lenton, Perry, and Saunders (1971) developed the quantitative GLC measurement of abscisic acid using an internal standard of t-ABA. In using t-ABA as an internal standard, there must be (1) no presence of a significant amount of t-ABA in the plant materials, (2) no isoemrisation occurring during the purification of extracts, and (3) recovery of the two isomers in the same fraction during the purifications. They found Epon 1001 stationary phase gave the best chromatographic performance compared to 1.5 % QF-1 and 2.5 % OV-17 stationary phase.

Adding 15 µg of synthetic 2-trans ABA to the plant material as an internal standard at the beginning of extraction procedure to determine % recovery of ABA through purification, Simpson and Saunders (1972) found an estimated 0.234 mg of ABA/kg dry weight in the tall pea variety Alaska grown in the dark and 0.644 mg under 16 hour daily photo-period, while in the dwarf pea variety Meteor 0.186 mg and 0.647 mg respectively, after correcting for 56 % recovery of ABA during the purification.

Barnes (1972), however, reported no significant difference of ABA either between tall and dwarf nor between dark and red light condi-

tions, estimating 1020 μg in kg dry weight under dark treatment and 870 μg under red light in the dwarf pea *Gradus* and 820 $\mu\text{g}/\text{kg}$ and 700 $\mu\text{g}/\text{kg}$ respectively in the tall pea variety progress No.9.

Shindy and Smith (1975) estimated 2.62 ng of ABA/g fresh weight of 8-day-old cotton ovules in the acidic fraction of the solvent partition without further purification, confirming the identification of ABA by a computer-controlled GC-MS.

7.5.2.2. Determination of ABA by an electron capture detector

The most sensitive assay of abscisic acid with the methylated esters of ABA in the plant extracts was achieved by using electron capture-gas chromatography without being converted to a halogenated derivative (Seeley and Powell, 1970 ; Powell, 1972). 10 picograms (10^{-11} g) of ABA could be detected quantitatively with linearity from 1 picogram to 1 nanogram by employing 2 % SE-30 as a stationary phase.

Since then electron capture gas chromatography has been widely adapted, particularly for the measurement of ABA in plant extracts, 573 pg of cis-ABA/mg dry leaves was found in salinized plants of Nicotiana rustica compared with 90 pg/mg dry weight leaves from nonsalinized plants (Mizrahi, Blumenfeld, Bittner, and Richmond, 1971); 1 μg and 10.75 μg of ABA in 100 g fresh weight of leaves with and without treatment of Ethephon to the five leaf stage of monoecious cucumber respectively and 6.43 and 2.23 $\mu\text{g}/100\text{g}$ fresh weight of gynoecious cucumber with the same conditions mentioned above (Rudich, Halevy, and Kedar, 1972); 10/1 ratio of bound to free abscisic acid (80 : 7.7 $\mu\text{g}/\text{kg}$ fresh weight) in the senescence of citrus fruit peel (Goldschmidt, Goren, Even-Chen, and Bittner, 1973); 6.8 μg of ABA in one gram dry weight in the tissue at the time of acropetal calyx abscission to compare with 1.0-2.3 $\mu\text{g}/\text{g}$ dry weight in the early stages such as tight bud to petal abscission in Hibiscus rosa-sinensis L.; ABA contents in relation to ripening of grape (Coombe and Tate, 1972).

In related compound, 0.11 mg of phaseic acid and 5.95 mg of 4-dihydrophaseic acid/kg dry weight of non-imbibed Phaseolus vulgaris seed respectively was determined by Walton, Dorn, and Fey (1973).

7.5.2.3. Identification of ABA

7.5.2.3.1. GC-MS for ABA

In 1968, Gaskin and MacMillan explored the identification and estimation of ABA in a cider apple juice by GC-MS, using the stationary phases as mentioned in section 7.5.3. They identified ABA and trans-ABA from a methylated crude acidic fraction of cider apple juice, but not phaseic acid and estimated 30 μg abscisic acid per litre as compared

with 20 µg estimated with bioassay by Pieniażek and Rudnicki (1967). In 1970 Most, Gaskin, and MacMillan confirmed the presence of ABA in the inhibitors B₁ and C from immature fruit of Ceratonia siliqua L. (Carob) and in commercial carob syrup, by TLC, GLC and GC-MS.

After confirming the presence of Phaseic acid in the seed of Phaseolus multiflorus by GC-MS, Durley, MacMillan and Pryce (1971) estimated 0.3 mg/kg fresh weight of seed by GLC. With the same techniques, 5.5 nmoles of ABA/g fresh weight in the immature fruit of Ceratonia siliqua L. was estimated by Most, Gaskin, and MacMillan (1970); 19.0 nmoles/g dry weight for the testa, 1.4 nmoles/g for the pericarp, and 0.09 nmoles/g dry weight for the embryo in newly harvested nuts by Williams, Ross, and Bradbeer (1973); 2.62 ng/g fresh cotton ovules by Shindy and Smith (1975).

7.5.2.3.2. Identification of ABA by racemisation with UV irradiation

On prolonged exposure to light the plant hormone ABA is isomerized to give a 1 : 1 equilibrium mixture cis, trans ABA and trans, trans ABA (Mousseron-Canet, Mani, Dalle, and Olive, 1966). Lenton, Perry, and Saunders (1971) used this characteristic to help confirm the presence of ABA in the plant extract; the extract was irradiated with a Hanovia Model 16 UV lamp and 50 % of each isomer was obtained after 4 hour irradiation. Williams, Ross, and Bradbeer (1973) showed that the gas chromatographic behaviour of mixed isomers of ABA appeared as two peaks with retention times of 8.7 and 12.1 minute which agreed with the results of Lenton, Perry, and Saunders (1971). They obtained a 52.6 % of the 8.7 minute peak and a 47.4 % of the 12.1 minute from the methylated extract from testas of freshly harvested seeds after 8 hour irradiation with UV. With the methylated extract before 8 hour irradiation with UV, they obtained 90.8 % of the large peak corresponding to the cis, trans isomer and 9.2 % to the trans, trans isomer. The 90.8 % of the peak at the 8.7 minute retention time was identical to cis, trans ABA by GC-MS. Therefore, they proceeded to identify ABA in plant extract by gas chromatography before and after UV racemisation.

7.5.3. Gibberellins by GLC and GC-MS

Ikekawa, Sumiki, and Takahashi (1963) first separated and identified the methyl esters of gibberellins A₁ to A₉, using 1.5 % SE-30 and 2 % QF-1. They found that the decomposition GA₈ gave a broad peak and partially decomposed, although SE-30 was better than QF-1.

After Cavell, MacMillan, Pryce, and Sheppard (1967) established a gas chromatographic separation for 17 gibberellins of the methyl esters and trimethyl silyl ether of the methyl esters on both 2 % of QF-1 and SE-33 columns, they identified the presence of gibberellins A₁, A₄, A₅, A₆, A₈, and A₁₃ from the crude acidic extract of immature seed of Phaseolus multiflorus. A new dimension to the investigation of plant hormones was the development of the powerful technique of combined gas chromatography-mass spectrometry (GC-MS) which both separate and identifies well resolved compound in one operation. For example MacMillan, Pryce, Eglinton, and MacCormick (1967) unequivocally confirmed the previous tentative identification of gibberellin by GC alone (Cavell, et al., 1967; Pryce, et al., 1967). In their continuous investigations of gibberellins by GC-MS, a new gibberellin, GA₁₇, was identified from extracts of young seeds of Phaseolus multiflorus (Pryce and MacMillan, 1967) and also many gibberellins that had been found in other species e.g., GA₁₉ (Pryce, MacMillan, and McCormick, 1967); gibberellin A₂₀ (MacMillan and Pryce, 1968); GA₁, GA₅, GA₆, and GA₈ (MacMillan, 1968); GA₁, GA₃, GA₅, GA₆, GA₈, GA₁₇, GA₁₉, GA₂₀, and possibly another gibberellin, phaseic acid and some other compounds (Durley, MacMillan, and Pryce, 1971). GA₄ was found in dark-grown Phaseolus coccineus seedlings (Crozier, Bowen, MacMillan, Reid, and Most, 1971); and traces of GA₉ during the stratification of apple seeds (Sinska, Lewak, Gaskin, and MacMillan, 1973); and GA₁, GA₃, GA₄, GA₇, GA₉, and GA₁₃ from cotton ovules (Shindy and Smith, 1975).

Limitations of using GC-MS for plant hormones is that conjugate-plant hormones cannot be directly identified and a minimum of 5 ug for derivatisation is required (MacMillan, 1970).

According to the retention time or cochromatography with known gibberellins, several gibberellins were tentatively identified after solvent partition or elution from thin-layer plate; e.g., GA₃ in immature seedless apple fruits (Hayashi, Naito, Bukovac, and Sell, 1968); GA₃ in immature seeds of Pharbitis nil (Tamura, Takahashi, Murofushi, Yokota, and Kato, 1968); GA₄ and GA₇ in immature apple seed (Luckwill, Weaver, and MacMillan, 1968); GA₁, GA_{4/7}, GA_{5/20}, and GA₈ in germinating chilled seed of Corylus avellana L. with growth retardant treatments (Ross and Bradbeer, 1971 a and b); possible presence of GA₁, GA₅, GA₈ and GA₉ in the shoots and GA₅, GA₉, and GA₁₃ in the roots of Tulipa generiana (Aung, De Hertogh, and Staby, 1971). GA₂₀ in Bryophyllum deigremontianum under different photoperiodic conditions (Zeevaart, 1973).

Using gas-liquid radiochromatography with three stationary phases 2 % QF-1, 2 % SE-30 and 1 % XE-60, Durley, Railton, and Pharis

(1973) identified GA_3 formed from GA_5 in seedlings of dwarf Pisum sativum.

7.5.4. Cytokinins by GC-MS

In gas-liquid chromatography of cytokinins, Most, Williams, and Parker (1968) characterized the trimethylsilyl derivatives of pure compounds of cytokinins, using 3 % SE-52 stationary phase. Bui-Dang-Ha and Nitsch (1970) identified zeatin riboside from the root of chicory (Cichorium intybus L.) by GC-MS.

Upper, Helgeson, Kemp, and Schmidt (1970) reported the retention times of 20 purines or purine nucleosides on 2 % QF-1 on Gas-Chrom Q and identified 6-(3-methyl-2-butenylamino)-9- β -D-ribofuranosylpurine from t-RNA hydrolysates and 6-(3-methyl-2-butenylamino)purine in culture filtrates of Agrobacterium tumefaciens. Babcock and Morris (1970) reported also a possible quantitative and qualitative measurement of cytokinin ribosides from t-RNA hydrolysates using isothermal chromatography on a DC-11 column. Upper, Helgeson, and Schmidt (1970) demonstrated the feasibility of identification and measurement of cytokinins in plant extracts by GC-MS. Horgan, Hewett, Purse, and Wareing (1973 b) identified zeatin riboside in sycamore sap by GC-MS.

Most recently Shindy and Smith (1975) demonstrated the presence of dihydrozeatin, zeatin, 2iP, 2iPA, and zeatin riboside in cotton ovules by a computer controlled GC-MS.

7.6. Loss of authentic plant hormones during extraction procedure

7.6.1. Loss of IAA

Indole-3-acetic acid has been known to be a labile compound during extraction procedures. The indole nucleus itself is very easy to destroy by exposure to strong mineral acids (Dannenburg and Liverman, 1957). There is a number of papers that deal with the recovery of IAA during extraction procedures. Thimann and Skoog (1940) in the early period of auxin study reported that over 100 per cent recovery could be obtained by three times ether extractions of duckweed. One of the earliest recovery tests through a whole extraction procedure was reported by Vlitos and Meudt in 1954. The loss of IAA during the extraction procedure varied with species and solvent used. For ether partitioning from acidified water, 62-75 % recovery of IAA was obtained with or without tomato tissues. In spinach water extraction gave very poor recovery, whereas the ethanol extraction was very high.

In several trials, one of the poorest recoveries for authentic IAA and ^{14}C -IAA was 25-30 % and average 14 per cent respectively, when

Hamilton, Bandurski, and Grigsby (1961) tested the recovery of IAA in the following procedure: 80-95 % ethanol extraction, concentration of ethanol extract, ether extraction, celite partition column, and paper chromatography in etiolated corn shoots. However 77 % recovery from the only celite column test has been shown (Hamilton, et al., 1961). Burnett, Audus, and Zinsmeister (1965) found that a high recovery of 85-97 % was obtained by ether extraction of the acidic aqueous phase and further purification was made by DEAE cellulose eluted with 0.05 M Na_2SO_4 without using 5 % NaHCO_3 . The method involving extraction of 5 % sodium bicarbonate, appeared to give only 50 per cent recovery of IAA, indole-3-carboxylic, indolebutyric, indolepropionic, and indolelactic acid as determined by spectrophotofluorometry. The conventional method of Larson (1955) with an ether extract-partitioned with 5 % sodium bicarbonate and the acidified aqueous solution, then extracted with ether gave 60-85 % recovery IAA-2- ^{14}C added to 0-200 μg of authentic IAA in 350 mg of lyophilized cucumber leaves (Galun, Izhar, and Atsmon, 1965). Although there were no indicated data of the percentage loss of authentic IAA in the paper, a determination of IAA was obtained by cochromatography with the aid of authentic IAA addition (Tafuri, 1966; Dullaart, 1967).

Ueda and Bandurski (1969) also examined the loss of IAA by destruction or absorption with 15 gram of ground corn kernels, fortified with 400 μg authentic IAA. The recovery of IAA obtained was 65-79 % from the 80 % acetone extraction procedure, concentration of acetone, and reextraction with water.

In a preliminary test, Mann and Jaworski (1970) investigated the extraction procedures described by Kefford (1955). They investigated each step of the extraction procedures with plant material and ^{14}C -IAA. Less than 10 % loss of IAA occurred in the ether extraction of the acid aqueous extract with or without tissue. Further purification with sodium bicarbonate from ether solution gave a 70-85 % recovery. A consistent decrease of IAA occurred with a subsequent purification, giving about 58-63 % recovery. A drastic decrease was observed, when IAA in ether was reduced during thin film evaporation of ether. They recommended to not dry the ether completely. The final recovery of IAA was 10-34 %.

Goldschmidt, Monselise, and Goren (1971) obtained about 96 % and 88 % of IAA-2- ^{14}C with and without plant tissue respectively. Their extraction procedure was methanol extraction, concentration of MeOH extract, extraction with di-isopropyl ether at pH 7.5, extraction with diethyl ether at pH 6.0 and reextraction with ether at pH 3.0.

Knegt and Bruinsma (1973) corrected the loss of IAA during the purification after crude methanol extract, assuming the 50 % tolerance in the loss. They obtained 92 % of the IAA accumulation in the ether phase and 96 % after careful rewashing of fractions.

Glenn, Kuo, Durley, and Pharis (1972) obtained 93 % recovery of IAA from PVP column using pH 8.0 buffer.

7.6.2. Loss of other plant hormones

Quantitative analysis of plant hormones has generally been studied by comparative methods using the same extraction procedures and therefore values in the literature are not absolute values corrected neither for differences in percentage recovery nor for in sensitivity of the bioassays used. However a number of papers have reported that the estimation of plant hormones was corrected for by determining the loss of added authentic plant hormones during the extraction procedures, as reviewed in the previous section for IAA.

The loss of ABA during the extraction procedure was determined by the racemate dilution method, analogous to the isotope dilution method (Milborrow, 1967). Milborrow obtained an average of 68 % recovery of ABA in the ether solvent partitioning and 98 % recovery in silica gel TLC. Lenton et al. (1971) used the 2 trans isomer ABA since plant materials do not contain this isomer i.e. in GLC Simpson and Saunders (1972) used the isomer as an internal standard. 45-70 % recovery of ABA in the various tissues by this method was obtained by Lenton, Perry, and Saunders (1971), 56 % by Simpson and Saunder (1972) and 75 % during purification from dormant birch buds by Harrison and Saunders (1975). 34 % recovery of 2-¹⁴C-ABA, 18 % recovery of 2-¹⁴C-phaseic acid, and 10 % recovery of 2-¹⁴C-4'-dihydrophaseic acid were obtained during extraction procedures by Walton, Dorn, and Fey (1973), and 60 % recovery of xanthoxin through the full extraction procedure by Firn, Burden, and Taylor (1972).

In gibberellin recovery tests, 90 % of GA₃ during solvent partitioning at pH 2.5 was obtained by Tautvydas (1965), 90-97 % recovery from a PVP column for GA₁, GA₃, GA₄, GA₅, GA₇, GA₈, GA₉, and GA₁₃ was obtained by Glenn, Kuo, Durley, and Pharis (1972) and a 90 % recovery of GA₃ from a silica gel column using gradient elution by Powell and Tautvydas (1967).

In a cytokinin recovery test, Skene (1972) obtained no loss of 10 µg of added zeatin during the extraction procedure.

CHAPTER 8

Materials and Methods8.1. Loss of ^{14}C -IAA during extraction and purification procedures8.1.1. The plant materials

The leaves taken from only the clusters and the shoots chopped by hand secateur were put into dry ice and then lyophilized. The lyophilized sample was stored in a preserving jar in a deep freeze until the plant material was extracted.

Initially 30.9 mg of ^{14}C -IAA contained 0.5 mCi of radioactivity, however, due to the impurity of the ^{14}C -IAA it was first purified by cellulose thin layer chromatography with methyl acetate: acetonitrile: 28 % ammonia: water (40 : 10 : 5 : 4 by v) solvent system (The solvent system-MAAW)(Ohwaki, 1966). The band of ^{14}C -IAA which coincided with the Rf of cold IAA under UV light was scraped off and eluted with EtOH using a sintered-glass funnel (10 x 1.5 cm). If the purified ^{14}C -IAA was stored for longer than 3 months, the hormone was repurified.

In experiment 1, 411,517 dpm representing 11.25 μg of ^{14}C -IAA in 2ml of MeOH was added to the MeOH solvent in which 30 g of apple stem tissue was then macerated. In Exp.II, ^{14}C -IAA in EtOH (499,335 dpm; 13.65 μg) was added to MeOH solvent in 10 g of apple stem tissue before macerating the sample with a M.S.E. homogenizer (Measuring Scientific Equipment Ltd). The solvents were redistilled before use.

8.1.2. Solvent partition chromatography

The extraction procedures were followed as shown in Table 7-A and B. The sample were macerated with 100 ml of cold 80 % MeOH containing 0.02 % sodium diethyldithiocarbamate for 5 minutes in a M.S.E. Homogenizer below 0°C . The macerated sample was extracted with 80 % MeOH containing 0.02 % sodium diethyldithiocarbamate for 12 hours at $1-2^{\circ}\text{C}$. The extracted solution was filtered with Millipore-filter which was stored in a deep freezer before used. The residues were extracted again for 12 hours at $1-2^{\circ}\text{C}$. After filtering the extract, the residues were rinsed with fresh cold 80 % of MeOH several times. The pooled 80 % MeOH extract was evaporated in vacuo at 35°C with a Buchi rotary evaporator. The evaporated solvents were trapped by a dry ice cold trap. The aqueous phase was stored at $1-2^{\circ}\text{C}$ over night to precipitate phloridzin (Tautvydas 1965). The aqueous phase then was centrifuged at 0°C with 10,000 rpm in 30 minutes. The supernatant was decanted and the residues were rinsed

with cold water. The residues were discarded. The combined aqueous phase then was extracted with petroleum ether (40-60°) until colorless (Phillips and Wareing, 1958b; Alden and Eliasson, 1970). The aqueous solution adjusted to pH 2.5 by 50 % HCl or first adjusted to pH 8.0 with diluted KOH and extracted with 1/3 volume of ethyl acetate (eight times; Crozier, Aoki, and Pharis, 1969). This aqueous phase was used for cytokinins determination. The pooled ethyl acetate was extracted with 1/3 - 1/4 v of 5 % sodium bicarbonate (5 x). The combined sodium bicarbonate solution which was adjusted to pH 2.5 with 50 % HCl was extracted with 1/3 v ethyl acetate (8 x). The ethyl acetate fraction was evaporated under reduced pressure at 35 °C with a rotary evaporator. The residue in the round flask was lyophilized by a Freeze-drying Unit Model 10 p (Edwards High Vacuum Ltd). the residue was then redissolved in a small quantity of 0.1 M phosphate buffer and loaded on a Sephadex G-10 column.

8.1.3. Column chromatography

8.1.3.1. Sephadex gel chromatography

Sephadex G-10 (85 g) was allowed to stand with 0.1 M phosphate for 30 minutes in a hot water bath to de-gas and swell. The slurry was poured into the column (3.5 x 40 cm) and was packed with a stainless steel rod with a perforated disc. The column was eluted with 1,000 ml of 0.1 M phosphate buffer and further with 500 ml. The flow rate of the eluant was 25 ml per 10 minutes.

8.1.3.2. Adsorption column chromatography

Silica gel (80-200 mesh, May & Baker Co.Ltd) and Celite 535 or 545 were washed with 6 N HCl separately. After 24 hours, the slurry of each compound was poured into a large column (3.5 x 100 cm) and eluted with distilled water and MeOH. The Compounds were washed until the effluent was free of acid. The silica gel and celite were then dried in a vacuum oven (Parkinson Cowan (N.Z) Ltd) at 100°C for 24 hours. The mixture of 10 g of silica gel and 10 g of celite was packed by the method of Khalifah, Lewis, and Coggins (1965). The mixture in chloroform was gradually poured in the column (2 x 20 cm; Quickfit CR 32/20), while a stainless steel packing rod (terminating in a perforated disc matching the column diameter) was moved up and down. The samples in 100 - 1,000 µl aliquots were added to 2 g of silica gel in a weighing boat and dried overnight in a vacuum oven at room temperature. The silica gel with the samples were loaded on the top of the silica gel-celite column. The solvent system was

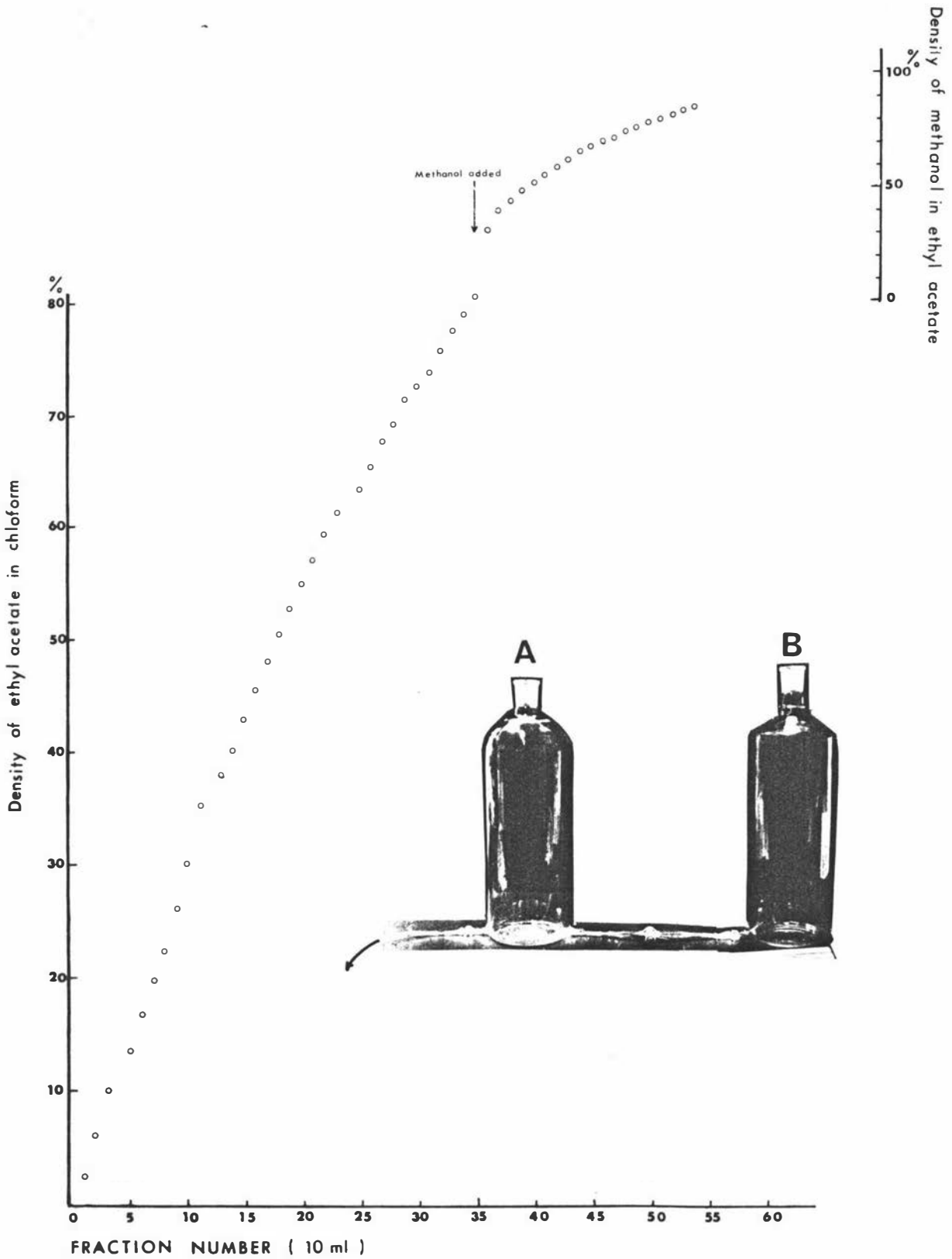


Fig.13. Density of ethyl acetate in chloroform and methanol in ethyl acetate in each 10 ml fraction leaving the B cylinder flask to the A cylinder flask in the gradient elution.

a gradient of 200 ml of chloroform and 330 ml of ethyl acetate (equal weights), followed by 200 ml of MeOH. Two 1,500 ml cylinders (A and B) were connected together at the bottom with an interflow P.T.F.E. stopcock. The bottom of cylinder A rested upon a magnetic stirrer and was connected through a ground-glass joint to the top of the silica gel column. As soon as ethyl acetate solvent in the B cylinder flask got through the interflow P.T.F.E. stopcock to the A cylinder flask, the mixture of two solvents went into the column. When the mixture of two solvents was about 130 ml (i.e., about 50 ml in B), 200 ml of MeOH was added to the B cylinder flask. MeOH-ethyl acetate gradient started as soon as the stopcock opened. 10 ml fractions were collected directly into scintillation vials by an automatic fraction collector. The flow rate was 20 ml per 10 minutes. The gradient elution pattern was calculated by density of ethyl acetate in chloroform and methanol in ethyl acetate for each 10 ml fraction leaving the B cylinder to A cylinder (See Fig.13).

8.1.4. Cellulose thin layer chromatography

Cellulose powder was washed with 6 N HCl followed by distilled water and then dried. 0.3 mm thick cellulose thin layer plates (20 x 20 cm) was made with a Stahl type applicator (Upjohn Co.). The plates were left overnight in air for drying. The activity of ^{14}C -IAA as 14,909 dpm (0.2038 μg) in 50 μl of MeOH was applied as 3 cm x 3 bands for each plate and was developed with MAAW up to the 10 cm line on the plate. The plates were equilibrated for 30 minutes in the saturated solvent tank before the development. Each Rf was scraped off into a vial and then eluted in the vials by 1 ml of MeOH at 1.0 to 2.0°C in the refrigerator overnight. A scintillant was added and counted by a scintillation spectrometer (See 8.1.5.).

Each percentage recovery was derived from two or three replications.

8.1.5. Determination of radioactivity

Estimation of recovery for ^{14}C -IAA for each step of the extraction and purification procedure was made in duplicate. Radio-activity was determined, using a Model 3320 automatic Tri-Carb Liquid Scintillation Spectrometer (Packard Inst. Comp. Inc), in terms of disintegration of ^{14}C -IAA per minute (dpm), corrected for counting efficiency (Regression coefficient, $y = 1 - 418.6x + 149.47$). The scintillation cocktails used were 800 ml dioxane; 160 ml ethylenglycolmonoethyl ether; 48 g naphthalene, 9.6 g 2,5-

diphynylorazolyl 1-benzene (POPOP) (Veen 1966; Veen and Jacobs, 1969) for alkaline aqueous solutions and POPOP-PPO-toluene triton x-100 (1 I toluene, 4g PPO, 100 mg POPOP, 500 ml Triton x-100; Patterson and Greene, 1965) for organic solvents. The amount of scintillant per vial was 10-20 ml. The background was always subtracted.

8.2. Behaviour of ^3H -zeatin in chromatography

8.2.1. Solvent partition chromatography

8.2.1.1. Preparation and determination of ^3H -zeatin

The specific activity of ^3H -zeatin for 0.18 mg (Donated by Letham, 1972) was 1 mCi/mg. In order to purify ^3H -zeatin, an aliquot in EtOH was spotted on a cellulose thin layer plate and developed by the solvent system MAAW. The band of ^3H -zeatin at the same Rf as cold zeatin was scraped off and was eluted with MeOH and thereafter the purified ^3H -zeatin was used. ^3H -zeatin was often repurified before use.

^3H -zeatin (46,669.7 dpm, 0.01969 μg) in absolute EtOH was put in a 25 ml cornic flask. After drying in a vacuum oven at room temperature, the residue of ^3H -zeatin was dissolved in 30 ml aqueous solution at either pH 2.5 with 0.5 M formic acid or pH 8.3 with 0.1 M phosphate buffer. The pH 2.5 solution was partitioned with 1/3 volume of ethyl acetate either saturated or non-saturated with 0.5 M formic acid while the pH 8.3 solution was partitioned against n-butanol saturated with 0.1 M phosphate buffer.

Each fraction of the partitions was put directly into scintillation vials and the solvents were removed in a vacuum oven at 35-40°C. After drying the solvents, 2 ml of MeOH was added to each scintillation vial and several series of aliquots were counted. After the 8th partition, the aqueous phase at pH 2.5 was adjusted to pH 8.3 with 1.0 M phosphate to make finally a 0.5 M phosphate buffer adjusted with KOH. The aqueous phase was then partitioned with BuOH saturated with 0.1 M phosphate buffer. The aqueous phase at pH 8.3 which was partitioned by ethyl acetate up to the 8th fraction was extracted with the saturated BuOH in order to determine total recovery of ^3H -zeatin. When the saturated BuOH was used for the partition of the aqueous phase at pH 8.3, the final residue of ^3H -zeatin in the aqueous phase was estimated by partitioning with saturated BuOH.

Each experiment was replicated three times.

The partition coefficient was calculated by

$$K_d = \frac{100 - p}{p}$$

where p is the percentage of recovery by the first partition from the aqueous phase and K_d is the partition coefficient.

Estimation of radioactivity for the recovery of ^3H -zeatin from extraction procedures was essentially the same as that for ^{14}C -IAA. Disintegration per minute to ^3H -zeatin was obtained by using the correction $y = -76.73678 X + 61.7564$.

8.2.1.2. Back washing from ethyl acetate phase

30 ml of the aqueous solution at pH 2.5 which had 47,755.8 dpm was partitioned 8 times with 1/3 ethyl acetate. The pooled ethyl acetate phases were back washed five times with 10 ml of aqueous phase at pH 2.5. Each 10 ml of back washed aqueous solution was collected separately in scintillation vials and dried in a vacuum oven at 35-40°C. The residue was dissolved with 1.0 ml of absolute MeOH and aliquots counted for radioactivity.

The aqueous phase which was partitioned with ethyl acetate was adjusted with 1.0 M phosphate buffer solution to be pH 8.3 and 0.5 M phosphate buffer and was partitioned with 1/3 v of BuOH (4 X). The pooled BuOH extracts were evaporated by a rotary evaporator. The aqueous phase was partitioned once more with BuOH and the BuOH extract was counted for the remaining radioactivity in the aqueous phase.

8.2.1.3. Back washing from BuOH phase

30 ml of the aqueous phase with 47,775.8 dpm (0.020139 ug) at pH 8.3 was partitioned four times with 1/3 v of BuOH saturated with 0.1 M phosphate buffer. The BuOH phase was back washed 3 times with 10 ml of 0.1 M phosphate buffer. Each 10 ml was collected into scintillation vials and dried in a vacuum oven. The BuOH phase was dried with a rotary evaporator and the residue was dissolved with 2 ml of MeOH and the aliquots were counted. The aqueous phase after partitioning 4 X with saturated BuOH was once more partitioned and counted to determine residual activity.

8.2.2. ^3H -zeatin in column chromatography

8.2.2.1. Sephadex G-10

Sephadex G-10 column was used as mentioned in column chromatography of ^{14}C -IAA (See 8.1.3.1.). The glass wool containing 686,336.5 dpm (0.289428 μg) of ^3H -zeatin was loaded on the top of Sephadex G-10 and each 100 ml fraction was eluted with 0.1 M phosphate buffer up to the 16th fraction. A 0.5-1.0 ml aliquot taken from each 100 ml fraction was counted with dioxane scintillant and dpm estimated. The pooled fractions from the 1st to the 10th, 11th-16th, and 17th-21st were extracted with BuOH saturated with 0.1 M phosphate buffer (4 X). The residue was dissolved in 2 ml MeOH and the aliquots were counted. The experiments were repeated two times.

8.2.2.2. Sephadex LH-20

Sephadex LH-20 column methods essentially followed those of Steen and Eliasson (1969). A Sephadex LH-20 column (2.5 x 30 cm ; 40 g of gel) was packed with the swollen gel by the usual method. ^3H -zeatin (337,386 dpm, 0.142276 μg) in 500 μl ethanol was loaded on to the top which was covered with a filter paper disc. Cold cytokinins such as zeatin, zeatin riboside, dihydrozeatin, and 6-(β,β -dimethylallylamino) purine (300 μg in 300 μl EtOH) were loaded on the top of column respectively. The solvent was 95 % EtOH with 0.001 M HCl. A 5 ml fraction was collected into the scintillation vials with a flow rate of 30 ml/h. Each fraction was detected by transmission at 280 nm with a 101 Model Hitachi spectrophotometer for cold cytokinins. ^3H -zeatin was evaporated to dryness in a vacuum oven and the residue was dissolved with 1 ml absolute MeOH. Each aliquot was counted to determine its radioactivity.

Each experiment had one or two replications.

8.2.2.3. Dowex 50 W x 8

The method using a Dowex 50 W x 8 column (3.0 x 50 cm) was essentially the same as Miller (1965). Dowex 50 W x 8 resin was washed by cycling before packing the column.

The glass wool containing 686,336.5 (0.289428 μg) of ^3H -zeatin was loaded on the top of Dowex 50 W x 8. The loaded samples were rinsed with 300 ml of distilled water and labelled zeatin eluted with 6 N ammonia solution. A 100 ml fraction was collected in a 100 ml conical flask. The aliquots of 0.5 and 1 ml from 100 ml were counted with dioxane fluid by a scintillation counter. The means of the samples were used to estimate total activity in the 100 ml fraction.

The experiment was duplicated.

8.2.2.4. Behaviour of ^3H -zeatin in paper and thin layer chromatography

50 μl of ethyl alcohol with 51,773.1 dpm (0.021833 μg) of ^3H -zeatin was applied on a 2.5 cm zone of Whatman 3 MM, DEAE cellulose, and silica gel GF 254 (S.G. GF 254). Paper sheets were washed by allowing N formic acid to flow down them for several days (Letham, 1966a). The thickness of the DEAE cellulose was 0.3 mm. 5 plates usually were made with 8 g powder MN DEAE + 2 g cellulose powder MN 300 and dried at 50°C for 40 minutes (Stahl, 1969). The thickness of cellulose and Silica gel GF 254 was 0.3 mm. Three replicates were spotted on the same paper or thin layer plates. Thin layer plates were developed up to a 10 cm line marked before development and the paper chromatography to 20 cm.

The solvent systems were: (1) Isopropanol : Ammonium Soln. (Sp.g. 0.880) : H_2O (10:1:1 v/v/v) for Whatmann paper 3 MM, (2) Methyl acetate : Acetonitrile : Ammonium : Water (40 : 10 : 5 : 4 v/v/v/v) (solvent system MAAW) for DEAE-cellulose and cellulose, and (3) Butanol saturated with water for Silica gel GF 254. Each Rf of the thin layer chromatograms was scraped off by a razor blade directly into scintillation vials containing 1 ml MeOH and were shaken overnight (Griffin Flask Shakers). The paper was cut into Rfs and eluted by the same methods.

8.3. Bioassay techniques for IAA, ABA and GA

8.3.1. The combination of 5 mm oat and 10 mm wheat coleoptile sections for IAA and ABA

Because of different sensitivity of oat and wheat coleoptile sections to IAA and ABA, the combination of oat and wheat coleoptile bioassay was tested.

The wheat variety Aotea and the hullless oat variety Brighton were used. The procedures used to grow oat and wheat seedlings were essentially the same as Sirois, (1966). The uniform seeds selected were sterilized for 5 minutes by 2 % of sodium hypochlorite solution and rinsed by running tap water for 2 hours. The seeds spread on the filter paper in the plastic trays (30 x 43 x 6.5 cm) were inclined about 45° and distilled water remained in the inclined bottom edge of plastic trays covered by a glass sheet. The trays were exposed for 24 hours to a red light (Fluorescent tubes were wrapped by the thin red cellophane paper ; Nitsch and Nitsch, 1956). Thereafter the trays were transferred into the dark room at 25°C for a further 48 hours. Before transferring the trays into the dark room, pumice was spread

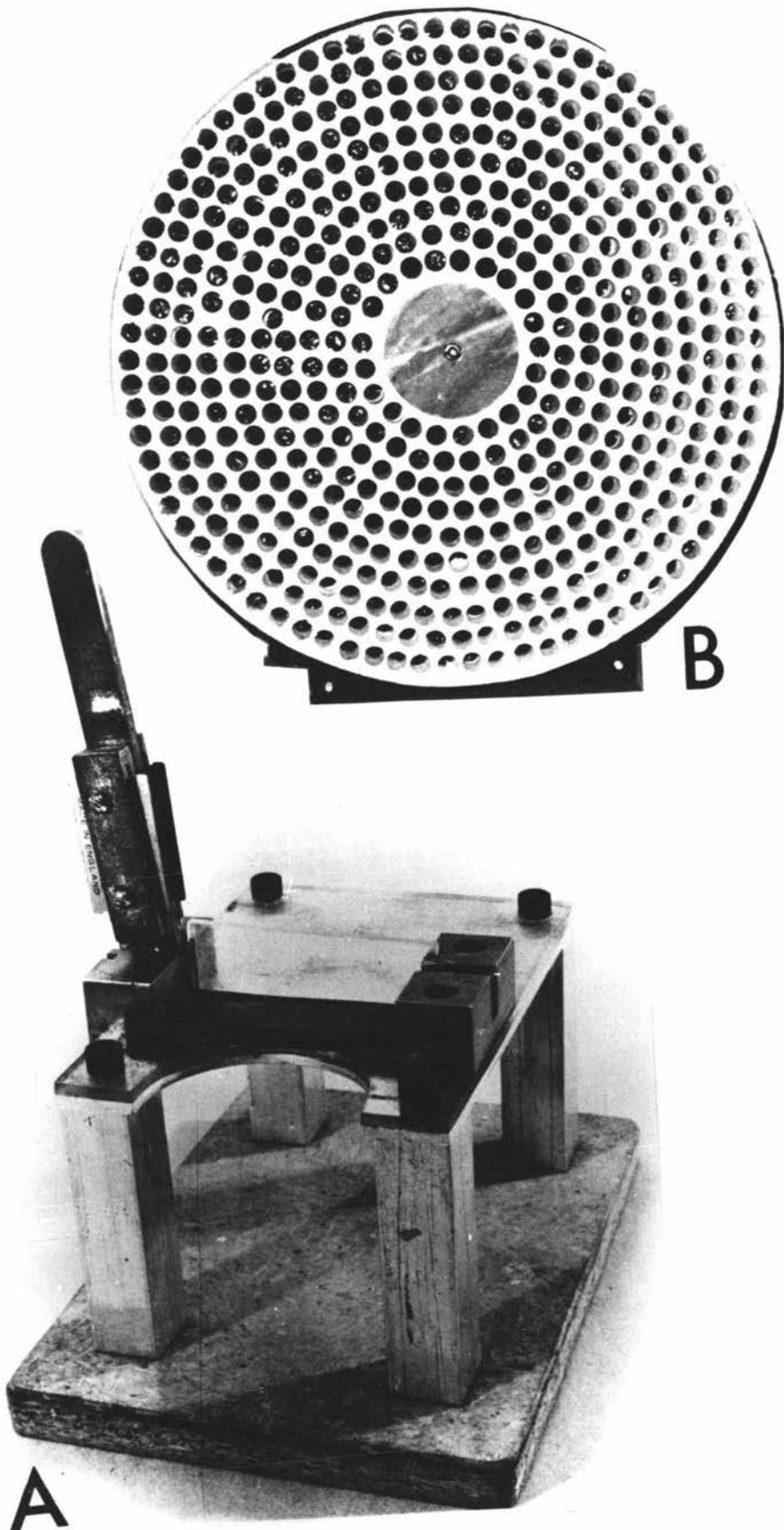


Fig. 14-A. Cutter of coleoptile sections.
14-B. Turn-table for coleoptile bioassays.

on only the oat seeds to prevent an upward growth of roots. A coleoptile length of about 20 - 30 mm was used. In order to get a similar size of seedlings for both oat and wheat coleoptile sections, the oat seeds were sown 12 hours before sowing the wheat seeds. 5 mm length of oat coleoptiles and 10 mm of wheat coleoptiles were cut 3 mm behind the tips by the coleoptile cutter (Crowe, 1963), slightly modified as follows : (1) To set the shield to cut 3 mm of tips instead of the scribe line on the plexiglass, (2) Attached the rubber piece on the plexiglass for the razor blade to be inserted into the rubber when the coleoptile were cut, (3) a 5 mm supplemental metal piece into the blade holder when required to be cut 10 mm length sections, (4) Used both 'Valet' and Gillete' types of blade (See Fig. 14-A).

The cut sections were floated on distilled water before use. All procedures to cut coleoptiles were done under a green safe light (Nitsch and Nitsch, 1956). 3 sections of oat and 5 sections of wheat coleoptiles were put into the test tube (150 x 2.0 mm) in 1 ml of phosphate citrate buffer at pH 5.0 (1.794 gm K_2HPO_4 , 1.019 gm citric acid to 1 litre, 20 gm of sucrose, Nitsch and Nitsch, 1956). Tubes were rotated at 1 rpm for 20 hrs by a vertical turn table (See Fig.14-B). The coleoptile sections were fixed by one ml of 10 % of alcohol (Hopping, 1968) after growing for 20 hours. The length of coleoptiles were measured by a Mitutoyo dial caliper under a 4 x magnification with a photographic enlarger.

A statistical analysis was made of the 2 x 5 factorial with 4 replicates (Federer, 1955). In order to compare data the least significant difference (LSD) at the 5 % level was used. The same data were also analyzed separately as a randomized complete block design to compare LSD at the 5 % level.

8.3.2. Tan-ginbozu dwarf rice seedlings and lettuce hypocotyl bioassay for gibberellins

8.3.2.1. Dwarf rice bioassay with glass tubes

The bioassay methods were essentially similar to that of Martin (1971). However, several conditions of the bioassay were slightly modified.

The method of applying seeds in the glass tubes is that the embryo side of seeds were placed at the bottom of pyrex tubes (6 mm internal diameter at the top and 2 mm internal diameter at the constricted zone and total length 90 mm of glass tube). The glass tubes with seeds were inserted into the holes of plexiglass plates (800 holes) on a plastic tray lined with Whatman 3 MM paper in a thin layer of

water. The plastic tray was placed above the water surface in aluminium trays (5 x 66 x 80 cm) under a polyethylene frame box (38 x 83 x 95 cm). Polyethylene frame box was not sealed completely in order to keep the temperature of water at about 32°C. The light intensity was 750 fc with a HLRG lamp (High-pressure mercury lamp, Phillips). After about 64 hours, 3 seedlings about 3-5 mm coleoptile length, 1-2 mm length of primary root, were transferred into a vial containing 2 ml of distilled water and a disc of filter paper. The GA₃ concentrations tested were 0.0, 0.001, 0.005, 0.05, 0.5 µg per ml. The glass tubes with seedlings in a vial were wrapped nearly completely with Glad-wrap (clear plastic). The vials were left in the aluminium tray with about 1.5 cm depth of water. After 3 days the length of the coleoptile to the first leaf sheath of the seedlings was measured by a dial caliper. There were four replications for each concentration. Data were analyzed by the randomized complete block design.

8.3.2.2. Modified dwarf rice bioassay

Tan-ginbozu dwarf seeds were spread in a thin layer of water on Whatman 3 MM paper in a tray (65 x 30 x 43 cm) covered with a glass sheet under day light fluorescent tubes (two tubes, 120 cm, of 80 watt from 42 cm). After growing 55-64 hours the seedlings were selected as follows.

	<u>Length of coleoptiles (mm)</u>	<u>Length of roots (mm)</u>
A series	1.545 ± 0.07	2.23 ± 0.16
B series	2.19 ± 0.06	7.57 ± 0.37
C series	4.30 ± 0.61	12.70 ± 2.46

5 seedlings were placed into a vial (4.7 x 5.7 cm, scintillation vial) which had 1.0 ml of 2 % of agar and 1.0 ml of distilled water. The 2 % agar at about 40-45°C were added into a vial kept in a deep freezer. The vials containing a standard concentration of GA₃ in EtOH as 0.0, 0.001, 0.005, 0.05, and 0.5 µg/ml were dried in a vacuum oven and then were kept in a deep freezer until adding the agar solution. After transferring the 5 seedlings, the top of vials were sealed with Glad-wrap. The vials were placed on the bench the 'day-light' fluorescent tubes at 30-32°C for 3 days. The first sheath measurement was the same as mentioned above.

8.3.2.3. Lettuce hypocotyl bioassay

This bioassay was essentially that followed by Frankland and

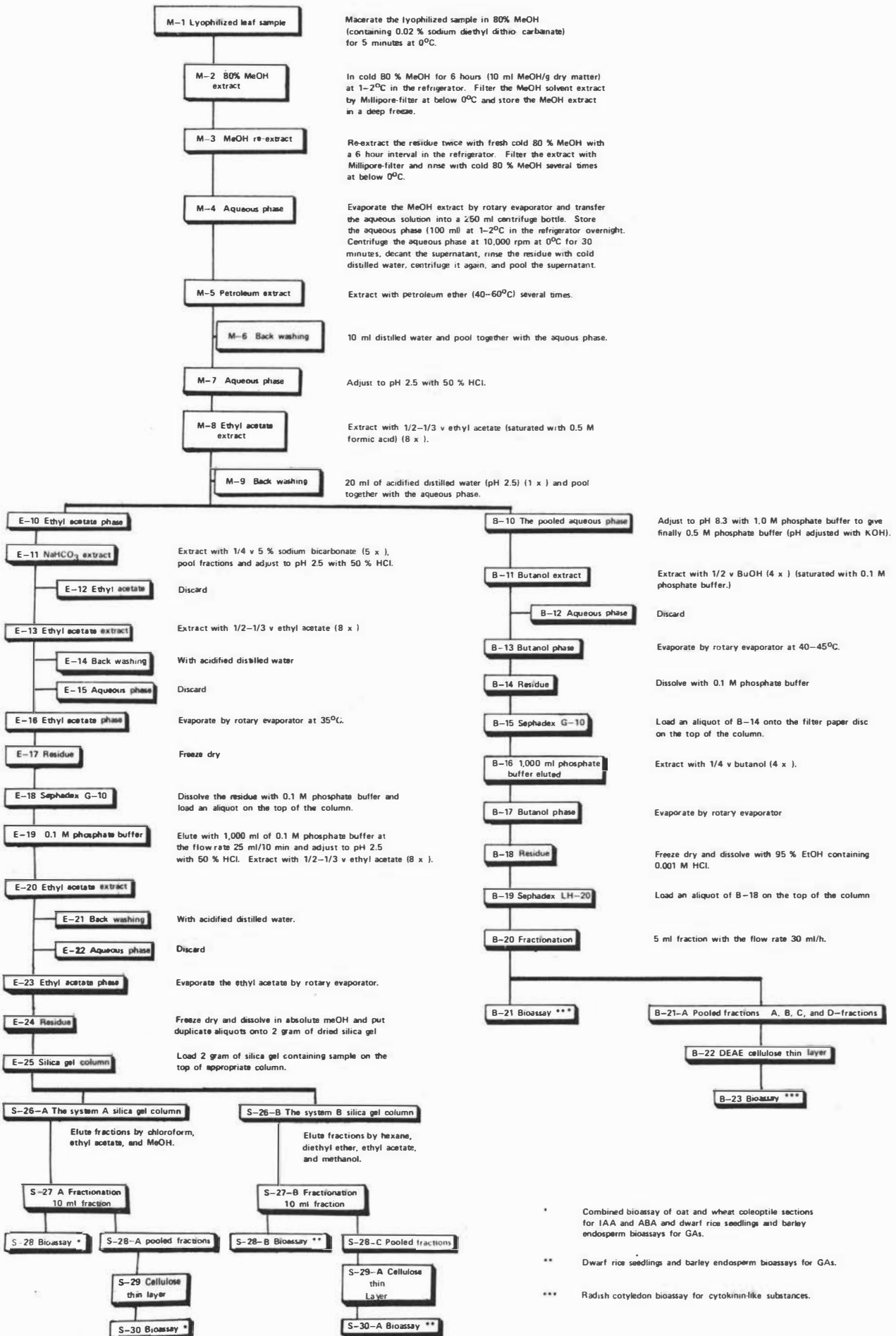


TABLE 6. A FLOW CHART OF EXTRACTION PROCEDURE FOR IAA-, ABA-, GA-, AND Ck-LIKE SUBSTANCES IN APPLE LEAVES.

Wareing (1960). The lettuce seeds were spread on the wet Whatmann 3 MM paper on a tray covered with a glass sheet. The tray was kept in the dark at 25°C. After 2 days seedlings with about 6-8 mm radicles were selected and 10 seedlings were placed in a vial (2.7 x 4.2 cm) with a filter paper moistened with 1 ml of 0.1 M phosphate buffer solution at pH 6.5. The vials sealed with Glad-wrap, were placed on the bench under 30 cm 'day-light' fluorescent tubes mentioned above at 25°C in the bioassay room. After 3 days hypocotyl length was measured by a dial caliper. Arctic, Arctic King, Green Mignonette, and Buttercruch lettuce varieties were tested and Gibberellic acid was used at concentrations of 0.001, 0.01, 0.1 and 1 µg/ml. There were three replications for each treatment. A 4 x 5 factorial experiment was used.

8.4. Endogenous plant hormones in apple leaves

8.4.1. Extraction procedures of IAA, ABA, and GAs

8.4.1.1. Leaf samples and extraction procedures

Leaves were collected only from the cluster bases bearing fruits on 12/11/70, were immediately frozen with dry ice, and later were lyophilized. The lyophilized leaf samples were ground with a Glen Cresten mill and were kept in preserving bottles in a deep freezer at - 20°C until used.

The extraction procedures used by various workers were examined in a preliminary experiment (See 9.1-9.2) and then plant hormones in apple leaf samples were analysed as shown in Table 6. 60 g of lyophilized leaf samples were extracted by the procedures in Table 6.

8.4.1.2. Ethyl acetate phase

8.4.1.2.1. The system A silica gel-celite column

In Table 6, the steps from E-10 to S-26 A and B were extraction procedures for IAA, ABA, and Gibberellins from the system A silica gel-celite column, as acidic fractions. Therefore the residue of ethyl acetate phase at the step E-24 was dissolved with MeOH. The aliquots equivalent to 30 g dry weight of leaf samples and to 15 g dry weight of leaf samples were added to each batch of 2 g of silica gel in the weighing glass boat respectively. The former was loaded on the system A silica gel column and the latter was for the system B column. The system A column was eluted by chloroform, ethyl acetate, and methanol and the system B column was eluted by hexane (160 ml), diethyl ether (330 ml), ethyl acetate (160 ml), and MeOH (200 ml). 53 fractions were collected from the system A and 100 fractions from the system B

column, each fraction being 10 mls.

2 ml of MeOH was added to each fraction. Several aliquots based on dry weight mentioned later were added to vials (2.7 x 5.7 cm) for gibberellins and test tubes (2.0 x 15.0 cm) for IAA and ABA. The aliquots from each fraction were pooled together as follows:

A Fraction = 1 -25 fraction (250 ml)

B Fraction = 26-35 fraction (100 ml)

C Fraction = 36-45 fraction (100 ml)

D Fraction = 46-53 fraction (80 ml)

The pooled fractions for A,B,C, and D in the vials respectively were dried in a vacuum oven.

8.4.1.2.1.1. Determination of IAA- and ABA-like substances

Each fraction collected from the system A silica gel-celite column eluted to 53 fractions was dissolved with 2.0 ml of MeOH and the aliquots equivalent to 0.2, 1.0, and 2 g dry weight of leaf samples were added to the test tubes and were dried in a vacuum oven for the bioassay of IAA- and ABA-like substances.

For cellulose thin layer chromatography the solvent system-MAAW (Methyl acetate : Acetonitrile : Ammonium : Water)(40 : 10 : 5 : 4 v/v/v/v) was used. Each chromatogram was bioassayed by the combination of oat and wheat coleoptile sections.

8.4.1.2.1.2. Determination of gibberellins

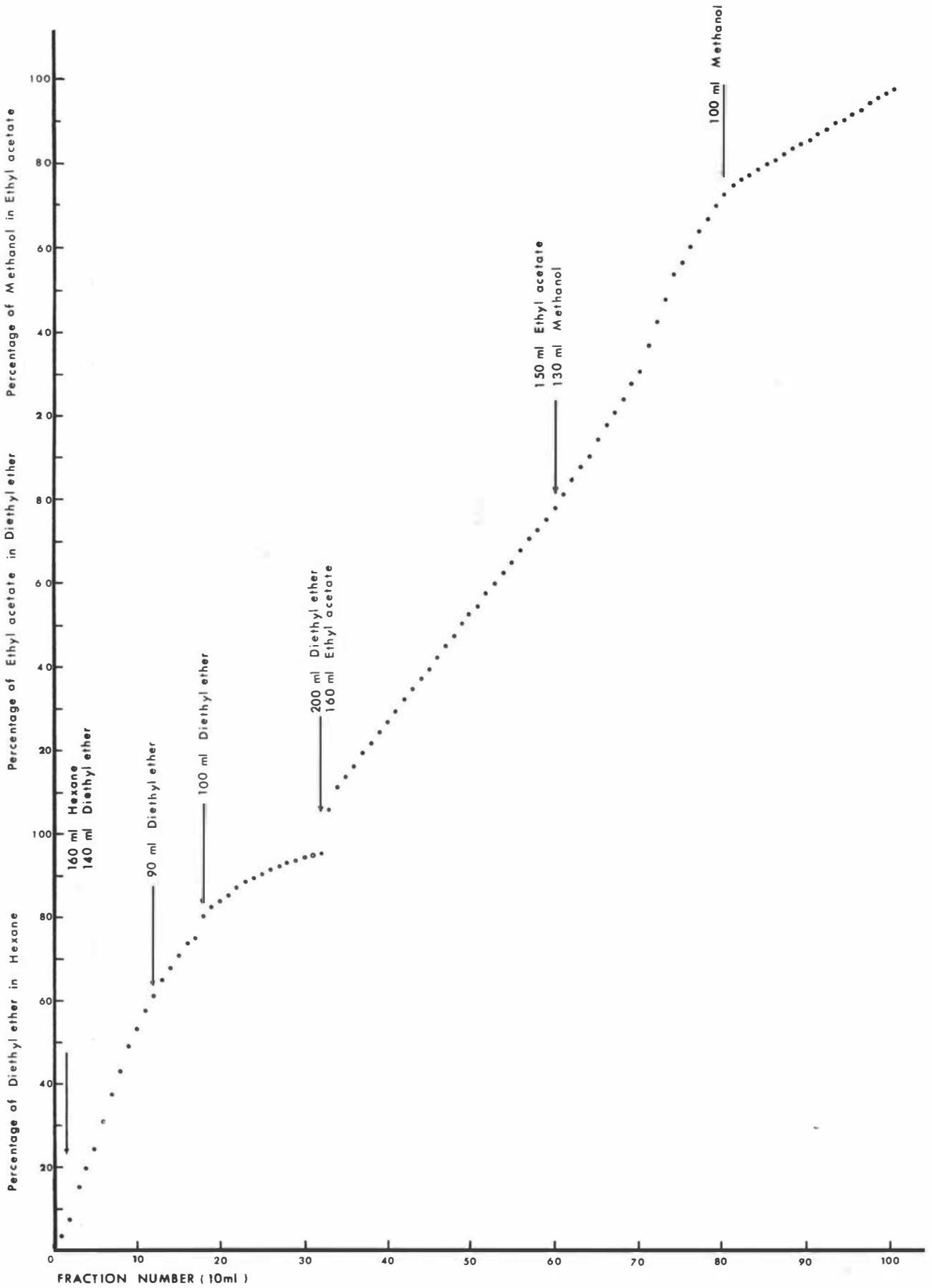
The aliquots equivalent to 1.0, 3.0, and 5.0 gram dry weight of leaf samples from each fraction of 53 fractions were added to the vials (scintillation vial) were dried in a vacuum oven for the use of dwarf rice bioassay. 1.0 g dry weight equivalent was added into a vial (2.7 x 4.2 cm) for the embryoless half barley bioassay test (See 8.4.-1.4.2.)

For cellulose thin layer chromatography, the aliquots equivalent to 1.0 and 5.0 g dry weight were applied as 2.5-5.0 cm bands and the plates developed to 15 cm from the origin by the solvent system-MAAW as for IAA and ABA determinations. 1 cm bands of cellulose were scraped off, by a razor blade, into the vials. The vials were stored in a deep freezer until the bioassays were made.

8.4.1.2.2. The system B silica gel-celite column chromatography

A three step gradient elution in the system B silica gel-celite column was used. The first step in the gradient solvent system was 160 ml of hexane in the A cylinder flask and 140 ml of diethyl ether in

Fig.15. Density of diethyl ether in hexane, ethyl acetate in diethyl ether, and methanol in ethyl acetate in each 10 ml fraction leaving the B cylinder flask in the gradient elution.



the B cylinder flask (See 8.4.1.2. ethyl acetate phase). After eluting 10 fractions, 100 ml of diethyl ether was added to the B cylinder flask and again at the 20th fraction. Then the second step was started from the 33rd to 59th fractions with the 200 ml (the remainder of mixture with hexane and diethyl ether) in the A cylinder flask and 160 ml of ethyl acetate in the B cylinder flask. In the third step the ethyl acetate/diethyl ether mixture was made up to 150 ml with ethyl acetate and 130 ml of MeOH added to the B cylinder. After eluting the 60th to 79th fractions 100 ml of MeOH was added at the 80th fraction and the column was eluted up to the 100th fraction (See Fig.15)

Each 10 ml fraction was dried and dissolved with 1 ml of MeOH. The aliquots equivalent to 1.0, 3.0, and 5.0 g dry weight were used for the dwarf rice seedling bioassay, and an aliquot containing 1.0 g dry weight was bioassay with the embryoless half barley test.

8.4.1.3. Bioassay for IAA and ABA

For the estimation of IAA-like and ABA-like substances, the bioassay was the combination of 3 sections of oat and 5 sections of wheat coleoptile (See 8.3.1.) (the combination bioassay).

The bioassays were made at three levels of concentration from the same fraction as mentioned above, but were not duplicated at the same concentration. A standard calibration for IAA and ABA was made for each batch of bioassays, obtaining the least significant difference (LSD) at the 5 % level with four replications as mentioned in section 8.3.1. A shaded area for the oat coleoptile bioassay and a dotted area for the wheat bioassay represents a significant difference at the 5 % level from the control (0.0 μ g) of the standard solution.

8.4.1.4. Bioassay for gibberellins

The gibberellins from the aliquots equivalent to 1.0 and 5.0 gram dry weight of leaf sample from the system A silica gel column were determined by dwarf rice bioassay with glass tubes containing 2 ml of distilled water (See 8.3.2.1.).

The aliquots containing 1.0, 3.0, and 5.0 g dry weight from the system B silica gel column were used in a dwarf rice bioassay with a culture medium of 1.0 ml 2 % of agar + 1.0 ml distilled water (See 8.3.2.2.) except the aliquot equivalent to 5.0 gram dry weight which was bioassayed by glass tubes. The statistical analyses were the same as calculated the LSD at the 5 % level from the standard calibration unless otherwise mentioned.

8.4.1.4.1. Barley endosperm test

Aliquots equivalent to 1.0 gram dry weight from the system A and B silica gel column respectively was used in the barley endosperm tests.

Seeds of the barley variety Research were dropped in 75 % ethanol for 30 second and then were soaked in 50 % H_2SO_4 for 3 hours at the room temperature (Coombe, Cohen, and Paleg, 1967). The seeds were dehulled, thoroughly washed in the tap water and were rinsed with distilled water. The seeds were dried by air and were cut into two parts with a razor blade. The embryoless halved barley seeds were stored in plastic bags until the seeds were used. The embryoless halved-seeds were soaked in 75 % ethanol for 30 sec and then in 1 % NaOCl for 15 minutes. After thoroughly washing the halved-seeds with sterilized distilled H_2O , the seeds were incubated in distilled water for 24 hours at $1-2^{\circ}C$ in the refrigerator. Four embryoless halved seeds in two milliliters of buffer solution containing 2 micro-moles acetate buffer and 20 micro-moles of calcium chloride with $10 \mu g/ml$ of chloramphenicol (Jones and Varner, 1967) and 0.01 M $FeCl_3$ (Goodwin and Carr, 1970) were incubated in a capped vial (2.7 x 4.2 cm). The test vials were shaken by an oscillation shaker (less than 40 oscillation per minute) at $25^{\circ}C$ in the bioassay room for 24 hours. The embryoless halved seeds were rinsed and the solution made up four milliliters for testing. 1 ml aliquot from the 4 ml testing solution was measured by the α -amylase test (Jones and Varner, 1967) and glucose test (Miller, Blum, Glennon and Burton, 1960). 1 ml of freshly prepared potato starch solution was added to the aliquot of testing solution. After 1 minute the solution was fixed with 1 ml of iodine reagent (Take 1 ml of 6.0 g KI and 600 mg of I_2 in 100 ml of distilled water and add to 100 ml of the 0.05 N HCl) for α -amylase test, or 1 ml of 3, 5-dinitrosalicylic acid reagent (1.2 g of phenol, 0.3 g of sodium sulfite, 120 g of Rochelle salts in 2 % of NaOH 300 ml with 300 ml of distilled water; Miller, Blum, Glennon, and Burton, 1960) for glucose test. 5 ml of distilled water was added to the fixed solution and then the optical density at 620 nm for α -amylase, and 640 nm for glucose test, determined by a model 101 Hitachi spectrometer.

8.4.2. Extraction procedure for cytokinins

8.4.2.1. Aqueous phase

In Table 6, the steps from B-10 to B-20 represent the purification at cytokinin-like substances. The aqueous phase after ethyl acetate extraction at pH 2.5 was adjusted to pH 8.3 and was then extracted 4 x with 1/2 volume of BuOH saturated with phosphate buffer.

The combined BuOH phase was reduced in vacuo at 45°C with a rotary evaporator. The residue was dissolved in 0.1 M phosphate buffer and passed through Sephadex G-10. The 1000 ml volume phosphate buffer eluant was extracted with 1/4 v BuOH (4 x) and was evaporated in vacuo at 45°C with a rotary evaporator. The residue dissolved in ethanol was loaded on a Sephadex LH-20 column and eluted with 95 % 0.001 M HCl. All of the procedures were the same mentioned in the preliminary test in Chapter 8. Each fraction was dried in a vacuum oven and the aliquots equivalent to 1 and 5 g dry weight of leaf sample from 1 ml of EtOH added to each fraction were bioassayed with radish cotyledons. The fractions from 1 to 15th fraction were discarded from the bioassay, because the major cytokinins activity tested from Sephadex LH-20 occurred from the 16th to 40th fraction. The fractions 16 to 85 were grouped into 4, as A, B, C, and D as follows:

- A Fraction = 16 - 43rd fraction (140 ml)
- B Fraction = 44 - 63rd fraction (100 ml)
- C Fraction = 64 - 78th fraction (75 ml)
- D Fraction = 79 - 85th fraction (35 ml)

For DEAE cellulose thin layer chromatography, aliquots containing 0.5, 1.0 and 5.0 g dry weight in EtOH from the combined fractions of A, B, C, and D was spotted as 2.5 - 5.0 cm bands on DEAE cellulose thin layer plates which were developed with the solvent system-MAAW. Each Rf was scraped off into a petri dish and stored in a deep freezer until radish cotyledons were ready to use.

8.4.2.2. Bioassay for cytokinin-like substances

The radish cotyledon bioassay was essentially the same as described by Letham (1968). Seeds of radish (Raphanus sativus L. cv Long Scarlet) were spread on well-wetted paper in plastic trays covered with a glass plate and were kept at 25°C in the dark bioassay room. After about 24-36 hours, the smaller cotyledon of the pair was excised from the medium sized seedling. Cotyledons were excised in the light and were pooled in distilled water and then distributed amongst petri dishes (diameter 5.0 cm) containing a filter paper (4.5 cm) with 1.5 ml of distilled water. 8 cotyledons were used in each dish. The dishes were placed on a sheet of wetted filter paper in the tray covered with a glass sheet. The trays were placed on a bench under continuous 'day light' fluorescent tubes. After 3 days each batch of cotyledons was blotted between filter papers and then was measured a fresh weight.

In statistical analysis, a single chromatogram from each fraction and each Rf thin layer was compared with the LSD at the 5 % level from the 0.0 μ g zeatin concentration in the standard calibration with 4 replications, this LSD being represented by the shaded and dotted area in Fig. 24.

8.5. Authentic plant hormones and plant materials in gas liquid chromatography

8.5.1. General procedure of GLC

A Pye 104 chromatograph temperature programmed with Model 64 and fitted with flame ionization detectors was used. A Phillips Model 8000 recorder or a Servoscribe voltage potentiometric recorder was used operating at a chart speed of 1 cm/minute.

8.5.1.1. Packing column

The silanized columns (Ross and Bradbeer, 1971 a) (150 cm x 4 mm ID glass tubes or 180 cm x 2 mm ID glass tubes attached with metal connector) were packed with 3 % OV-1 or 3 % SE-52 on 80-100 mesh Gas-Chrom Q (Applied Sciences Laboratories, Inc) using the method in the Pye Unicam manual as follows: The supporting material of Gas-Chrom Q was placed into chloroform containing 3 % OV-1 or 3 % SE-52 in a 250 ml round bottom flask. The flask was connected to a vacuum and gently shaken in vacuo with a shaker (The Griffin Flask Shakers, Griffin & George LTD). The flask in vacuo was often shaken by hand for even distribution of the stationary phase. When most of the solvent had been evaporated the remaining material was heated on a water bath at 100°C for 30 minutes to 1 hour under moderate vacuum.

The end of the column was plugged with silanized glass wool. Then ½ gram of 3 % OV-1 or 3 % SE-52 coated on Gas-Chrom Q was poured into the inlet of column. A 10 lb pressure of nitrogen pushed the materials to the end of the column which was vibrated by a vibrator. This procedure was repeated several times until the column was packed. The column was preconditioned at 300°C for 24 hours and then carrier gas was passed through continuously at about 10-20 ml/min. for 24-48 hours.

8.5.1.2. The GLC conditions

The conditions were injector zone about 160°C; detector oven 300°C, nitrogen at 20 lb, hydrogen at 13 lb, and air at 10 lb. The flow rate was 50 ml/min. for a temperature programmed procedure, the instrumental conditions were 4 minute initial hold at 160°C and after a manual change of temperature to 200°C for 1½ minute a 3°C/min. temperature programme from 200°C to 280°C or 300°C was started. This is called the 160° - 200°C temperature programme.

8.5.1.3. Preparation of derivatives

Methyl esters derivatives (ME) were prepared by Horning's

Method (1968). Samples containing 10 μ g of authentic hormones such as NAA, IAA, IPA, ABA, GA₉, GA₅, GA₄, GA₁, and GA₁₃ were quantitatively pipetted from a MeOH stock solution and placed in Macro culture vials lined with teflon cap (1.6 x 7.5 cm) (Gehrke and Lakings, 1971). MeOH solution containing authentic plant hormones was exposed to excess ethereal diazomethane (1-2 ml) for one minute or less, and then ether and MeOH were removed by a stream of nitrogen (Horning, 1968).

Methyl esters-trimethylsilyl ether derivatives (ME-TMS) were prepared by the addition of the ratio 6 : 4 : 2 with dried pyridine (PYR) (0.15 ml) : hexamethyldisilazane (HMDS) (0.10 ml) : trimethylchlorosilane (TMCS) (0.05 ml) in order to the residue of methylated compounds (Horning, Knox, Dalgliesh, and Horning, 1966). The reagents to make the ME-TMS derivatives are called PYR-HMDS-TMCS silyl reagents in this text. After standing overnight at room temperature, the reagents were removed by evaporation under vacuum in a similar fashion to Lehrfeld (1971), using a pickle jar. The residue was dissolved in MeOH for injection. In the earlier experiment toluene was used, but later the use of toluene was abandoned because of the contamination of detectors.

In the injection technique 1 μ l-5 μ l of ME-TMS derivatives in MeOH which was taken to the 10 μ l Hamilton syringe plus 1 μ l of pure MeOH was injected to the top of the column.

These general procedures were used in the following experiments, unless otherwise mentioned.

8.5.2. Gas chromatographic separation of hormones from apple leaves and comparison with bioassay results

8.5.2.1. Plant extract

168 gram lyophilized samples taken on 22/10/70 were extracted by the steps from M-1 to E-17 in Table 6. The aliquot equivalent to 80 gram dry weight of leaf sample was loaded on Sephadex G-10 and followed the steps from E-18 to E-24 in Table 6, but extracted by diethyl ether instead of ethyl acetate. The residue of diethyl ether phase at the step E-24 in Table 6 was dissolved with 2 ml of MeOH and the aliquot was used in the following experiment.

8.5.2.2. GLC of plant extracts

The methylated residue equivalent to 20 gram dry weight of leaf sample was dissolved in 40 μ l of MeOH. The 2 μ l aliquot equivalent to the 1.0 gram dry weight of leaf sample was injected with 1 μ l of pure MeOH using a 10 μ l Hamilton syringe. The 1.5 μ g of authentic plant hormones such as 5-methyl indole, NAA, IAA, ICA, IPA, ILA, ABA, GA₄, and

GA₁ in 1 ml of MeOH was co-chromatographed with and without the plant extract.

GLC conditions were the same as mentioned in 8.5.1. The stationary phase was 3 % of SE-52 in a 180 cm x 2 mm ID column. Temperature programmed separation was from 120°C-300°C with an initial hold for 4 minutes at 120°C and then 3°C/min.

8.5.2.3. The IAA- and ABA-like substances in the aliquot of ether acidic fraction by bioassay

The aliquots equivalent to 0.1, 0.5, 1.0, and 1.5 gram dry weight in 5, 25, 50, and 75 µl MeOH respectively were spotted as a 3 cm band on cellulose thin layer plates except for 1.5 gram sample in which a 5 cm band was applied. The IAA and ABA markers were spotted on the middle of a plate. The plates were developed by the solvent system MAAW. Each concentration of dry leaf sample mentioned above was chromatogrammed by three replications except the 1.5 gram dry weight sample which was not replicated.

The principle bioassay was the same as mentioned in section 8.3.1. i.e., the combined coleoptile bioassay with 2 sections of 5 mm oat and 3 sections of 10 mm wheat coleoptile. The means from three chromatograms were compared with the LSD at the 5 % level from the IAA and ABA standard calibration with 4 replications.

8.5.3. GLC calibration of natural plant hormones and related compounds

The aliquots equivalent to 10 µg in MeOH for NAA, IAA, IPA, IIA, ABA, GA₄, GA₅, GA₁₃, and GA₁ were silylated by the PYR-HMDS-TMCS silyl reagents (see 8.5.1.3.). The residue of ME-TMS derivatives was dissolved with MeOH and dilute to 1.0 µg, 1.0, 0.5, 0.1, 0.05, 0.01, 0.0075, and 0.0025 µg/µl. Each concentration was chromatographed by 10 x, 20 x, 50 x, 1 x 10²x, 5 x 10²x, 10 x 10²x, 20 x 10²x, and 50 x 10²x attenuation respectively.

A single injection was chromatographed in most cases and the programme was the 160°C-200°C programme (See 8.5.1.2.).

8.5.4. GLC of natural plant hormones and related synthetic plant hormones after cellulose thin layer chromatography

Cellulose MN 300 (Machery Nagel Co.) was washed with 6 N HCl, 2 % EDTA, MeOH, and ether consequentially.

50 µl MeOH containing 10 µg each of NAA, IAA, IPA, ABA, GA₉, GA₄, GA₁₃, and GA₁ was spotted on a cellulose thin layer plate.

Two plates were developed to a 15 cm line from the origin by the solvent

system MAAW. One plate (B) was removed as soon as the 15 cm line was reached while the second plate (A) was removed 3 minutes later. The A plate was scraped off in 1.0 cm bands and the B plate 1.5 cm bands. The cellulose was scraped off by a Gillette blade into a sintered disc funnel lined with filter paper and eluted with 15 ml of MeOH/0.25 M formic acid. The solvent was dried in the vacuum oven at room temperature. The residue was methylated by the usual method. The residue was dissolved with 100 μ l of MeOH and 1 μ l of methylated material plus 1 μ l of pure MeOH was injected.

Standard calibration of authentic plant hormones was made by the same procedure but without the development of the cellulose thin layer plates. The diluted concentration of 0.1, 0.05, and 0.025 μ g/ μ l in 100 μ l MeOH was injected onto 3 % OV-1 on 80-100 mesh Gas-Chrom Q with a 150 cm x 4 mm ID column.

8.5.5. Effect of different silylation methods on retention time and peak height of natural plant hormones

8.5.5.1 Gibberellin A₃

10 μ g of GA₃ was silylated by three different methods. Treatment 1 was 150 μ l of pyridine, 100 μ l of hexamethyldisilazane, and 50 μ l of trimethylchlorosilane (TMCS) standing over-night at room temperature. Treatment 2 was 100 μ l pyridine (PYR) and 100 μ l of N.O.-bis(trimethylsilyl)trifluoroacetamide (BSTFA) standing overnight at room temperature. Treatment 3 was 40 μ l of PYR, 40 μ l of BSTFA, and 20 μ l of TMCS over-night and then 100 μ l of BSTFA. 2 μ l was injected with each 1 μ l of pure reagent, i.e., methanol in Treatment 1 and BSTFA in Treatment 2 and 3.

The column was a 180 cm x 2.0 mm ID with a 3 % OV-1 stationary phase coated on Gas-Chrom Q (80-100 mesh). The detector response was at 1×10^2 attenuation.

Retention times were measured from the solvent peak and quantity determined by peak height. The number of theoretical plates (N) was calculated by $N = \left(\frac{X^2}{Y}\right)$, where 'Y' is the length of the baseline cut by the two tangents, 'X' is the distance from starting of solvent peak to peak maximum (McNair and Bonelli, 1969).

8.5.5.2. Several authentic plant hormones

In order to make a ME-derivative, an aliquot of 10 μ g each of NAA, IAA, IPA, ABA, GA₉, GA₅, GA₄, GA₁₃, and GA₁ in 100 μ l MeOH was pooled and methylated by the method used in 8.5.1.3. 1 μ l of the ME-derivatives were injected. The MeOH was evaporated with nitrogen from

the remaining derivatives the residue of which was silylated with BSTFA + TMCS as described in section 8.5.5.1. The chromatographic conditions were same except the detector response was at 2×10^2 attenuation. The experiment was performed in triplicate.

Table 7. Effect of pH in the aqueous phase on loss of ^{14}C -IAA in the extraction procedure.

A. At pH 8.0				B. At pH 2.5			
Plant sample	Dpm of ^{14}C -IAA	% recovery between two steps	% recovery from the original	Plant sample	Dpm of ^{14}C -IAA	% recovery between two steps	% recovery from the original
10 g of lyophilized stem	499,335 (13.65 ug)		100	30 g of lyophilized stem	411,517 (11.25 ug)		100
80% MeOH extract with 0.02% sodium diethyl dithiocarbamate	451,500	90.4	90.4	80% MeOH extract with 0.02% sodium diethyl dithiocarbamate	399,931	97.2	97.2
Aqueous phase	438,333	97.1	87.9	Aqueous phase	352,984	88.3	85.8
Centrifuge (omitted)				Centrifuge at 0°C with 10,000 rpm for 30 minutes			
Petroleum ether extract	4,353	1.0	0.9	Petroleum ether extract	3,786	1.1	0.9
Aqueous phase adjust to pH 8.0	147,750	33.7	29.6				
Ethyl acetate extract (8 x)	1,650	1.1	0.3				
Aqueous phase adjust to pH 2.5	32,536	22.0	6.5	Aqueous phase adjust to pH 2.5	338,983	110.4	94.7
Ethyl acetate extract (8 x)	30,128	92.6	6.0	Ethyl acetate extract (8 x)	364,604	93.5	88.6
NaHCO_3 extract (5 x)	30,747	102.1	6.2	NaHCO_3 extract (5 x)	348,286	95.5	84.6
Ethyl acetate phase	594	2.0	0.1	Ethyl acetate phase	4,977	1.4	1.2
Aqueous phase acidify to pH 2.5				Aqueous phase acidify to pH 2.5			
Ethyl acetate extract	28,656	93.2	5.7	Ethyl acetate extract (8 x)	347,402	99.7	84.4
Aqueous phase	80	0.3	0.0	Aqueous phase	4,321	1.2	1.1
Residue to ethyl acetate	22,573	78.8	4.5	Residue of ethyl acetate after evaporation	309,049	89.0	75.1
Loaded on Sephadex G-10				Loaded on Sephadex G-10			
1000 ml fraction				1000 ml fraction	292,300	94.6	71.0
				1000-1500 ml	12,510	4.0	3.0
Ethyl acetate extract (8 x)				Ethyl acetate extract (8 x)	352,464	120.5	85.7
				Aqueous phase	1,852	0.5	0.5
Residue to ethyl acetate after evaporation	18,914	83.8	3.8	Residue of ethyl acetate after evaporation	333,822	94.7	81.1

CHAPTER 9
Experimental Results

9.1. Loss of ^{14}C -IAA during extraction and purification procedures

9.1.1. Effect of pH in the aqueous phases on the degradation of ^{14}C -IAA in the extraction procedure

9.1.1.1. At pH 8.0 in the aqueous phase

A number of research worker have used extraction procedures at pH 8.0 in the aqueous phase, after evaporation of methanol extract, with ethyl acetate partition so that some inhibitors which interfered in the bioassays were removed from the aqueous phase.

Table 7-A indicated each percentage of recovery for ^{14}C -IAA at different steps during purification. The values in Table 7-A represent one trial, although a duplicate experiment confirmed the results. The greatest loss of ^{14}C -IAA occurred when the aqueous phase was adjusted to pH 8.0 with 1.0 M phosphate buffer and extracted with ethyl acetate, i.e., only 29.6 % of ^{14}C -IAA was recovered from the original dosage of IAA. A further extraction at the same pH with ethyl acetate gave only an additional 6.5 % from the alkaline aqueous phase. There was little loss of ^{14}C -IAA when partitioned with bicarbonate (pH 8.5) after first extracting into ethyl acetate at pH 2.5. This surprising result suggests that some factor, possible enzyme was causing degradation of IAA but that this factor could be removed by first partitioning the auxin into ethyl acetate at pH 2.5 (See next section and discussion).

9.1.1.2. At pH 2.5 in the aqueous phase

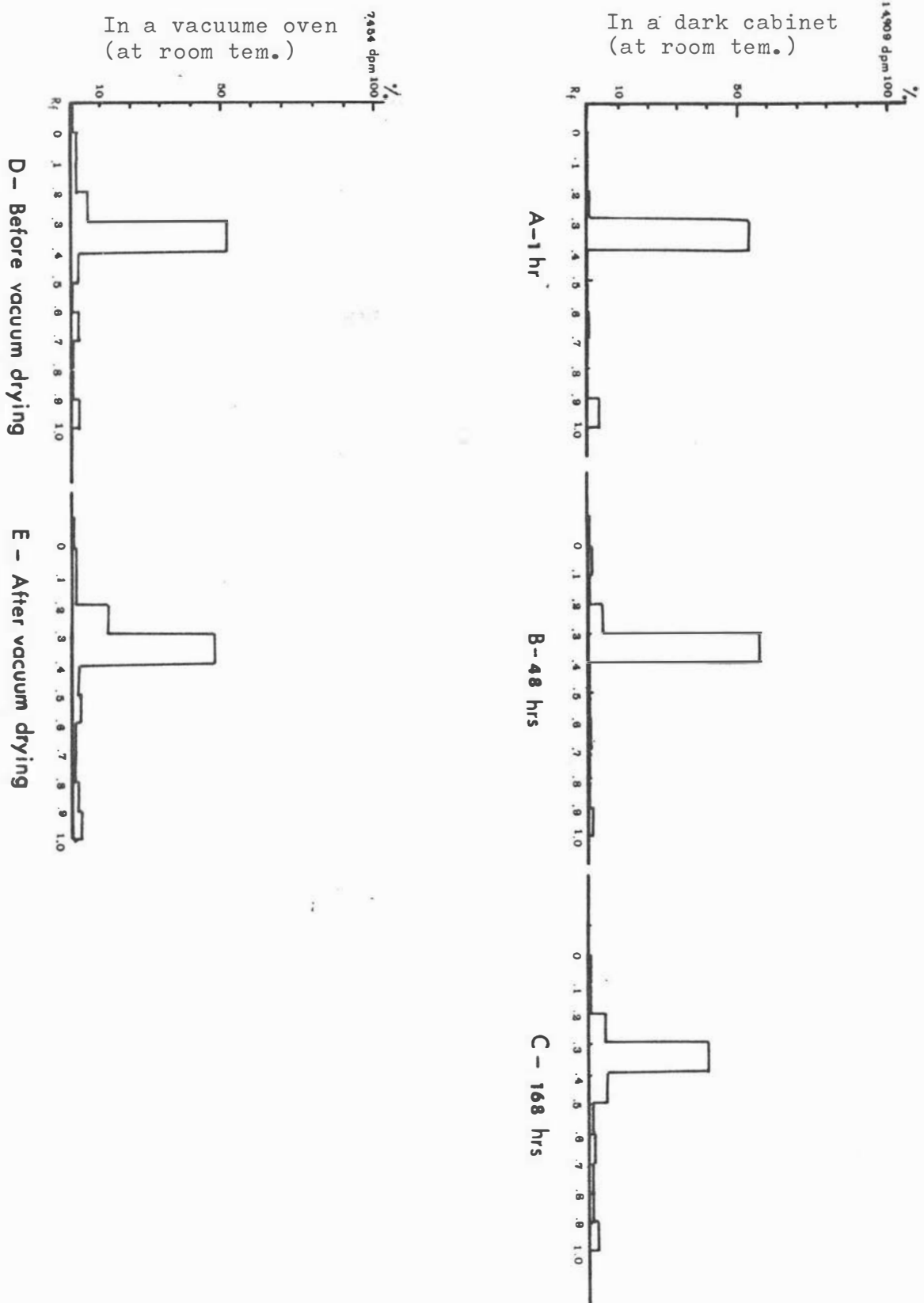
The values in Table 7-B are means from duplicate trials. The extraction procedures in Table 7-B are the same as in Table 7-A except for the initial pH of the aqueous phase. In contrast to the loss at pH 8.0 there was little loss of ^{14}C -IAA in the acidified aqueous phase.

Almost 80 % recovery was achieved through the whole extraction procedures, including the Sephadex G-10 column chromatography, as shown in Table 7-B. The solvent extraction procedure gave 75 % recovery. However, when the residue was loaded and eluted from Sephadex G-10 81 % recovery was obtained. Probably because interfering plant components were removed.

9.1.2. Column chromatography

9.1.2.1. Recovery of ^{14}C -IAA from Sephadex G-10 column chromatography

The exact position of elution of ^{14}C -IAA from a Sephadex G-10 column was not studied. A 1,000 ml fraction using 0.1 M phosphate

Fig. 16. Recovery of ^{14}C -IAA after cellulose TLC

buffer should be sufficient to elute ^{14}C -IAA, on the basis of its molecular weight compared to GAs (Crozier, Aoki, and Pharis, 1967). After evaporation of the ethyl acetate phase with which ^{14}C -IAA was extracted from the acidified phosphate buffer solution of the final residue of 333,822 dpm (9.1267 μg) 309,049 dpm (8.4494 μg) was recovered from the solvent partition column.

9.1.2.2. Behaviour of ^{14}C -IAA in the gradient elution of the silica gel-celite column chromatography

The methods are described in section 8.1.3.2. The original applied ^{14}C -IAA was 18,450.6 dpm (0.5044 μg) of ^{14}C -IAA which was applied to on the top of the silica gel-celite column. Each 5 ml fraction collected from the column eluted by a gradient of chloroform, ethyl acetate, and methanol is shown as dpm, percentage recovery and a cumulative percentage of recovery of ^{14}C -IAA in Table 8. The highest recovery of ^{14}C -IAA was at the 24th fraction after 115 ml volume of elution. About 60 % of ^{14}C -IAA occurred within 15 ml around the 24th fraction which was the highest peak. The 50 ml from the 21st to 31st fractions exhibited an 88 % recovery of ^{14}C -IAA, while the first 250 ml volume from the 1st to 50th fraction gave 98 % recovery, gave only an additional 10 % increase. Therefore if the IAA fraction is the only component of interest in the plant materials, the 50 ml from the 21st to 31st fractions could be collected from the silica gel-celite column.

9.1.3. Recovery of ^{14}C -IAA from cellulose thin layer chromatography

In order to know the fate of ^{14}C -IAA on cellulose thin layer during storage of plates in the dark cabinet at room temperature and during a dry condition in vacuum, ^{14}C -IAA with the apple leaf samples from Sephadex G-10 obtained in Table 7-A and B was used. Aliquots equivalent to 14,909 and 7,454 dpm was spotted on cellulose thin layer. After the development in the solvent system-MAAW, each Rf was counted and converted to the percentage of radioactivity recovered from the thin layer was shown in Fig. 16-A,B,C,D, and E.

The means of recovery of ^{14}C -IAA from the cellulose thin layer were 50.0 ± 6.9 at IAA position of Rf 0.3-0.4, regardless of the conditions of storage. The duplicate experiment as in the Fig. 16-A,B,-C, D, and E indicated 57.22 ± 3.58 . The means of recovery included the lower and upper from Rf 0.3-0.4 IAA position were calculated 58.2 ± 4.5 . Because some plates were not well separated as shown in Fig. 16-C-168 hrs. The duplicated experiment gave a similar result, 63.37 ± 10.45 . Total

Table 8. Recovery of ^{14}C -IAA from a silica gel-celite column
by the gradient elution method (18,450.6 dpm = 0.50444 μg).

No. of fraction	Dpm of 5 ml fraction	% recovery of ^{14}C -IAA	Cumulative % recovery
1	0.0	0.000	0.000
2	2.1	0.011	0.011
3	0.0	0.000	0.011
4	4.1	0.022	0.033
5	1.0	0.0025	0.038
6	0.0	0.000	0.038
7	17.1	0.093	0.131
8	35.3	0.191	0.322
9	75.3	0.41	0.732
10	127.2	0.69	1.422
11	82.0	0.44	1.862
12	50.0	0.27	2.132
13	34.1	0.18	2.312
14	24.1	0.13	2.442
15	22.2	0.12	2.562
16	31.6	0.17	2.732
17	33.9	0.18	2.912
18	43.8	0.24	3.152
19	34.7	0.19	3.342
20	39.9	0.22	3.562
21	47.6	0.26	3.822
22	880.4	4.77	8.592
23	3576.1	19.38	27.972
24	4087.2	22.15	50.122
25	3282.4	17.79	67.912
26	1753.4	9.50	77.412
27	1104.4	5.99	83.402
28	651.8	3.53	86.932
29	400.1	2.17	89.102
30	296.8	1.61	90.712
31	146.8	0.80	91.512
32	167.9	0.91	92.422
33	198.5	1.08	93.502
34	130.2	0.71	94.212
35	86.2	0.47	94.682
36	64.8	0.35	95.032
37	56.7	0.31	95.342
38	44.9	0.24	95.582
39	48.8	0.26	95.842
40	102.6	0.57	96.412
41	70.6	0.38	96.792
42	50.4	0.27	97.062
43	31.9	0.17	97.232
44	31.1	0.17	97.402
45	26.1	0.14	97.542
46	12.9	0.07	97.612
47	22.7	0.12	97.732
48	20.2	0.11	97.842
49	19.7	0.11	97.952
50	5.3	0.03	97.982
51	18.0	0.10	98.080
52	22.3	0.12	98.20
53	13.6	0.07	98.27
54	16.7	0.09	98.36
55	13.5	0.07	98.43
56	16.9	0.09	98.52
57	10.1	0.05	98.57
58	9.9	0.05	98.62
59	11.8	0.06	98.68
60	4.5	0.03	98.71
61-80	299.8	1.62	100.33
81-100	150.3	0.81	101.14
Total	18664.3	101.14	101.14

Table 9. Effect of solvent on the partition of ^3H -zeatin at pH 2.5 and pH 8.3. and partition co-efficients with ethyl acetate and butanol.

P.	Ethyl acetate extraction						Butanol extraction			
	pH 8.3		pH 2.5				pH 8.3		pH 2.5	
	saturated with 0.1M phosphate		saturated with		Non-saturated		saturated with		saturated with	
	DPM	%	DPM	%	DPM	%	DPM	%	DPM	%
1st	3,304.6	7.08	419.4	0.90	430.4	0.92	32,248.6	69.06	2,631.5	5.64
2nd	3,009.0	6.44	242.4	0.52	295.4	0.63	8,727.6	18.69	2,874.3	6.16
3rd	3,974.7	8.51	240.4	0.52	319.2	0.68	2,110.7	4.52	3,815.2	8.17
4th	2,376.8	5.09	130.7	0.29	293.8	0.63	537.0	1.15	3,005.8	6.44
5th	2,300.6	4.93	314.0	0.67	273.4	0.59	140.1	0.30	1,567.4	3.36
6th	2,736.4	5.86	177.0	0.38	233.6	0.50	23.4	0.05	2,372.5	5.08
7th	2,418.9	5.18	280.8	0.60	241.4	0.52	18.7	0.04	2,160.8	4.63
8th	2,092.0	4.48	130.7	0.28	299.4	0.49	18.7	0.04	1,261.8	2.70
Sub. Tot.	22,213.0	47.57	1,936.4	4.15	2,316.6	4.96	43,824.8	93.85	19,689.3	42.16
R.	21,477.4	45.99	44,189.8	94.63	43,045.9	92.18	18.7	0.04	16,630.6	35.61
Total	43,690.4	93.56	46,126.2	98.78	45,362.5	97.14	43,843.5	93.89	36,319.9	77.78
P.C.	13.12		108.89				0.488		16.73	

Values are means of three replications in ethyl acetate extraction but one replicate at pH 8.3 and two replications at pH 2.5 in BuOH extraction.

Abbreviations: P = Partition; P.C. = Partition coefficient; Sub. Tot. = sub-total; R. = Remainder

counting of ^{14}C -IAA through the Rfs in cellulose thin layer was $67.45 \pm 4.9\%$ and the duplicated experiment was almost the same, $68.51 \pm 10.63\%$.

Therefore it may be concluded that there was about 40-50 % recovery of ^{14}C -IAA through the whole extraction procedures such as methanol extraction, solvent partition, Sephadex G-10 chromatography and cellulose thin layer chromatography.

9.2. Behaviour of ^3H -zeatin in chromatography

9.2.1. Solvent partition chromatography

9.2.1.1. Effect of pH in the aqueous phase on partition of ^3H -zeatin by ethyl acetate and butanol (See section 8.7.1.1.)

Radioactivity of each partition fraction and the partition coefficients at pH 8.3 and 2.5 between water and ethyl acetate or butanol are shown in Table 9. The partition coefficients are expressed as

$$K_d = \frac{\text{Percentage in aqueous phase}}{\text{Percentage in organic phase}}$$

Where percentage of ^3H -zeatin was calculated by % recovery from the original dosage.

The partition coefficient with ethyl acetate at pH 8.3 and at pH 2.5 was 13.12 and 108.89 respectively. About 48 % of ^3H -zeatin was found in the ethyl acetate after partitioning eight times at pH 8.3. Therefore, ^3H -zeatin in the aqueous phase at pH 8.3 would be considerably partitioned into the ethyl acetate phase. On the other hand, little ^3H -zeatin partitioned into the ethyl acetate phase at pH 2.5. As can be seen in Table 9, 4.15 % partitioned into the ethyl acetate phase saturated with 0.5 M formic acid and 4.96 % into the pure ethyl acetate.

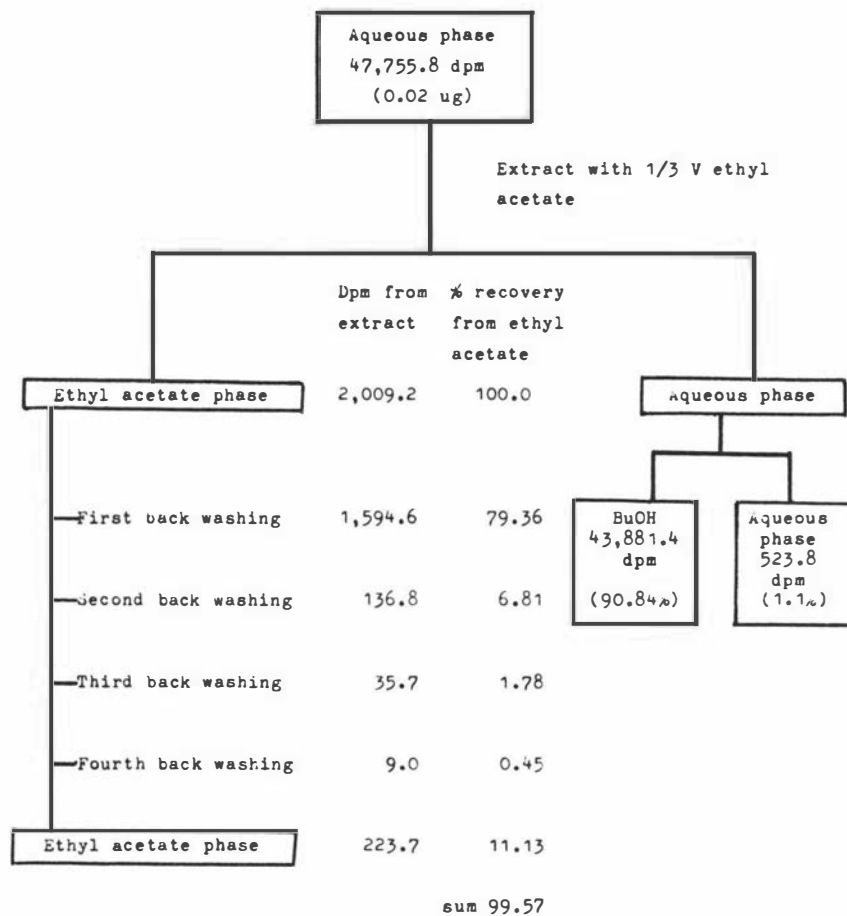
Butanol had an appreciable capacity to remove zeatin at pH 8.3 and 2.5, with a partition coefficient of 0.448 and 16.73 respectively. In other words, 93.42 % of ^3H -zeatin could be removed from the aqueous phase at pH 8.3 by partitioning 4 times with butanol. 8 times partition could also remove 42.16 % of ^3H -zeatin from the aqueous phase at pH 2.5.

9.2.1.2. Back washing from ethyl acetate phase

The partition coefficient in ethyl acetate for ^3H -zeatin in the acidified aqueous phase was very high, after 8 partitions with the aqueous phase at pH 2.5 the ethyl acetate contained only 2,009 dpm (4.21 % as shown in Table 10-A. 80 % of the ^3H -zeatin in the ethyl acetate phase could be removed by back washing with an acidified aqueous solution. Further back washing did not give significantly better recovery of ^3H -zeatin from the ethyl acetate phase.

Table 10 A and B. Back washing from ethyl acetate and butanol phase.

A. Back washing from ethyl acetate phase



B. Back washing from Butanol phase

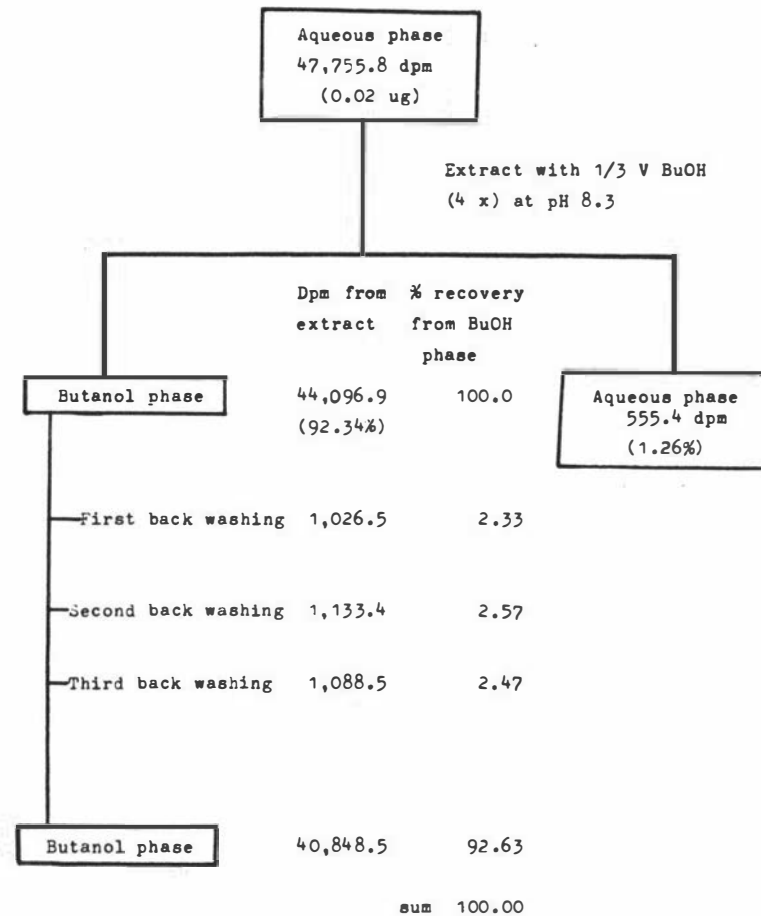


Table 11. Partition of ^3H -zeatin from Sephadex G-10 column chromatography
(686,336.5 dpm = 0.289428 μg)

Fraction number	Dpm of 100 ml* fraction	% recovery*	Cumulative % recovery	% recovery** from extract by BuOH
Blue dextran 50 ml	1,233	0.18	0.18	
1st	5,700	0.83	1.01	
2nd	29,770	4.34	5.35	
3rd	26,255	3.83	9.18	
4th	31,805	4.63	13.81	
5th	31,060	4.53	18.34	81.33 (588,220 dpm)
6th	87,420	12.74	31.08	
7th	169,830	24.74	55.82	
8th	140,715	20.50	76.32	
9th	43,165	6.29	82.61	
10th	10,700	1.56	84.17	
11th	5,800	0.74	84.91	
12th	3,685	0.54	85.45	
13th	4,505	0.66	86.11	2.87
14th	3,235	0.47	86.58	(19,729.9 dpm)
15th	2,390	0.35	86.93	
16th	880	0.13	87.06	
17th	870	0.13	87.19	
18-21st	4,698**	0.68**	87.87	0.68
Total	5,990,018.0		87.87	84.88

(1) * Estimation from the aliquots 100 ml fraction.

(2) ** Extraction from the effluent by BuOH saturated with 0.1 M phosphate buffer.

(3) Values are calculated from two replications.

Table 12. Pattern in the fractionation of ^3H -zeatin from Dowex 50 W x 8
(686,336.5 dpm = 0.289428 μg)

No. of fraction	Dpm of 100 ml fraction	% recovery	Cumulative % recovery	Recovery from extraction Dpm	%
1	153.4	0.02	0.02		
2	168.3	0.03	0.05		
3	191.8	0.03	0.08	50.8	0.07
4	2,430.0	0.35	0.43		
5	395,535.0	57.63	58.06		
6	85,970.0	12.53	70.59		
7	50,745.0	7.39	77.98		
8	46,710.0	6.81	84.79		
9	24,785.0	3.61	88.40		
10	13,800.0	2.01	90.41		
11	7,170.0	1.04	91.45		
12	9,445.0	1.38	92.83		
13	4,580.0	0.67	93.50	557,001.4	81.16
14	6,865.0	1.00	94.50		
15	5,860.0	0.85	95.35		
16	4,700.0	0.68	96.03		
17	4,005.0	0.58	96.61		
18	5,205.0	0.76	97.37	18,817.9	2.74
19	3,545.0	0.52	97.89		
20	2,125.0	0.31	98.20		
21	2,500.0	0.36	98.56	18,095.7	2.64
Total	676,488.5	98.56	98.56	593,965.8	86.61

Values are from single replication.

9.2.1.3. Back washing from butanol phase by 0.5 M phosphate buffer

Back washing from butanol phase with 10 ml of 0.5 M phosphate buffer gave an average 2.46 % recovery of ^3H -zeatin at each back washing. That is three back washings gave 7.37 % loss from the partitioned butanol and therefore it may not be a desirable procedure.

9.2.2. ^3H -zeatin in column chromatography

9.2.2.1. Sephadex G-10

The behaviour of ^3H -zeatin in partition column chromatograph on Sephadex G-10 is given in Table 11. The main peak was 24.74 % in a 100 ml fraction. A 300 ml fraction around the main peak contained nearly 60 % of the radioactivity. In a 1,000 ml fraction, 84 % of ^3H -zeatin was obtained. A figure of 81.33 % of ^3H -zeatin was obtained when the aqueous phase was extracted with butanol. An additional 700 ml gave 2.87 % of labelled zeatin, while a further 400 ml from the 18th-21st fractions gave only 0.68 % recovery. Total radioactivity estimated in 2,100 ml fraction, therefore, was about 85 %. Only an extra 3.7 % of ^3H -zeatin was obtained beyond 1,000 ml, thus 1,000 ml would be a reasonable amount for estimating zeatin.

9.2.2.2. Dowex 50 W x 8

The pattern of elution for ^3H -zeatin using a 50 W x 8 column exhibited a tailing peak as shown in Table 12. There was no activity in the distilled water rinse (300 ml) but as soon as 6 N ammonium solution was used the ^3H -zeatin appeared in the fractions. The first and second fractions of 200 ml represented 57-63 % of the activity and the third fraction had 12.53 %. Therefore a cumulative percentage recovery of ^3H -zeatin was over 70 %. The pooled 1,000 ml fraction partitioned with BuOH gave 81.16 % recovery, while 93.50 % was obtained direct from the ammonium effluent. However total radioactivities partitioned by BuOH from 1,800 ml ammonium fraction was 86.61 %.

9.2.2.3. Sephadex LH-20

The elution pattern of ^3H -zeatin and unlabelled cytokinins such as 6-(β , β -dimethylallylamino)-purine, dihydrozeatin, zeatin, and zeatin-riboside in Sephadex LH-20 eluted with 95 % EtOH with 0.001 M HCl is given in Table 13.

The 6-(β , β -dimethylallylamino)-purine and dihydrozeatin peaks overlapped while the peaks of zeatin and dihydrozeatin were almost separated. That is the peak of dihydrozeatin occurred at the 21st fraction, whereas that of zeatin showed at the 24th fraction.

Table 13. Behaviour of different cytokinins in Sephadex LH-20 column chromatography.

No. of fraction (5 ml)	Optical Density				³ H-Zeatin				
	DPA (300 µg)	Zeatin (300 µg)	Dihydro-zeatin (300 µg)	Zeatin riboside (300 µg)	Dpm of 5 ml fraction	% recovery	Cumulative % recovery	Dpm of extract	% recovery from extract
1	0.000	0.000	0.008	0.000	0.0	0.00	-		
2	0.000	0.000	0.007	0.038	0.0	0.00	-		
3	0.000	0.000	0.000	0.005	0.0	0.00	-		
4	0.000	0.009	0.000	0.026	0.0	0.00	-		
5	0.000	0.012	0.000	0.022	0.0	0.00	-		
6	0.000	0.008	0.000	0.025	11.1	0.00	-		
7	0.000	0.009	0.000	0.022	11.7	0.00	-		
8	0.000	0.006	0.000	0.018	66.7	0.00	-		
9	0.000	0.007	0.000	0.020	78.1	0.00	-		
10	0.000	0.024	0.008	0.024	74.4	0.00	-		
11	0.009	0.012	0.007	0.017	71.1	0.00	-		
12	0.010	0.007	0.013	0.017	65.8	0.00	-		
13	0.010	0.010	0.015	0.017	30.4	0.00	-		
14	0.010	0.009	0.012	0.014	33.3	0.00	-		
15	0.013	0.006	0.013	0.015	31.2	0.00	-		
16	0.015	0.013	0.011	0.013	508.8	0.15	0.15	368.9	0.1
17	0.014	0.009	0.016	0.015	518.3	0.15	0.30		
18	0.018	0.011	0.022	0.022	1,670.0	0.50	0.80		
19	0.028	0.017	0.142	0.027	1,515.0	0.50	1.30		
20	0.285	0.020	0.588	0.018	1,875.0	0.56	1.86		
21	1.190	0.093	1.020	0.018	2,113.3	0.63	2.49		
22	1.150	0.323	0.940	0.020	6,000.0	1.78	4.27		
23	0.379	0.760	0.457	0.020	55,691.7	16.51	20.78		
24	0.032	1.180	0.422	0.022	116,223.3	34.45	55.23		
25	0.007	0.790	0.300	0.026	95,536.7	28.32	83.55		
26	0.000	0.298	0.294	0.030	31,035.0	9.20	92.75		
27	0.000	0.057	0.147	0.030	6,355.0	1.88	94.63		
28	0.000	0.018	0.094	0.046	2,805.0	0.83	95.46		
29	0.000	0.011	0.023	0.097	2,618.3	0.78	96.24	311,211.3	92.24
30	0.000	0.009	0.012	0.178	2,520.0	0.75	96.99		
31	0.000	0.017	0.008	0.283	2,266.7	0.67	97.66		
32	0.000	0.009	0.009	0.340	2,181.7	0.65	98.31		
33	0.000	0.007	0.009	0.313	2,190.0	0.65	98.96		
34	0.000	0.012	0.008	0.241	1,367.8	0.41	99.37		
35	0.000	0.008	0.009	0.165	1,109.0	0.33	99.70	7,658.7	2.27
36	0.000	0.008	0.007	0.089	464.8	0.14	99.84		
37	0.000	0.007	0.007	0.046	335.0	0.10	99.94		
38	0.000	0.006	0.002	0.033	184.4	0.05	99.99		
39	0.000	0.006	0.006	0.026	162.0	0.05	100.04		
40	0.000	0.006	0.002	0.018	105.7	0.03	100.07		
41	0.000	0.006	0.002	0.022	69.5	0.00	100.07		
42	0.000	0.006	0.002	0.027	56.6	0.00	100.07		
43	0.000	0.006	0.002	0.020	30.3	0.00	100.07		
44	0.000	0.006	0.002	0.026	49.4	0.00	100.07	506.1	0.15
Total					338,032.1			319,745.0	94.77

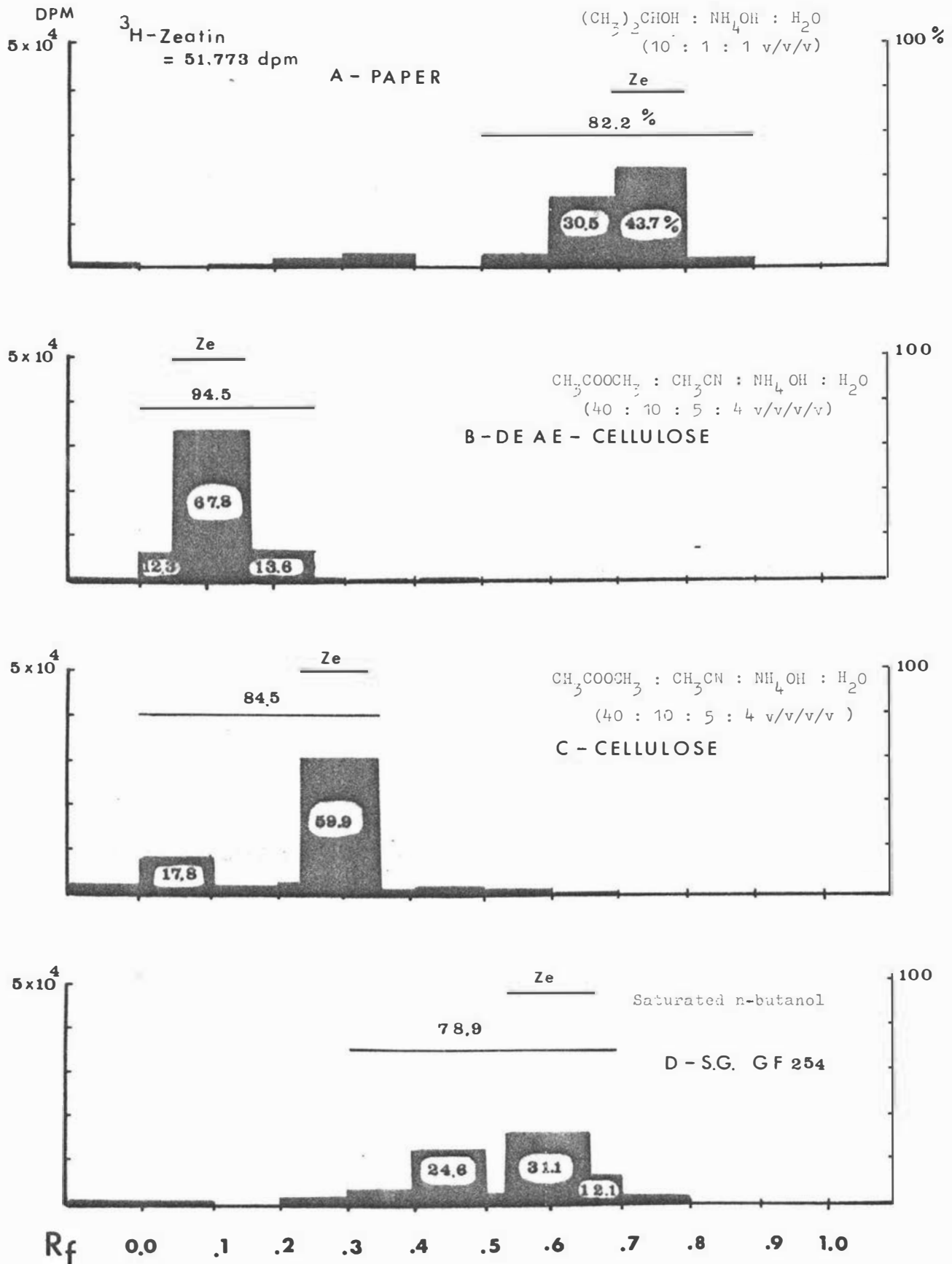


Fig. 17. Paper and thin layer chromatography of ${}^3\text{H}$ -zeatin.

Cold zeatin eluted from the same column gave a similar pattern to ^3H -zeatin, 92.24 % within 75 ml eluant from the 16th to 27th fractions. Further fractions within 45 ml had 5.35 % of additional radioactivity. The remainder from the 37th to the 50th fraction exhibited only 0.15 % in 70 ml.

9. 2. 3. ^3H -zeatin in paper and thin layer chromatography

The behaviour of ^3H -zeatin on paper, cellulose, DEAE cellulose, and silica gel GF-254 is shown in Fig. 17. Almost total recovery was obtained, but the labelled zeatin was spread over various Rfs especially with silica gel GF-254 and paper. The paper chromatogram exhibited 74.26 % at Rf 0.6-0.8 and 82.15 % through Rf 0.5- 0.9. In silica gel GF-254 thin layer chromatography, there were narrow band between Rf 0.5-0.525 under UV light. The band was scraped off and contained 3.47 % of the total radioactivity. Within Rf 0.4-0.65 there was 59.17 % and 78.9 % within Rf 0.3-0.6. On the other hand DEAE cellulose and cellulose had better characterized zones than those on paper and silica gel. Especially, 94.35 % recovery was found from Rf 0.0-0.25 in DEAE cellulose developed by the solvent system-MAAW. In the cellulose thin layer chromatogram developed by the solvent system-MAAW, 59.86 % of ^3H -zeatin was counted from Rf 0.24-0.34 and one particular peak was noticed at Rf 0.0-0.10 which gave 17.82 %.

9.3. Bioassay techniques for IAA, ABA, and GAs

9.3.1. The combination of 5 mm oat and 10 mm wheat coleoptile sections

Oat coleoptile sections are known to be specific to IAA with less interference from inhibitors ~~than wheat coleoptile sections~~. On the other hand wheat coleoptiles respond markedly to inhibitory substances and their IAA response may be masked by inhibitors at the same Rf. A standard calibration with single and combinations of oat and wheat coleoptiles at the concentrations of 0.0, $5 \times 10^{-8}\text{M}$, $5 \times 10^{-7}\text{M}$, $5 \times 10^{-6}\text{M}$, $5 \times 10^{-5}\text{M}$ IAA, and $4.5 \times 10^{-8}\text{M}$, $4.5 \times 10^{-7}\text{M}$, $4.5 \times 10^{-6}\text{M}$, and $5 \times 10^{-5}\text{M}$ ABA is given in Fig. 18 and Table 14 shows the analyses of variance.

There is almost a linear response to log concentrations whether single or combination of oat and wheat coleoptile sections are used in the test tubes. A combination of oat and wheat coleoptile sections in the same tube gave no interference to the response to IAA and ABA in all treatments, although there was a slight tendency that IAA promoted oat coleoptile sections with the absence of the wheat coleoptile section, whereas ABA inhibited wheat coleoptile sections slightly more with the

Table 14. Standard calibration of the oat and wheat coleoptile bioassays either singly or combined in the test tube for the response to IAA and ABA.

A. Analyses of variance of IAA in oat coleoptile and wheat coleoptile sections calculated by the 2 x 5 factorial respectively.

B. Analyses of variance of ABA in oat coleoptile and wheat coleoptile sections calculated by the 2 x 5 factorial respectively.

	IAA					Abscisic acid			
	Oat coleoptile			Wheat coleoptile		Oat coleoptile		Wheat coleoptile	
	Degree of freedom	Mean Square	F ratio	Mean square	F Ratio	Mean Square	F Ratio	Mean Square	F ration
Treatments	9	4.262	17.420***	14.826	18.336***	1.223	7.346***	29.564	89.237***
Replications	3	0.0937	0.383	1.198	1.481	0.1556	0.935	0.702	2.119
Concentrations (Conc)	4	9.258	3.784*	32.893	40.679***	0.1754	10.533***	65.114	196.543***
Combination (Com)	1	0.517	2.113	0.144	0.178	0.0127	0.076	2.551	7.700**
Conc x Com	4	0.203	0.830	0.4295	0.531	0.9951	5.9718**	0.7678	2.318
Errors	27	0.244667		0.80859		0.16649		0.3313	

C. Analyses of variance of IAA calculated by the randomised complete block design.

D. Analyses of variance of ABA calculated by the randomised complete block design.

Source of Variation	IAA					Abscisic acid			
	Single			Combination		Single		Combination	
	Degree of freedom	Mean Square	F ratio	Mean square	F ratio	Mean square	F Ratio	Mean square	F ratio
Treatments	4	3.65825	25.419***	1.867	13.5617***	37.0593	81.345***	23.0576	22.822***
Replications	3	0.036	0.250	0.236	1.714	0.82183	1.8031	0.09733	0.413
Errors	12	0.142917		0.137667		0.45558		0.23567	

*** = 0.001 > P, ** = 0.01 > P > 0.001, * = 0.05 > P > 0.01

absence of oat coleoptile section at the higher concentrations of both IAA and ABA.

With oat coleoptile sections there was no significant difference between 5×10^{-8} M IAA and the control. However, all other concentrations of IAA were significantly different from each other at the 5 % level of LSD calculated by the randomized complete block design from the same data, but not with the 2 x 5 factorial. With wheat coleoptile sections for IAA response there were no significant differences between the nearest two concentrations.

For ABA bioassay, oat coleoptile growth was inhibited by ABA. However there were no sensitivity of log concentrations in the response of oat coleoptile section to ABA. On the other hand wheat coleoptile sections exhibited highly significant growth differences between 4.5×10^{-7} M and 4.5×10^{-6} M ABA. There was no significant difference between the control and 4.5×10^{-8} M ABA, and between 4.5×10^{-6} M and 4.5×10^{-5} M ABA.

9.3.2. The dwarf rice seedlings and lettuce hypocotyl bioassay for gibberellins

9.3.2.1. Tan-ginbozu dwarf rice seedling in the glass tubes and agar culture medium

Using glass tubes, the results of Tan-ginbozu dwarf rice bioassay (Martin, 1971) are shown in Table 15-A and B and Fig. 19. The dwarf rice seedling had no response to gibberellin at $0.001 \mu\text{g/ml}$. However, a 125.6 % increase in leaf sheath occurred at $0.005 \mu\text{g/ml}$ GA_3 concentration. At a higher concentration there was a pronounced response of leaf sheath growth to gibberellin.

The results using the agar culture medium are given in Table 16-A and B.

A preliminary test indicated that the best volume of culture medium was 1.0 ml of 2 % agar and 1.0 ml of distilled water for the dwarf rice bioassay and this was therefore used.

The coleoptile and primary roots were classified into three groups, A, B, and C series. As can be seen in Table 16 the younger stages give a better total response to gibberellin. A and B series gave a similar response, although there was a slight increase in sensitivity to gibberellin at $0.005 \mu\text{g/ml}$ in the A series.

The data between the results of glass tubes and agar medium could not be directly compared, because they were carried out at different times but the dwarf rice seedling bioassay using glass tubes seems to be more sensitive (See Table 15-A and B), although the agar

Table 15. Comparison of dwarf rice seedlings at different concentration of GA_3 in the glass tubes and agar culture medium.

A. Method of culture medium

Method of culture medium	Concentration of gibberellic acid					Means
	0.0	0.001	0.005	0.05	0.5	
	µg per millilitre					
Glass tube	100 (6.68)	102.25 (6.83)	125.60 (8.39)	156.70 (10.47)	210.03 (14.03)	138.92 (9.28)
Agar culture medium	100 (10.0)	103.50 (10.35)	113.50 (11.35)	141.10 (14.11)	214.80 (21.48)	134.58 (13.46)

1. Values are means of four replications.
2. Number in paranthesis indicates a length of first sheath of dwarf rice seedlings in mm.
3. Values within a solid underline are not significantly different at the 5% level (L.S.D.).

B. Analysis of variance.

Source of variation	Degrees of Freedom	Sum of Squares	Mean square	F Ratio
Treatments	9	70,110.8041	7,790.0982	457.0227***
Replications	3	134.8201	44.9400	2.6365
Method of culture (M)	1	187.2293	187.2293	10.9842***
Concentration (C)	4	69,272.1704	17,318.0426	1,016.0010***
M x C	4	162.8511	162.8511	9.5540***
Error	27	460.2243	17.0453	

*** = 0.001 > P ** = 0.01 > P > 0.001 * = 0.05 > P > 0.01

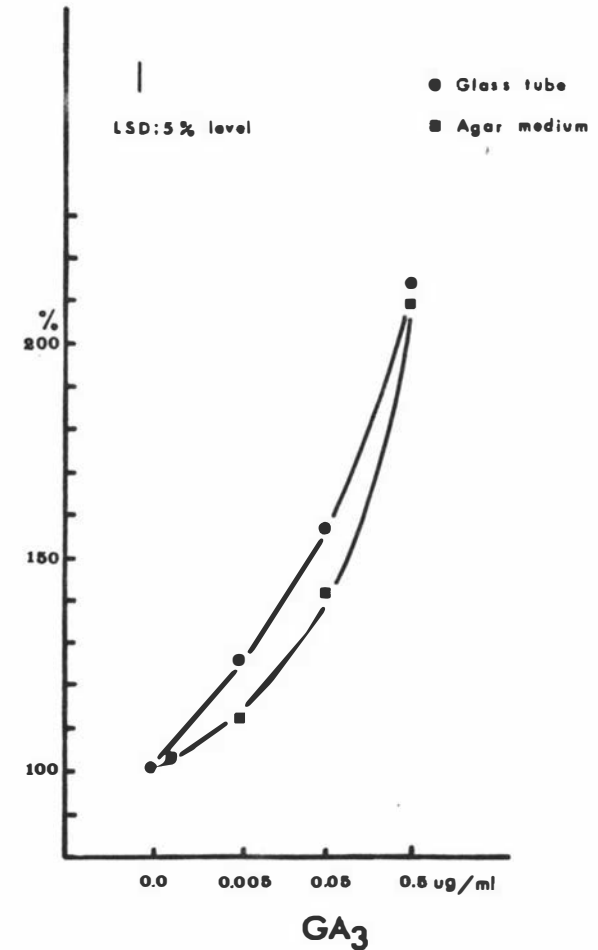


Fig. 19. Comparison of dwarf rice seedlings at a different concentration of GA_3 in the glass tubes and agar culture medium.

9.3.2.2. Comparison of different lettuce varieties for use in the lettuce hypocotyl bioassay for gibberellins

The response to gibberellin of the lettuce hypocotyl length in four varieties is presented in Fig. 20 and an analysis of variance of the data in Table 17.

Four lettuce varieties Arctic, Arctic King, Green Mignonette, and Buttercrunch were screened preliminarily from other lettuce varieties such as Webbs Wonderful, Neapolitan, Great Lakes, Tom Thumb, Butter Head, Imperial Summer-847, Mignonette, and Triumph.

From Table 17 and Fig. 20, it can be seen that in terms of sensitivity and linearity Arctic gave the best response for use in a bioassay; the response was statistically significant at 0.001 μ g and linear between 0.001 and 1.0 μ g.

9.4. Endogenous plant hormones in apple leaves

9.4.1. IAA- and ABA-like substances in apple leaves

The apple leaves taken on 12th November in 1972 were extracted by the method shown in Table 6. The ethyl acetate phase was initially purified by solvent partition and Sephadex G-10 chromatography. A portion of each fraction from the system A silica gel-celite column was then bioassayed. The remainder pooled and then subjected to cellulose TLC prior to bioassay.

9.4.1.1. IAA-like substances

Three levels of concentration equivalent to 0.2, 1.0 and 2.0 gram dry weight of leaves from each fraction (Fig. 21-A,B, and C respectively) and the 'A' fraction eluted by the system A silica gel column (See 8.4.1.2.1) were bioassayed with the combined oat wheat coleoptile sections. In Figure 21, a shaded area for oat coleoptile elongation and dotted area for wheat coleoptile elongation exhibited a significant promotion or inhibition of coleoptile length at the 5% level. The aliquot equivalent to 2 gram of leaf extract clearly promoted oat coleoptile sections at the fraction number 13, 14, and 15, the same fractions where ^{14}C -IAA had been shown to occur, as shown in Fig. 21-C₁ (See also Table 8). The aliquot equivalent to 0.2 g of dry weight of leaf sample exhibited many peaks as shaded areas from oat coleoptile extension in the fractions where most ^{14}C -IAA was eluted, although a few of the fractions where ^{14}C -IAA was eluted did not promote growth significantly. Surprisingly the aliquot of 1.0 gram dry weight did not

Table 17. Comparison of different lettuce varieties for use in the lettuce hypocotyl bioassay for gibberellins (mm in length).

A. Comparison of varieties

Varieties	Concentrations of GA ₃ ug/ml					Mean
	0	0.001	0.01	0.1	1.0	
Arctic	9.1 (100)	9.9 (108.8)	13.3 (146.2)	16.4 (180.2)	21.1 (231.9)	13.96
Arctic King	10.63 (100)	11.1 (104.4)	11.87 (126.2)	14.87 (158.1)	220 (188.2)	13.69
Green Magnonette	11.13 (100)	12.07 (108.5)	12.97 (116.5)	15.4 (138.4)	18.77 (168.6)	14.07
Butter Crunch	6.7 (100)	6.5 (96.14)	7.3 (108.9)	10.7 (159.4)	17.8 (264.5)	9.81

a) Numbers in paranthesis indicates percentage with control of length of seedlings.

b) Values within a solid underline are not significantly different at the 5% level and the values are means of 3 replicates, each replicate has 10 seedlings in the vial.

c) A test solution in the vials was 1 ml of buffer solution at pH 6.5.

B. Analysis of variance.

Source of Variation	Degrees of Freedom	Sum of Squares	Mean Square	F Ratio
Replications	2	0.0963	0.0482	0.138
Treatments	19	1051.21	55.3268	158.848***
Varieties (V)	3	189.607	63.2024	181.460***
Concentration (C)	4	819.2333	204.8083	588.204***
V x C	12	42.3694	3.5308	10.137***
Error	38	13.237	0.3483	

1) *** = 0.001 > P, ** = 0.01 > P > 0.001, * = 0.05 > P > 0.01, + = 0.1 > P > 0.05
 " = P > 0.1

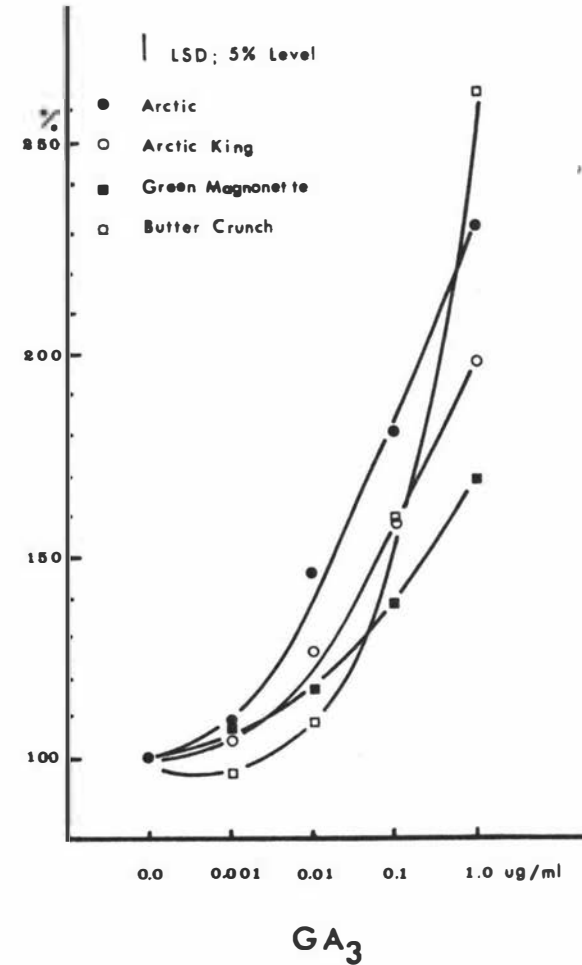


Fig. 20. Comparison of the lettuce bioassay at different concentrations of GA₃ between different varieties.

Table 18. Estimation of IAA- and ABA-like substances from silica gel-celite column as μg equivalents.

No. of fraction	IAA (μg) Oat (O)*			ABA (μg) Wheat (W)*		
	Dry matter (g)			Dry matter (g)		
	0.2	1.0	2.0	0.2	1.0	2.0
1	-	-	-	-	-	-
2	-	-	-	-	-	-
3	-	-	-	-	-	-
4	-	-	-	-	-	-
5	-	-	-	-	-	-
6	-	-	0.0653	-	-	-
7	-	0.0525	-	-	-	-
8	-	-	0.0613	-	0.04 (O)	-
9	0.0525	-	0.0569 (W)	-	-	-
10	0.0653	-	0.0525 (W)	-	-	-
11	-	-	-	0.25 (O)	-	0.055 (O)
12	0.1313	0.0653	-	-	0.075 (O)	0.015
13	-	-	0.0875	-	-	0.01
14	0.0653	-	0.525	-	-	-
15	0.0875	-	0.0613	-	-	-
16	-	-	-	-	-	-
17	0.1313	-	-	-	-	-
18	-	-	-	-	-	-
19	-	-	-	-	-	-
20	0.0625	-	-	-	-	-
21	-	-	-	-	-	-
22	0.0613	-	-	-	-	-
23	-	-	-	-	-	-
Total	0.657	0.1178	0.9098	0.25	0.115	0.080

* The estimated values are derived from the single chromatogram from each fraction eluted the silica gel A system up to the 23rd fraction and a significance from the standard calibration of the combination of oat and wheat coleoptile section for IAA and ABA.

Fig. 21. The activities of IAA and ABA-like substances in the apple leaves after silica gel-celite column and cellulose thin layer chromatography.

A, B, and C in Figure 21 indicate coleoptile length in the fractions eluted from the system A silica gel-celite column.

D, E, and F in Figure indicate its growth from cellulose thin layer chromatogram in the pooled A fraction (250 ml) eluted from the column.

A solid line represents oat coleoptile section and a dotted line, wheat coleoptile section.

A = D : 0.2 gram dry weight leaf sample

B = E : 1.0 gram dry weight leaf sample

C = F : 2.0 gram dry weight leaf sample

A shaded area for oat coleoptile elongation or a dotted area for wheat coleoptile length indicates a significance at the 5% level of LSD from the standard calibration.

Fig. 21-C₁ is derived from Table 8 in which ¹⁴C-IAA was fractionated.

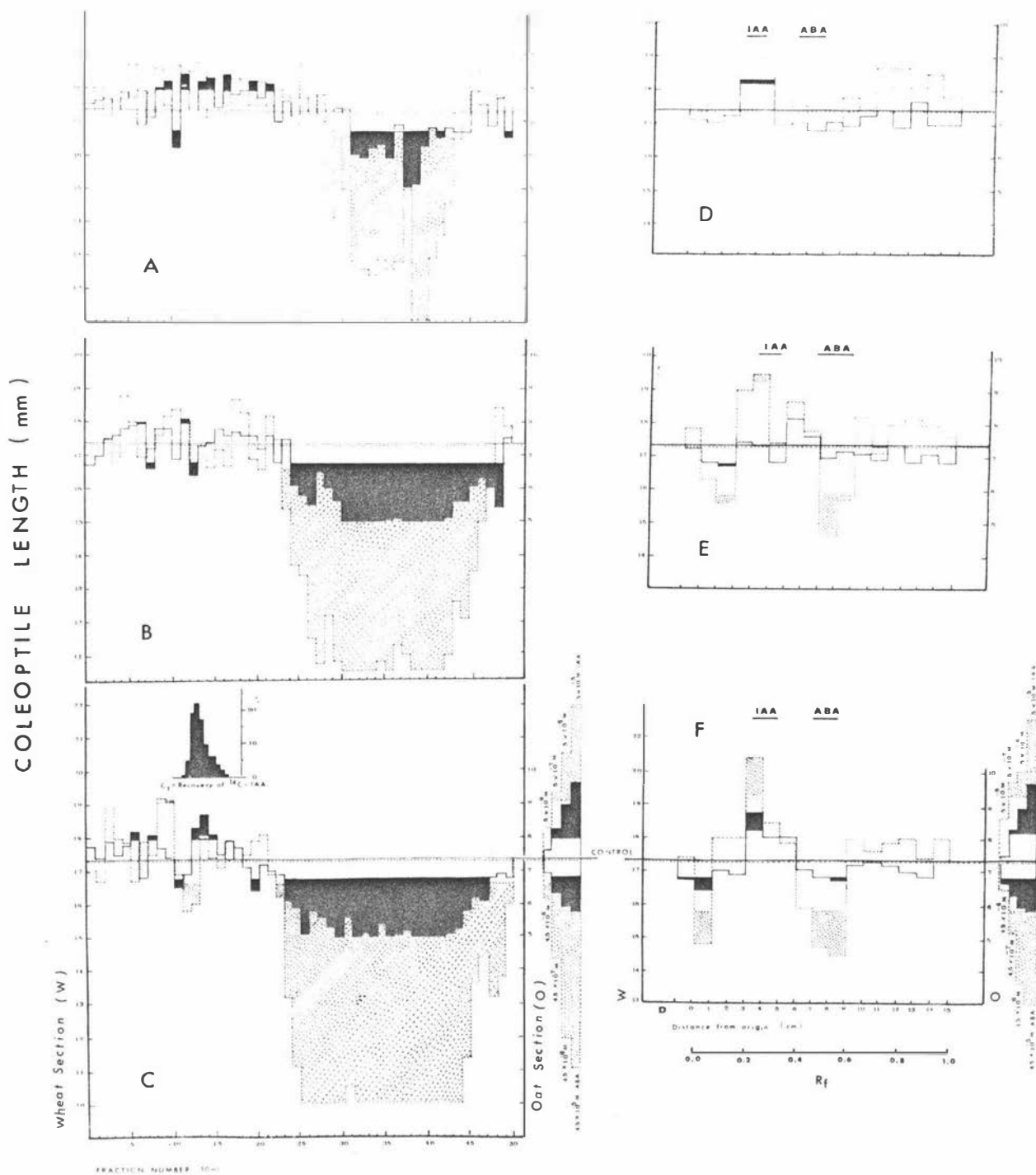


Fig. 21. The activities of IAA and ABA-like substances in the apple leaves after silica gel-celite column and cellulose thin layer chromatography.

show an active promotion of coleoptile sections as compared with the aliquots of 0.2 gram and 2.0 gram dry weight. This result may be a phenomena of counteraction between inhibitors and promoters in the same fraction. This is an idea which is supported by the results for the 2.0 g sample in which oat coleoptile growth was greatly promoted at the 13th fraction, while the wheat coleoptile growth was greatly inhibited.

The unknown stimulative components at the 6th to 10th fraction may be a compound slightly more polar than IAA if the response was not due to IAA. The results were that some highly promotive substances increased oat coleoptile sections at the 6th and 8th fractions with the aliquot containing 2 gram dry weight of leaf samples. The 7th with the aliquot containing 1.0 gram revealed its promotive activity and the 9th and 10th fractions with the aliquot containing 0.2 gram dry weight.

The quantitative estimation of promoters and inhibitors detected by oat and wheat coleoptile sections for the 1st - 23rd fraction is given in Table 18. Beyond the 23rd fractions in Fig. 21-A, B, and C, there were several yellow greenish components which caused great inhibition. These inhibitory components are not considered in the present studies.

From the 1st to the 23rd fraction the estimation of IAA-like substances with 0.2 gram dry weight was 0.657 μ g equivalents which was 7 times higher, weight for weight, than that for 2.0 gram dry weight. On the other hand the aliquot equivalent to 1.0 gram dry weight contained only 0.117 μ g.

The first 250 ml of eluant, which was free from most of the inhibitory greenish yellow pigments eluted from the 24th to the 47th fraction through the system A silica gel column, was chromatographed on cellulose thin layer plates. The results are shown in Fig. 21-D, E, and F. IAA-like substances, detected with the oat coleoptile sections at the same Rf as the IAA marker, were found in the 0.2 and 2.0 gram dry weight samples, but not in the 1.0 gram dry weight sample. From thin layer chromatography, the estimation of IAA-like substances was almost 10 x less in the aliquot equivalent to 0.2 gram dry weight compared to the 2.0 g sample as would be expected. The figures were 0.0875 and 0.6169 μ g respectively.

IAA-like substances detected by wheat coleoptile sections were found in the position of IAA marker with the aliquot containing 1.0 and 2.0 gram dry weight, but not with that of 0.2 gram dry weight, representing 0.074315 μ g and 0.6169 μ g equivalents respectively. Especially IAA-like substances at Rf IAA position estimated by oat and wheat coleoptile in the aliquot equivalent to 2.0 gram dry weight was

Fig. 22. Cellulose TLC of gibberellin-like substances in apple leaves before and after eluting from the system A silica gel-celite column.

The pooled fractions eluted from the column are chromatographed on cellulose thin layer as follows (The solvent system MAAW):

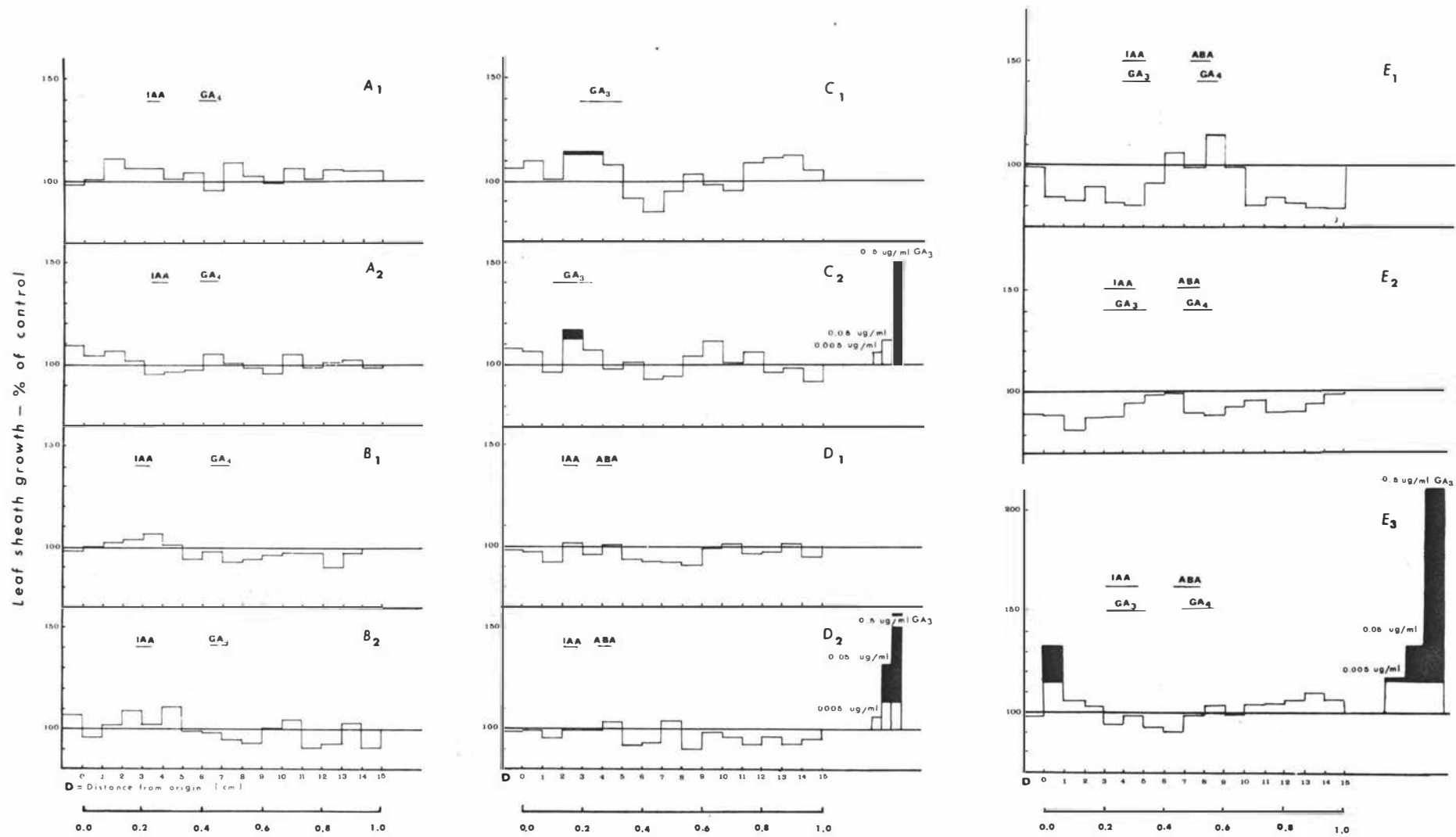
- A fraction (A_1 and A_2) = 1-25 fraction (250 ml)
- B fraction (B_1 and B_2) = 26-35 fraction (100 ml)
- C fraction (C_1 and C_2) = 36-45 fraction (100 ml)
- D fraction (D_1 and D_2) = 46-53 fraction (80 ml)

Tan-ginbozu dwarf rice bioassay was used with agar culture medium.

E_1 , E_2 , and E_3 in Figure are derived from the fraction eluted from Sephadex G-10 column(See M-1 to S-19 in Table 6).

- E_1 = 0.1 gram dry weight leaf sample
- E_2 = 0.5 gram dry weight leaf sample
- E_3 = 1.0 gram dry weight leaf sample

The dwarf rice bioassay in E was used with glass tubes. The shaded area represents a significance at the 5% level of LSD from the standard calibration.



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R_f

Fig.22. Cellulose TLC of gibberellin-like substances in apple leaves before and after eluting from the system A silica gel-celite column.

nearly quantitatively identical. The mean of the IAA-like substance from the different aliquot in Table 18 was 1,285.9 $\mu\text{g}/\text{kg}$ dry weight of leaf sample from the silica gel column chromatography, and 373.0 $\mu\text{g}/\text{kg}$ from the cellulose thin layer chromatography in Fig. 21-D and F.

9.4.1.2. ABA-like substances

An attempt was made to detect by GLC authentic ABA eluted from the system A silica gel column but the results were unclear. However peaks at the same retention time of ABA in GLC could occur at the 6th-10th fractions and not in the 11th-12th fractions which caused coleoptile inhibition. One point of interest was that the oat coleoptile sections at the 13th fraction were not affected by the inhibitors which reduced the growth of the wheat coleoptiles. Because the significantly promoted growth of oat coleoptile sections at the 13th, 14th and 15th fractions coincided with the ^{14}C -IAA as shown in Fig.21-C₁. It would seem that oats were mainly affected by the promoters and wheat by the inhibitors. Without consideration of any conditions such as a pattern of elution for ABA in the column or counteraction of IAA-like substance present in the same fraction, the mean of ABA-like substance in Table 18 was calculated as 468 $\mu\text{g}/\text{kg}$ dry weight. From cellulose thin layer chromatography the aliquot equivalent to 2.0 gram dry weight of leaf sample was detected at the Rf of ABA marker with the wheat coleoptile and oat bioassay. 0.1815 μg equivalent to ABA-like substance was detected at the Rf of 0.4-0.6 with the 2.0 gram dry weight of leaf extract and 0.11 μg with the 1.0 gram dry weight. Therefore the mean of those two aliquots was calculated as 100.4 $\mu\text{g}/\text{kg}$ dry weight of leaf sample.

9.4.2. Gibberellin -like substances in ethyl acetate phase

The extract was purified using the procedures in Table 6, M-1 to S-29 but omitting steps E-25 to S-28-A. The results obtained using aliquots equivalent to 0.1, 0.5, and 1.0 gram dry weight of leaf sample are shown in Fig.22-E₁, E₂, and E₃, bioassayed with dwarf rice seedling in the glass tubes.

The lowest concentration of dry weight (0.1 g) significantly promoted dwarf rice seedling growth at Rf 0.536-0.6, the position where ABA, GA₄, and GA₇ run. The estimated gibberellins equivalent to GA₃ from the dwarf rice bioassay was 0.005 μg on the 0.1 gram dry weight. However, there was no response at this Rf with the higher concentrations. No stimulative substance was found in the 0.5 gram sample. On the other hand the most profound stimulation was found at Rf 0.0-

0.0667 in the aliquot equivalent to 1.0 gram dry weight of leaf samples, representing 0.075 ug equivalent to GA₃.

9.4.2.1. Gibberellin-like substances in the fraction from the system A silica gel-celite column

9.4.2.1.1. Fractions from column

After purification with Sephadex G-10 the extract was placed on the silica gel-celite column and eluted with a chloroform, ethyl acetate, and methanol gradient.

The aliquots equivalent to 1.0 and 5.0 gram dry weight of leaf sample from each of 53 fractions were bioassayed by the dwarf rice seedlings using a glass tube and glucose test with barley endosperm. The activity of gibberellin-like substances exhibited as shaded areas at the LSD of 5 % level is shown in Fig. 23-B₁ and B₂.

An estimation of gibberellin-like substances in the 53 fractions from the silica gel-celite column was made in Table 19 with a comparison between the endosperm barley test and the dwarf rice bioassay.

Gibberellins were found in the 5th to the 10th fractions by the dwarf rice bioassay but not by the endosperm barley bioassay. The estimated amount for 5.0 g and 1.0 g dry weight equivalents was 0.007 μg and 0.0015 μg respectively. Another peak of gibberellin-like substances occurred at the 19th and 20th fractions with the aliquot equivalent to 5.0 gram but for the 1.0 gram dry weight aliquot only at the 19th fraction. This peak was also detected by the endosperm barley test, although the activities found were not significant. The major peaks occurred between the 25th to the 44th fractions. However, there were a number of fractions which inhibited the dwarf rice seedlings or barley endosperm. The gibberellin peaks could be divided into two groups, that is one that was not sensitive to the barley endosperm test at the 25th to the 35th fraction, and the other, very active not only in the barley endosperm bioassay but also in the dwarf rice seedlings at the 38th to the 44th fraction. However three fractions at the 40th to the 42nd fraction greatly inhibited the dwarf rice seedlings with the aliquot equivalent to 5.0 gram dry weight, this possibly being due to a higher content of inhibitors eluted at the same fractions. Two peaks at the 38th and the 40th fractions revealed greatly the activity of gibberellin-like substance with the dwarf rice seedlings using the aliquot equivalent to 1.0 gram dry weight. It was interesting to notice that some kind of yellow greenish coloured components were eluted

Table 19. The μg equivalent to GA_3 per gram dry weight of apple leaves from the elution of silica gel-celite columns by gradient elution with different solvents used.

Fraction number	The system A silica gel				The system B silica gel			
	Dwarf rice seedlings		Barley endosperm		Dwarf rice seedlings			Barley endosperm
	1.0 g	5.0 g	1.0 g		1.0 g	3.0 g	5.0	1.0
1	-	-	-	-	-	-	-	-
2	-	-	-	-	-	-	-	-
3	-	-	-	-	-	-	-	-
4	-	-	-	-	-	-	-	-
5	-	0.0025	-	-	-	-	-	-
6	-	-	-	-	-	-	-	-
7	-	0.0025	-	-	-	-	-	-
8	-	-	-	-	-	-	-	-
9	-	0.0020	-	-	-	-	-	-
10	0.0015	-	-	-	-	-	-	-
11	-	-	-	-	-	-	-	-
12	-	-	-	-	-	-	-	-
13	-	-	-	-	-	-	-	-
14	-	-	-	-	-	0.03	-	-
15	-	-	-	-	-	0.03	-	-
16	-	-	-	-	-	-	-	-
17	-	-	-	-	-	-	-	-
18	-	-	-	-	-	-	-	-
19	0.001	0.003	-	-	-	0.0175	-	-
20	-	0.002	-	-	-	0.0200	-	-
21	-	-	-	-	-	-	-	-
22	-	-	-	-	-	-	-	-
23	-	-	-	-	-	0.0175	-	-
24	-	-	-	-	-	0.02	-	-
25	-	-	0.004	-	-	-	-	-
26	-	0.0025	-	-	-	0.033	-	-
27	-	0.0025	0.008	-	-	-	-	-
28	-	-	-	-	-	-	-	-
29	-	0.0040	0.004	-	-	0.0275	-	-
30	-	0.005	-	-	-	-	-	-
31	-	0.0025	0.005	-	-	-	-	-
32	-	0.005	-	-	-	-	-	-
33	-	-	0.004	-	-	-	-	-
34	-	0.0045	0.005	-	-	-	-	-
35	-	0.0035	0.0045	-	-	-	-	-
36	-	-	0.004	-	-	-	-	-
37	-	-	0.006	-	-	-	-	-
38	0.0015	0.004	0.004	-	-	-	-	-
39	-	0.0022	1.000	-	-	-	-	-
40	0.011	-	1.500	-	-	-	-	-
41	-	-	0.015	-	-	-	-	-
42	-	-	0.005	-	-	-	-	-
43	-	0.0042	0.015	-	-	-	-	-
44	-	0.002	0.005	-	-	0.0325	-	-
45	-	-	-	-	-	-	-	-
46	-	-	-	-	-	-	-	-
47	-	-	-	-	-	-	-	-
48	-	-	-	-	-	0.0275	-	-
49	-	-	-	-	-	-	-	-
50	0.0145	-	-	-	-	-	-	-
51	-	-	-	-	-	-	-	-
52	-	-	-	-	-	-	-	-
53	-	-	-	-	-	-	-	-
54	-	-	-	-	0.025	-	-	-
55	-	-	-	-	-	-	-	-
56	-	-	-	-	-	-	-	-
57	-	-	-	-	-	-	-	-
58	-	-	-	-	-	-	-	-
59	-	-	-	-	0.0175	-	-	-
60	-	-	-	-	-	-	-	-
61	-	-	-	-	-	-	-	-
62	-	-	-	-	0.0430	-	-	-
63	-	-	-	-	-	-	-	-
64	-	-	-	-	-	0.02	-	-
65	-	-	-	-	-	0.045	0.005	-
66	-	-	-	-	-	0.035	-	-
67	-	-	-	-	-	-	0.075	-
68	-	-	-	-	-	0.020	-	0.00152
69	-	-	-	-	-	0.0375	0.0425	-
70	-	-	-	-	-	0.030	-	-
71	-	-	-	-	-	0.020	-	-
72	-	-	-	-	0.0200	-	-	-
73	-	-	-	-	-	0.020	-	-
74	-	-	-	-	-	0.0175	-	-
75	-	-	-	-	-	-	-	-
76	-	-	-	-	-	0.020	-	-
77	-	-	-	-	-	-	-	-
78	-	-	-	-	-	-	-	-
79	-	-	-	-	-	0.0175	-	-
80	-	-	-	-	-	-	-	-
81	-	-	-	-	-	-	-	-
82	-	-	-	-	-	-	-	-
83	-	-	-	-	-	-	-	-
84	-	-	-	-	-	-	-	-
85	-	-	-	-	-	-	-	-
86	-	-	-	-	-	-	-	-
87	-	-	-	-	-	-	-	-
88	-	-	-	-	-	-	-	-
89	-	-	-	-	-	-	-	-
90	-	-	-	-	-	-	-	-
91	-	-	-	-	-	-	-	-
92	-	-	-	-	0.0275	-	-	-
93	-	-	-	-	0.0200	-	-	-
94	-	-	-	-	-	-	-	-
95	-	-	-	-	-	-	-	-
96	-	-	-	-	-	-	-	-
97	-	-	-	-	-	-	-	-
98	-	-	-	-	-	-	-	-
99	-	-	-	-	-	-	-	-
100	-	-	-	-	-	-	-	-
TOTAL	0.0295	0.0549	2.5875		0.153	0.538	0.1225	0.00152

Fig. 23. Gibberellin-like substances in the fraction eluted from the silica gel-celite column with different solvent systems in the gradient elution.

Figure A represents the solvent B silica gel-celite column eluted by hexane, diethyl ether, ethyl acetate, and methanol.

A_1 = 1 gram dry weight leaf sample, using
glucose test in barley endosperm bioassay
 A_2 = 5 gram dry weight leaf sample
 A_3 = 3 gram dry weight leaf sample
 A_4 = 1 gram dry weight leaf sample

Tan-ginbozu dwarf rice bioassay in A_2 was used with glass tubes and in A_3 and A_4 , with agar culture medium.

Figure B represents the solvent A silica gel-celite column eluted by chloroform, ethyl acetate, and MeOH.

B_1 = 1 gram dry weight leaf sample, using
glucose test in barley endosperm bioassay
 B_2 was bioassayed with the dwarf rice seedlings
using glass tubes. A dotted line indicates 1.0
gram dry weight leaf sample and a solid line,
5 gram dry weight leaf sample

A shaded area shows a significance at the 5% level of LSD from the standard calibration.

Leaf sheath growth - % of control

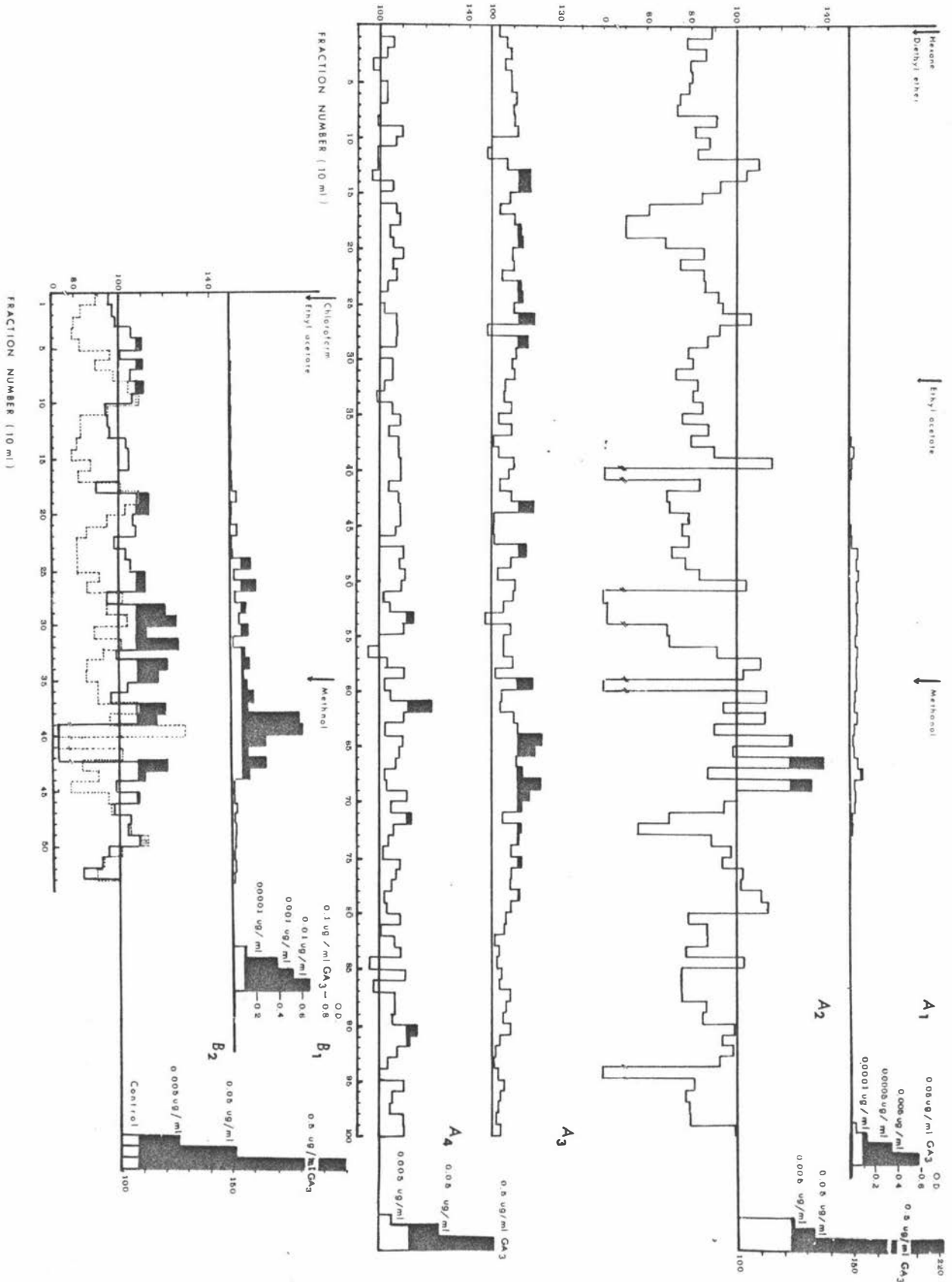


Fig.23. Gibberellin-like substances in the fraction eluted from the silica gel-celite column with different solvent systems in the gradient elution.

simultaneously with these fractions. The degree of intensities of coloured components coincided with that of the gibberellin-like substances. Some gibberellin-like substance which might be less polar than the others appeared also at the 50th fraction with both the aliquots equivalent to 1.0 and 5.0 gram dry weight and the dwarf rice bioassay but were not detected by the barley endosperm bioassay.

The activities of gibberellin-like substances from the mean of 5 gram and 1.0 gram dry weight of leaf extract were estimated as 20.24 $\mu\text{g}/\text{kg}$ dry weight of leaf sample by the dwarf rice seedling bioassay and 2,587.5 $\mu\text{g}/\text{kg}$ dry weight by the barley endosperm test. Thus there seems to be five different kinds of gibberellin-like substances present in the apple leaves eluted from the system A silica gel-celite column.

9.4.2.1.2. Cellulose thin layer chromatography from the pooled fractions of column

The pooled fractions A, B, C, and D (See section 8.4.1.2.1.2.) from the system A silica gel-celite column were tested by cellulose thin layer chromatography using the dwarf rice seedling bioassay with the agar medium culture. The results were shown in Fig. 22-A₁ & 2, B₁ & 2, C₁ & 2, and D₁ & 2, without replications.

Gibberellin-like substance was detected at the R_f of GA₃ position in the cellulose thin layer, representing 0.2 μg with the aliquot equivalent to 5.0 gram dry weight of leaf samples and 0.1 μg with the aliquot of 1.0 gram dry weight at the same R_f position in Fig. 22-C₁ & 2. With other fractions there were not clear to show an activity of gibberellin-like substances.

9.4.2.2. Gibberellin-like substances from the system B silica gel-celite column eluted by hexane, diethyl ether, ethyl acetate, and methanol

The system B silica gel-celite column was exactly the same mixture of silica gel and celite as used in the system A but the solvent system was changed to hexane, diethyl ether, ethyl acetate, and methanol. This was only one of the preliminary experiment to see whether the solvent system B may give a better separation of acidic plant hormones. Therefore detailed studies were not attempted to elucidate the behaviour of authentic plant hormones in the column by this solvent system.

The aliquot equivalent to 15 gram of lyophilized leaf sample was fractionated by the system B solvent. The aliquots equivalent to 1.0, 3.0, and 5.0 g dry weight of leaf sample from the first 100 fractions eluted from the column were bioassayed by the dwarf rice seedling using 1 ml of 2 % agar culture medium with 1 ml of distilled water except for the aliquot equivalent to 5.0 gram dry weight, in which glass tubes were used. The fractions were also bioassayed with the barley endosperm glucose test, using 1 ml for the glucose test from the 4 ml of the diluted incubation.

The results obtained from each fraction are shown in Fig. 23-A₁, A₂, A₃, and A₄. The estimations of gibberellin-like substances equivalent to GA₃ are given in Table 19.

Of the 100 fractions from the systems B silica gel-celite column the aliquot with 3.0 gram dry weight exhibited more significant peaks compared to the aliquots with 1.0 and 5.0 gram dry weight. The aliquot equivalent to 3.0 gram dry weight leaf sample (Fig 23-A₃), gave three areas which significantly promoted dwarf rice seedlings. However, the aliquot equivalent to 1.0 gram dry weight did not stimulate the dwarf rice seedlings to indicate the presence of GA-like substances in the fractions. The aliquot equivalent to the 5.0 gram dry weight sample gave only one area from the 65th to the 68th fractions, possibly because of inhibitory materials. Possibly areas of lesser inhibition in the 5.0 gram sample coincided with areas of gibberellin activity in the 3.0 gram sample, while the 1.0 gram sample showed activity at the 91st and 92nd fraction. Thus possibly four areas of gibberellin activity are present (See Table 19).

9.4.3. Cytokinin-like substances of apple leaves from Sephadex LH-20 and DEAE cellulose thin layer chromatography

9.4.3.1. On Sephadex LH-20 column chromatography

The aqueous fraction after extraction by ethyl acetate at pH 2.5 was alkalinized to pH 8.3 and extracted with butanol saturated with 0.1 M phosphate buffer. The butanol fraction was eluted with 0.1 M phosphate buffer through Sephadex G-10. The fraction eluted from the Sephadex G-10 was again partitioned with 95 % ethanol (0.001 M HCl) through the Sephadex LH-20. Each fraction equivalent to 1.0 and 5.0 gram dry weight of leaf sample was compared with the LSD at the 5 % level from zeatin standard calibration (0.0, 0.003, 0.03, 0.3, and 3.0 µg/ml with four replications). The results of chromatograms and the estimation of cytokinin-like substances are given in Fig. 24-E and Table 20.

Table 20. Determination of cytokinin-like activity in apple leaves using the radish cotyledon bioassay and purification by Sephadex LH-20 and DEAE cellulose chromatography (μg).

No. of fraction	Fractions of Sephadex LH-20		DEAE cellulose thin layer		
	Dry weight (g)		Rf.	Dry weight (g)	
	1.0	5.0		0.5	1.0
16	-	-	Pooled fractions from the 16th to 43rd fraction		
17	-	-			
18	-	-			
19	-	0.075			
20	-	-	0.0	0.021	0.255
21	-	-			
22	0.105	0.135	0.0-0.1	0.0225	0.04
23	-	-			
24	-	-	0.1-0.2	0.0255	0.024
25	-	-			
26	-	-	0.2-0.3	0.075	-
27	-	-			
28	-	-	0.3-0.4	-	-
29	-	-			
30	-	-	0.4-0.5	0.027	-
31	-	0.12			
32	-	0.24	0.5-0.6	-	-
33	-	-			
34	0.105	-	0.6-0.7	0.0195	0.0225
35	0.045	-			
36	0.06	0.06	0.7-0.8	0.0225	0.0195
37	-	-			
38	-	-	0.8-0.9	0.021	-
39	-	-			
40	-	-	0.9-1.0	-	-
41	0.06	-			
42	0.04	-			
43	0.165	-			
Sub-total	0.580	0.630		0.234	0.0435
44	-	-	Pooled fractions from the 44th to the 63rd fraction		
45	-	-			
46	-	0.04			
47	0.075	-			
48	-	-			
49	-	-			
50	-	0.045	0.0-0.0	-	-
51	-	-	0.0-0.1	0.027	-
52	-	0.09	0.1-0.2	-	-
53	0.075	-	0.2-0.3	-	-
54	-	-	0.3-0.4	-	-
55	-	0.09	0.4-0.5	-	-
56	0.06	-	0.5-0.6	-	-
57	-	-	0.6-0.7	-	0.15
58	-	-	0.7-0.8	-	0.075
59	-	0.045	0.8-0.9	0.016	0.0285
60	-	-	0.9-1.0	0.03	-
61	-	-			
62	-	-			
63	-	0.12			
Sub-total	0.210	0.43		0.073	0.2535
64	0.06	-	Pooled fractions from the 64th to the 78th fraction		
65	-	0.075			
66	-	-	0.0-0.0	-	-
67	-	0.18	0.0-0.1	-	0.0195
68	-	0.15	0.1-0.2	0.0195	0.0225
69	-	0.105	0.2-0.3	0.024	-
70	0.04	0.195	0.3-0.4	-	0.0225
71	-	-	0.4-0.5	-	0.04
72	-	0.31	0.5-0.6	-	-
73	0.045	0.18	0.6-0.7	-	-
74	-	0.135	0.7-0.8	0.0165	0.0225
75	0.054	0.135	0.8-0.9	-	0.12
76	-	0.12	0.9-1.0	-	-
77	-	-			
78	-	0.105			
Sub-total	0.199	1.69		0.06	0.247
79	0.054	4.50	Pooled fractions from the 79th to the 85th fraction		
80	0.03	0.045			
81	-	0.045	0.0-0.0	-	0.09
82	0.24	0.165	0.0-0.1	0.018	0.09
83	0.12	-	0.1-0.2	-	0.035
84	0.06	0.045	0.2-0.3	-	0.035
85	0.045	0.27	0.3-0.4	0.015	0.0225
			0.4-0.5	-	0.04
			0.5-0.6	-	0.0195
			0.6-0.7	0.0255	0.0255
			0.7-0.8	-	0.018
			0.8-0.9	-	-
			0.9-1.0	-	0.15
Sub-total	0.549	5.07		0.0585	0.0575
Total	1.538	7.82		0.4255	0.3480

Fig. 24. Cytokinin-like activities after Sephadex LH-20 column and DEAE cellulose thin layer chromatography as detected by the radish cotyledon bioassay.

The pooled fractions eluted from Sephadex LH-20 are as follows:

A fraction = 16-43rd fraction (140 ml)

B fraction = 44-63rd fraction (100 ml)

C fraction = 64-78th fraction (75 ml)

D fraction = 79-85th fraction (35 ml)

The pooled fractions were chromatographed on DEAE cellulose thin layer with the solvent system MAAW.

Each 5 ml fraction eluted Sephadex LH-20 column was bioassayed. A solid line indicates one gram dry weight leaf sample, and a dotted line 5 gram dry weight leaf sample. The cytokinin activity was divided into six groups for convenient reference. A shaded or dotted area indicates a significance at the 5% level of LSD from the standard calibration.

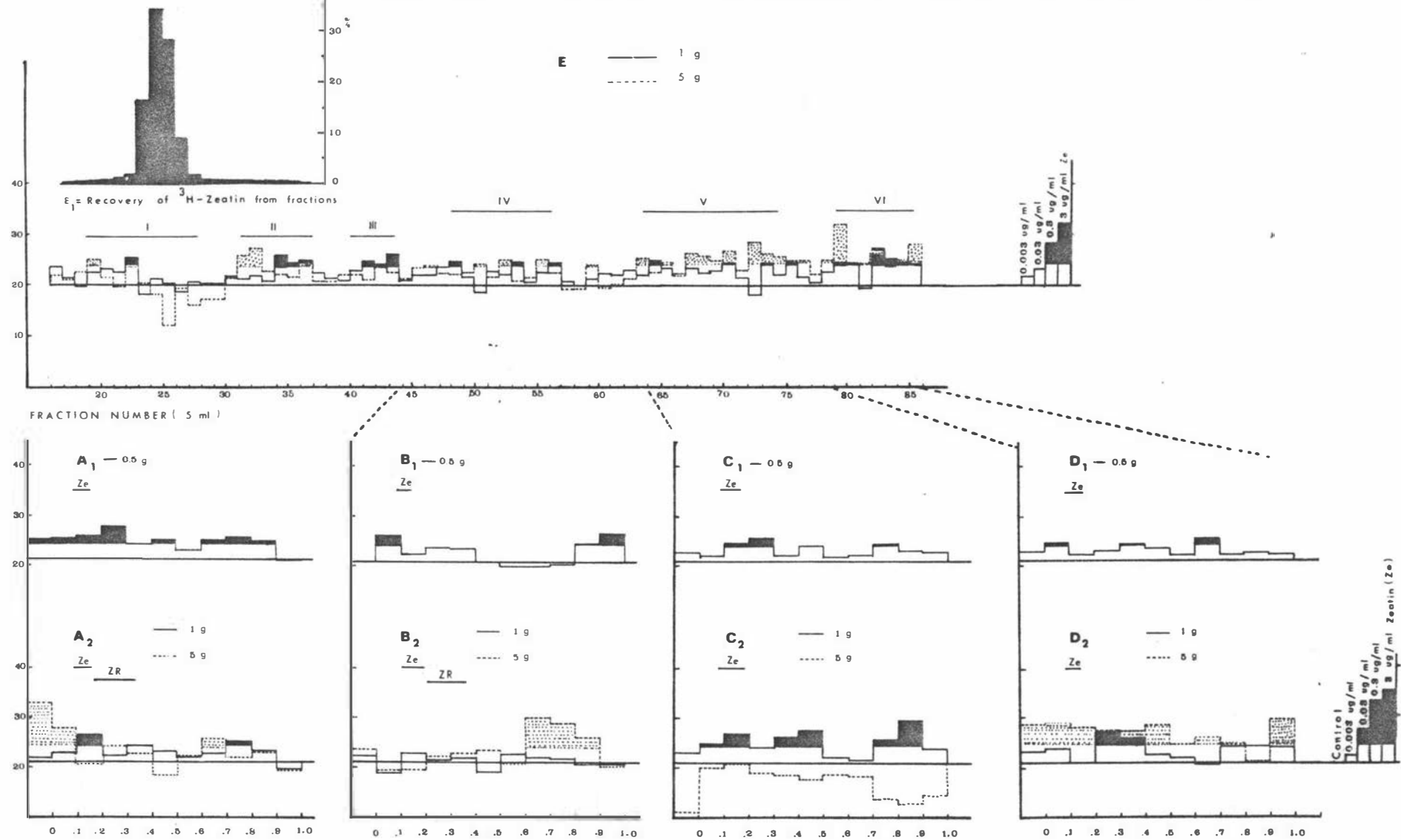
**R_f**

Fig. 24. Cytokinin-like activities after Sephadex LH-20 column and DEAE cellulose thin layer chromatography as detected by the radish cotyledon bioassay.

The cytokinins activity could be divided into six groups for convenient reference, Group 1 at the 19th-30th, Group II at the 31st-36th, Group III at the 41st-45th, Group IV at the 48th-57th, Group V at the 63rd-76th, and Group VI at the 79th-85th fractions.

^3H -zeatin and cold zeatin were eluted at the 22nd-27th fractions from the Sephadex LH-20 as shown in Fig. 24-E₁ and Table 13. However, there was no promotion of radish cotyledon growth in these fractions in Group I except at the 19th and 22nd fractions (Fig. 24-E). It seems likely that unknown inhibitors are present in the 23rd to the 29th fractions where the most of ^3H -zeatin was eluted. Especially marked inhibition of the radish cotyledon was shown at the 25th fraction, in which much of the ^3H -zeatin was eluted (Fig. 24-E₁). In fact, 6-(*r,r*-dimethyl allylamino)purine and dihydrozeatin peaked at the 20th and 21st fractions respectively as shown in Table 13 and therefore activities of cytokinin-like substances in Group I might be other than zeatin.

Possibly zeatin riboside eluted at the 28th to the 38th fractions (See Table 13) (Group II) and was estimated as 0.21 μg equivalent to zeatin for the 1.0 gram dry weight extract and 0.43 μg for the 5.0 gram dry weight. Group III and IV had slightly lower activity than other groups. In Group V the aliquot equivalent to the 5.0 gram dry weight extract showed cytokinin activity at all fractions except the 64th, 66th, 71st, and 77th. An intense yellow pigment at the 70th to the 76th fraction was observed and this pigment may be inhibitory to radish cotyledon growth. The slight inhibition on the 72nd fraction in the aliquot equivalent to the 1.0 gram dry weight could be due to the presence of the inhibitor, but the inhibition of radish cotyledon might be overcome in the same fraction with the aliquot equivalent to the 5.0 gram dry weight.

The cytokinin activities were extremely high in Group VI. The highest activity, at the 79th fraction, was estimated to be 4.50 μg with the 5.0 g aliquot, although 1.0 gram dry weight aliquot had less than 1/5 the activity. An average of cytokinin activities in Group VI from the aliquots equivalent to the 1.0 and 5.0 gram was estimated as 781.5 $\mu\text{g}/\text{kg}$ dry weight of leaf sample.

9.4.3.2. On DEAE cellulose thin layer chromatography

The fractions eluted from the Sephadex LH-20 column which were grouped as A, B, C, and D (See 8.4.2.1) were chromatographed on

DEAE cellulose thin layer. The results are represented as follows: aliquot equivalent to 0.5 gram dry weight in Fig. 24-A₁, B₁, C₁, and D₁, 1.0 gram dry weight by a solid line in Fig. 24- A₂, B₂, C₂, and D₂, and 5.0 gram dry weight by a dotted in Fig.24- A₂, B₂, C₂, and D₂. The estimation of cytokinin activities equivalent to zeatin is indicated in Table 20.

The A fraction containing 155 ml volume of eluted solution consisted of the Groups I, II, and III. With the 0.5 gram dry weight the cytokinin activity was spread over most Rfs. Cytokinin activity was greatest at Rf 0.2-0.3, the position of zeatin riboside, giving 0.075 μ g equivalent to zeatin. Cytokinin activity at the position of zeatin was less than that at the position of zeatin riboside, although zeatin and zeatin riboside overlapped at Rf 0.1-0.2. On the other hand the aliquot equivalent to the 1.0 and 5.0 gram dry weight exhibited a significant promotion of radish cotyledon from the origin to Rf 0.2 and Rf 0.6-0.8. Especially in the aliquot equivalent to the 5.0 gram dry weight, the highest cytokinin activity was at the origin suggesting that possibly the chromatogram had been over loaded. The next highest activity occurred at the Rf 0.0- 0.1. However, no promotive radish cotyledon was found at the Rf. 0.1- 0.2 where zeatin and zeatin riboside were overlapped. Possibly because either the plate was over-loaded or the inhibitor from the 26th-29th fraction was presents. Inhibition also occurred at Rfs 0.4-0.5 and 0.9-1.0. In contrast with the 1.0 gram dry weight, there was a significant promotion of radish cotyledon at Rf 0.1-0.2. The other cytokinin activity could be observed at the Rf 0.6-0.7 and 0.7-0.8 with the 5.0 and 1.0 gram dry weights respectively. Total cytokinin activities at the aliquots equivalent to the 0.5, 1.0, and 5.0 gram dry weight were 0.234 μ g, 0.0435 μ g, and 0.3175 μ g respectively in DEAE cellulose thin layer chromatography. An average of cytokinin activities from these aliquots was 192 μ g/kg dry weight of leaf samples.

The B fraction (Group IV) containing 95 ml of eluant from the Sephadex LH-20 exhibited cytokinin activity on DEAE cellulose as shown in Fig. 24-B₁ and B₂. There was significantly promoted radish cotyledon growth at three different Rfs with the aliquots equivalent to both the 0.5 and 5.0 gram dry weight. In the aliquot of 0.5 gram dry weight 0.027 μ g equivalent to zeatin was found at Rf 0.0-0.1 while at Rf 0.8-1.0, the total was 0.046 μ g equivalent to zeatin. The greater amount of leaf sample in the aliquot equivalent to 5.0 gram dry weight

showed activity at Rf 0.6-0.9 equivalent to 0.2535 μg of zeatin. No stimulation of radish cotyledons occurred with the aliquot equivalent to the 1.0 gram dry weight. Total cytokinin-like substances estimated from only the Sephadex LH-20 column in the Group IV was higher than that of DEAE cellulose thin layer with the aliquot equivalent to the 5.0 gram dry weight. An average of cytokinin activity equivalent to zeatin was 98.4 $\mu\text{g}/\text{kg}$ dry weight in the B fraction by DEAE cellulose thin layer chromatography.

In DEAE cellulose thin layer, the C fraction (Group V) containing 70 ml of eluant from the Sephadex LH-20 exhibited two groups of cytokinin activities in the aliquot equivalent to the 0.5 gram dry weight and three groups in the 5.0 gram dry weight. These groups had more or less similar Rf positions in two aliquots except the absence of activity at Rf 0.3-0.5 in the aliquot equivalent to the 0.5 gram dry weight. Cytokinin activities did not occur in any Rf with the aliquot equivalent to the 5.0 gram dry weight, most radish cotyledons being greatly inhibited especially at the origin and at Rf 0.7-0.9. This inhibition of radish cotyledon might be due to the yellow pigment which was eluted from the 70th to 76th fraction from the Sephadex LH-20 column. It was noticed that the radish cotyledons were more inhibited with DEAE cellulose than with fractions bioassayed directly from the column. With the mean of the aliquots equivalent to zeatin was calculated as 183.5 $\mu\text{g}/\text{kg}$ dry weight of leaves.

The D fraction (Group VI) containing 30 ml of eluant from the Sephadex LH-20 column exhibited three peaks with the aliquot equivalent to 0.5 gram dry weight, two peaks with that of the 1.0 gram dry weight, and activity at all Rfs, except the Rf 0.8-0.9 position, with the aliquot equivalent to the 5.0 gram dry weight. All samples showed activity at Rf 0.3-0.4 while activity was found at Rf 0.2-0.3 with the aliquots equivalent to the 1.0 and 5.0 gram dry weight of leaf samples. These cytokinin activities were at Rf 0.6-0.7 for both the 0.5 and 5.0 gram dry weight leaf sample, indicating 0.0255 μg equivalent to zeatin for both. Total cytokinin activities recorded from all Rfs in DEAE cellulose thin layer were 0.538 μg with the aliquot equivalent to the 5.0 gram dry weight, and 0.0585 μg and 0.0575 μg with the 0.5 and 1.0 gram dry weight respectively. An average activity for these three aliquots was estimated as 94 $\mu\text{g}/\text{kg}$ dry weight.

In summary the estimation of cytokinin activities equivalent to zeatin from the A, B, C, and D fractions by DEAE cellulose thin layer chromatography was 473.6 $\mu\text{g}/\text{kg}$ dry weight of apple leaves which was 1/3 of that using only Sephadex column chromatography.

Temperature programmed separation was from 120°C-300°C at 120°C with an initial hold for 4 minutes and then at 3°C/min.

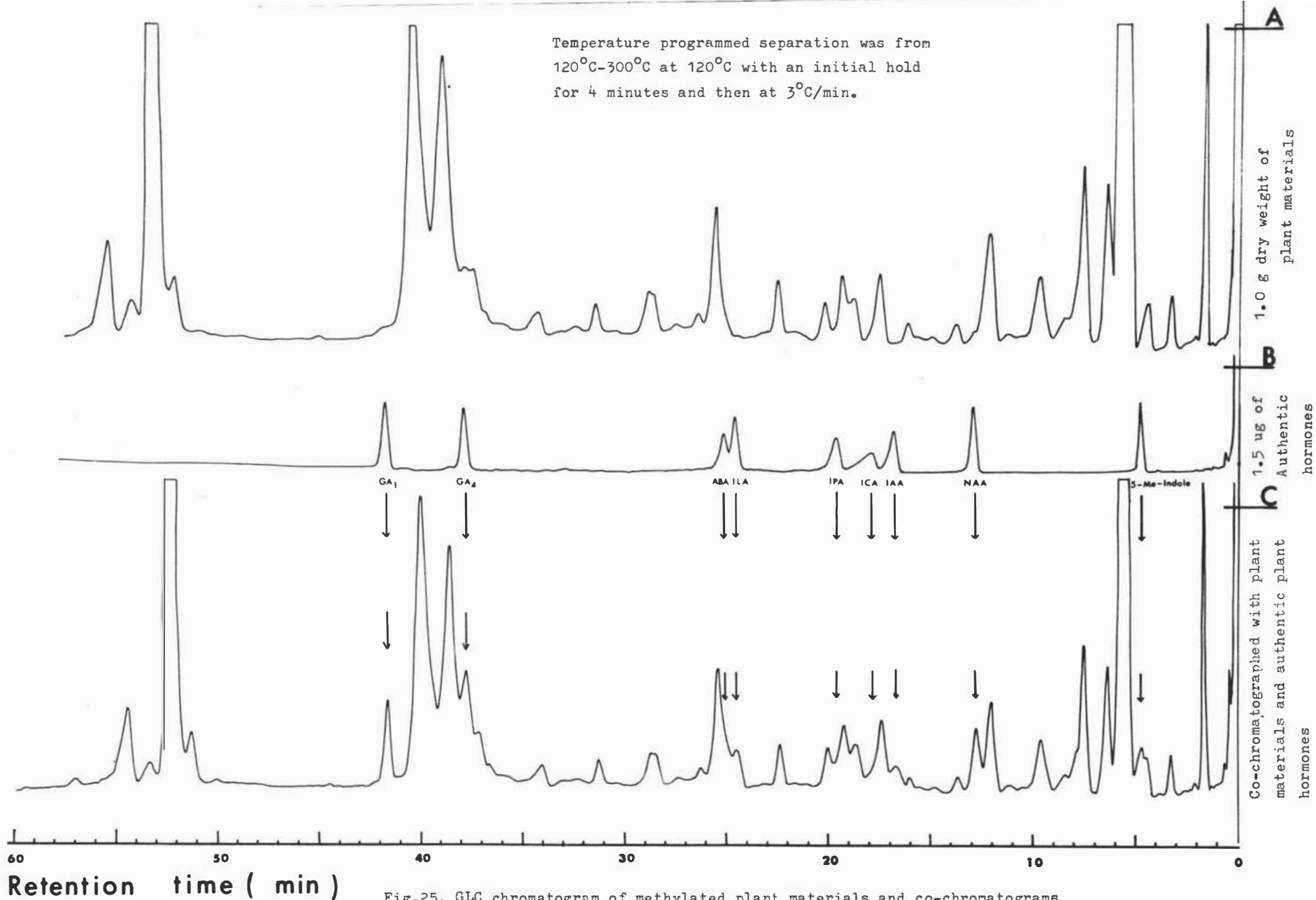


Fig.25. GLC chromatogram of methylated plant materials and co-chromatograms of authentic plant hormones

9.5. GLC of authentic plant hormones and plant materials

9.5.1. Gas chromatographic separation from the apple leaves and comparison with bioassay results

9.5.1.1. GLC of plant extracts

For the analysis of auxins, GAs, and ABA the acidic ether fraction was purified through Sephadex G-10 column without further purification by paper or thin layer chromatography. The acidic ether fraction of the apple leaves was methylated with excess ethereal diazomethane. The methylated residue was dissolved with MeOH and the aliquot equivalent to 1.0 gram dry weight of leaf sample was injected on to the 3 % SE-52 stationary phase (180 cm x 2 mm column) and chromatographed at 3°C/min from 120°C-300°C with 50×10^2 attenuation of the detector.

Fig. 25-A indicates the several peaks from plant extract; Fig. 25-B for the 1.5 ug equivalent of authentic plant hormones, and Fig. 25-C for co-chromatographed peaks with the plant extract and authentic plant hormones.

With the 50×10^2 attenuation, no peak of IAA appeared at the retention time of authentic IAA position (16.8 mm). The peaks of ICA, IPA, and ABA were overlapped with larger peaks at the same retention time. There was also a very small peak at the GA₄ retention time (27.88 mm) where two peaks were only partially resolved. Furthermore there were two big peaks close to the GA₄ peak.

The 160°C-200°C temperature programme was tried but further purification of the plant extract was required.

The present GLC studies did not investigate further quantitative and qualitative measurement of plant extracts.

9.5.1.2. The IAA and ABA-like substances in the aliquot of acidic ether fraction by bioassay

While the plant extracts were analysed by GLC, the aliquot of the same acidic ether fraction chromatographed on cellulose thin layer and bioassayed for IAA and ABA by the combined bioassay with oat and wheat coleoptile sections (See 8.5.2.1. and 8.5.2.3.).

The results for the aliquots equivalent to 1.5, 1.0, 0.5, and 0.1 gram dry weight of leaf sample are shown in Fig. 26-A, B, C, and D.

The Rfs of IAA and ABA exhibited a significant activity of promoters and inhibitors. The estimation of IAA-like substances was 0.07 µg, 0.11375 µg, and 0.0647 µg from the 1.5, 1.0, and 0.5 gram equivalent to dry weight respectively and the ABA-like substances 0.627 µg, 0.0615 µg, 0.0935 µg respectively.

Some promoters other than IAA-like substances detected by oat

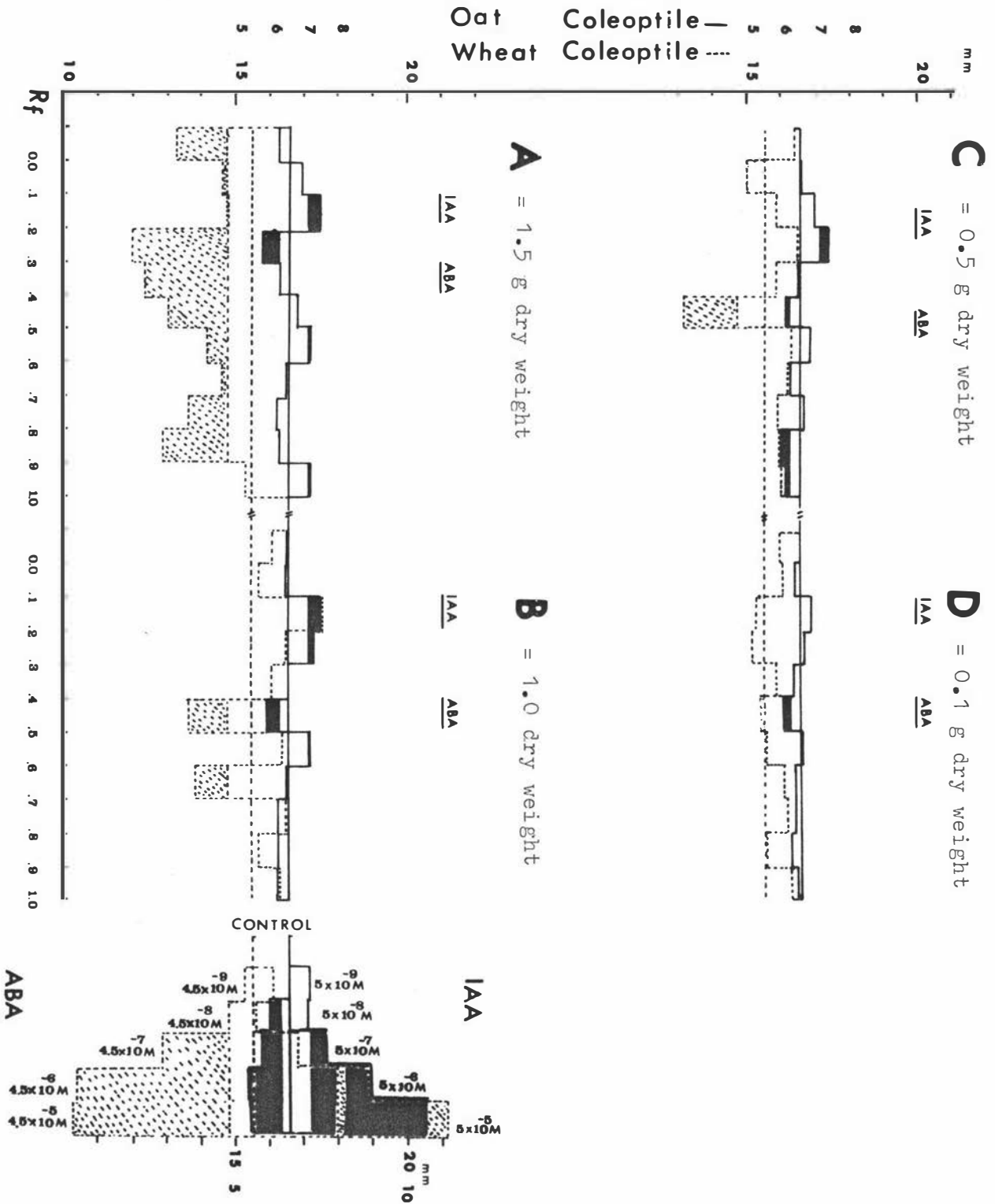


Fig. 26. Auxin- and ABA-like substances of apple leaves in the acidic fraction on cellulose thin layer chromatography.

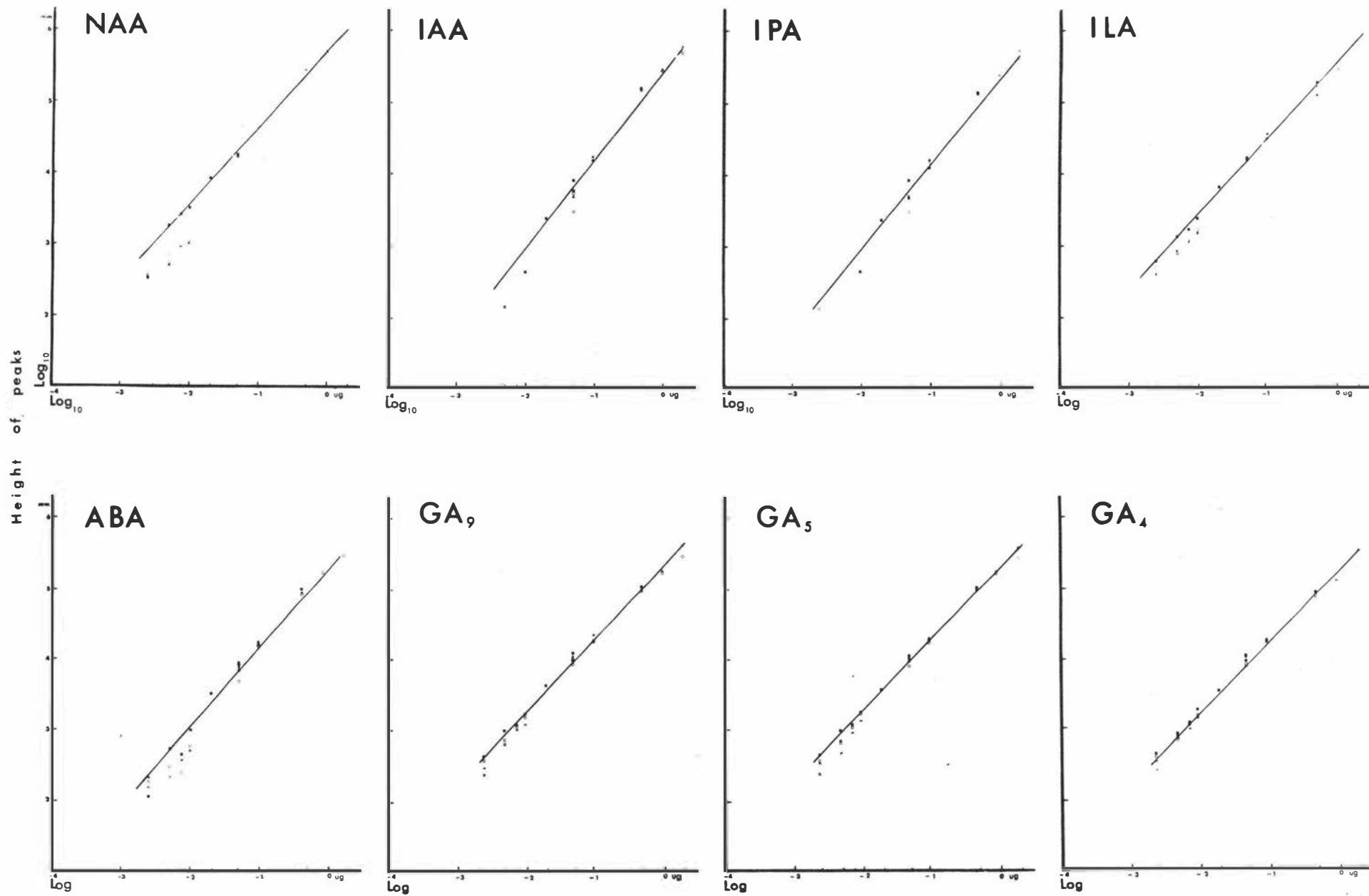


Fig.27-A

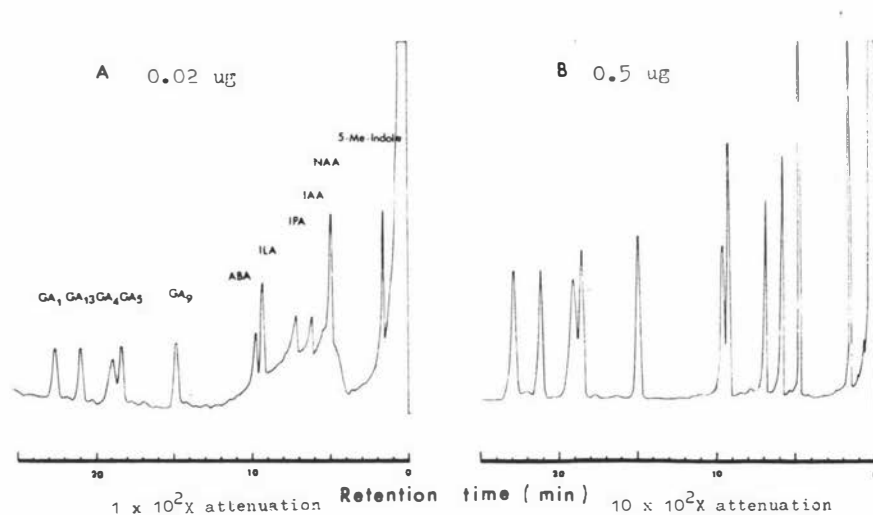
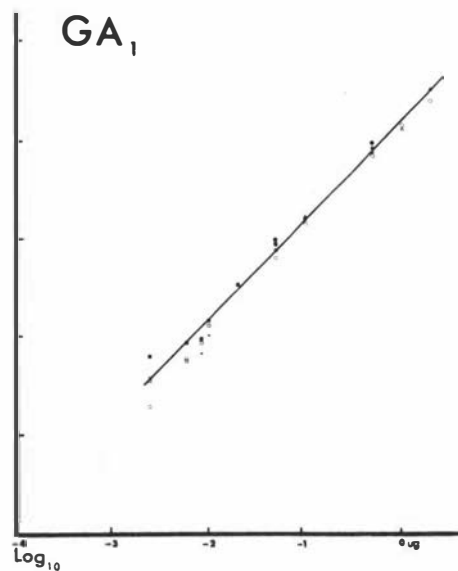
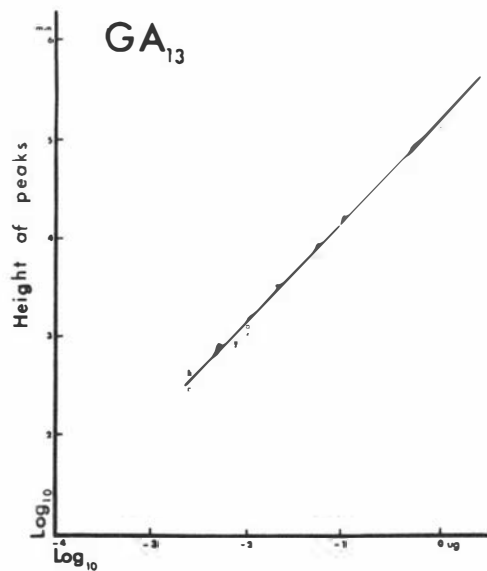


Fig.27-B

Attenuation

- 10 x
- × 20 x
- 50 x
- 1 x 10² x
- ▲ 5 x 10² x
- 10 x 10² x
- 20 x 10² x
- △ 50 x 10² x

Fig. 27. Standard calibration of authentic plant hormones by GLC.

The separation of methyl esters of authentic plant hormones was carried out with a 150 cm x 4 mm ID column packed with a 3 % OV-1 coated on 80-100 mesh Gas-Chrom Q.

The initial temperature hold was at 160°C for 4 minutes and then the dial was set to 200°C.

coleoptile sections occurred both in the 1.5 and 1.0 gram dry weight samples at Rf 0.5-0.6 and at Rf 0.9-1.0 position in the 1.5 gram dry weight but not in the 0.1 gram dry weight. There are also inhibitors other than ABA-like substances with the 1.5 gram dry weight.

Considering concentrations of IAA and ABA-like substances obtained above by bioassay, it was realized that further purification was necessary to use GLC for plant hormones. Since masking of the hormones by other components would otherwise occur.

9.5.2. Standard calibration of several authentic plant hormones by GLC

For the quantitation of plant hormone in plant material extracts by GLC, standard calibration of ME-TMS derivatives of NAA, IAA, IPA, ABA, GA₉, GA₅, GA₄, GA₁₃, and GA₁ was attempted from 0.0025 μ g to 2.0 μ g with a dilution technique, using a 3 % OV-1 stationary phase at several different attenuations (See 8.5.3).

The results are shown in Fig. 27-A and B. Most gibberellins, ABA and ILA were detectable down to 0.0025 μ g/ μ l, a concentration which can hardly be detected by the dwarf rice bioassay. However in order to detect lower concentration such as 0.0025 μ g/ μ l, a great deal of purification would be needed.

IAA and IPA are probably too unstable at very low concentrations. Linearity was quite good except for NAA, ILA and ABA at low concentrations as shown in Fig. 27-A and B.

In this experiment the best linearity was established with gibberellins from 0.0025 μ g to 2.0 μ g, possibly due to stability of the compounds in the gas chromatographic conditions used.

9.5.3. GLC of authentic plant hormones and related synthetic plant hormones after cellulose TLC

Cellulose thin layer chromatography with the solvent system MAAW has been used for auxins-, ABA-, and gibberellin-like substances in plant extracts in the previous sections. Although it was a useful method for good separation and convenience to handle, Rf positions tended to be variable, even in the same tank. In fact Rfs of a known compounds often vary in both paper and thin layer chromatography. Therefore markers of known compounds should be used on the same paper or thin payer plates.

A method of purifying plant materials by cellulose thin layer might also be useful for GLC. Therefore in order to study the behaviour and recovery of 10 μ g authentic plant hormones on cellulose thin layer

plates with the solvent system MAAW, plates were run for 15 cm from the origin and divided into 1 cm bands in the A plate which was left longer than the B plate (Fig. 28-A and 29-A) because of different speed of development and 10 sections with 1.5 cm band in the B plate (Fig. 28-B and 29-B) were tested by GLC. The results are shown in Fig 28 and 29.

GA₁, IAA, and GA₁₃ showed little variation in the A and B plates. Compounds such as IPA, NAA, ABA, GA₅, GA₄, and GA₉ were slightly different in behaviour between the two plates. NAA, ABA, GA₅, GA₄, and GA₉ appeared at the same Rf 0.7-0.8 in the B plate, but NAA and GA₅ were separated from NAA, ABA, GA₉, and GA₄ at Rf 0.8-0.93 in the A plate.

According to peak heights the recovery of each authentic plant hormone appeared to be nearly 100 % with a 10 µg sample on the cellulose thin layer as shown in Fig. 29-A and -B.

9.5.4. Effect of different reagents on formation of ME- and ME-TMS derivatives of several authentic plant hormones and on the retention time and peak heights

9.5.3.1. Gibberellin A₃

The behaviour of compounds in gas liquid chromatography is dependant on their volatile properties. Non-volatile substances can often be converted to volatile derivatives by using ethereal diazomethane and silyl reagents.

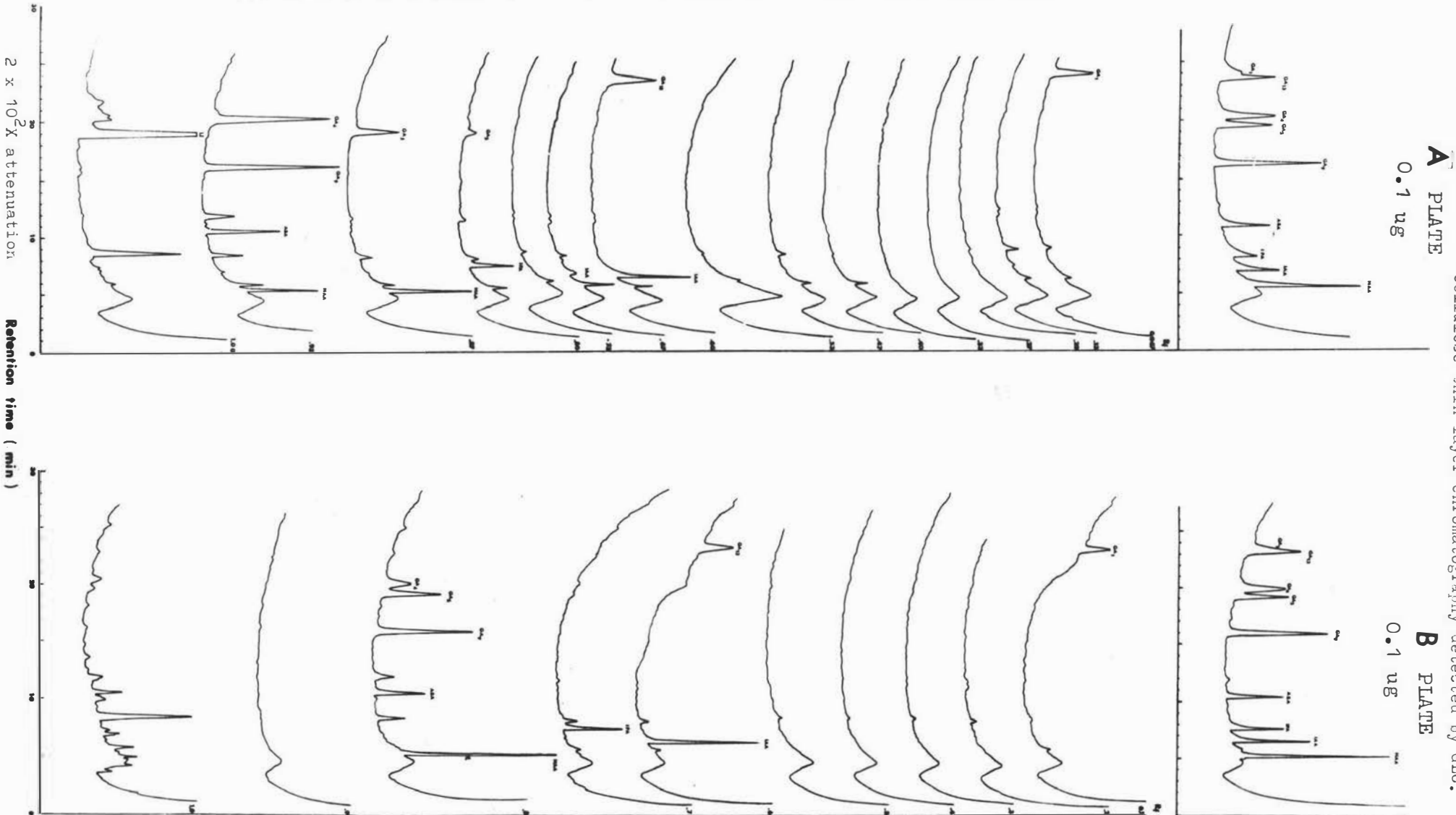
Methyl esters derivatives of some authentic plant hormones can be formed, but the gibberellin A₃ peak has a marked shoulder. In order to obtain a single peak of GA₃ in GLC, silyl reagents were tested. The results obtained by forming the ME-TMS derivative of 0.1 µg of GA₃ using three different methods are shown in Fig.30-A,-B, and-C. The ME-TMS derivative of GA₃ with PYR - HMDS - TMCS - silyl reagents exhibited two peaks at 23.5 and 24.9 mm retention time with 28.5 mm and 10.3 mm peak heights respectively. However, the ME-TMS derivative of GA₃ with PYR and BSTFA without TMCS prevented the shoulder. However peak height was not improved as much as when TMCS was used (Treatment 3), the peak height being nearly three and half times higher.

Therefore BSTFA together with TMCS reagent is a useful silyl reagent for gibberellin A₃.

9.5.4.2. Several authentic plant hormones

0.1 µg of NAA, IAA, IPA, ABA, GA₉, GA₅, GA₄, GA₁₃, and GA₁, the methyl esters and methylester trimethylsilyl ethers of these derivatives formed by using BSTFA with TMCS were chromatogrammed using 2 x 10² attenuation. The results are shown in Table 21 and Fig. 30-D and E.

Fig.28-A and -B. A behaviour of authentic plant hormones in cellulose thin layer chromatography detected by GLC.



The separation of methyl esters of authentic plant hormones was carried out with a 150 cm x 4 mm ID column packed with a 3 % OV-1 coated on 80-100 mesh Gas-Chrom Q.

The initial temperature hold was at 160°C for 4 minutes and then the dial was set to 200°C for ½ minutes by hand, before starting a 3°C/min. temperature programme from 200° to 300°C.

Fig. 29 Recovery of authentic plant hormones by GLC from cellulose thin layer chromatography*.

A		B	
Rf 0.067	GA ₁ (<100%)	Rf 0.1	GA ₁ (<100%)
0.13		0.2	
0.20			
0.27		0.3	
0.33			
0.40		0.4	
0.47		0.5	
0.53			
0.60	IAA (<100%)	0.6	IAA (<100%)
	GA ₁₃ (<100%)		GA ₁₃ (<100%)
0.67		0.7	IPA (<100%)
0.73			
0.80	IPA (88%)	0.8	NAA (<100%)
	GA ₅ (38%)		ABA (90%)
			GA ₅ (100%)
			GA ₄ (100%)
			GA ₉ (100%)
0.87	NAA (56%)	0.9	
	GA ₅ (84%)		
0.93	NAA (52%)		
	GA ₉ (100%)		
	ABA (<100%)		
	GA ₄ (<100%)		
1.0		1.0	

Fig.30. Comparison of chromatograms in methyl esters and TMS derivatives of several authentic plant hormones (See Table 22).

The separation of ME-YMS derivatives of authentic plant hormones was carried out with a 150 cm x 4 mm

ID column packed by a 3%

OV-1 coated on 80-100 mesh

Gas-Chrom Q by the 160°-200°C temperature programme.

D = 0.1 ug ME-TMS derivatives

A = 0.1 ug ME-TMS of GA₃ by Pry-HMDS-TMCS silyl reagents

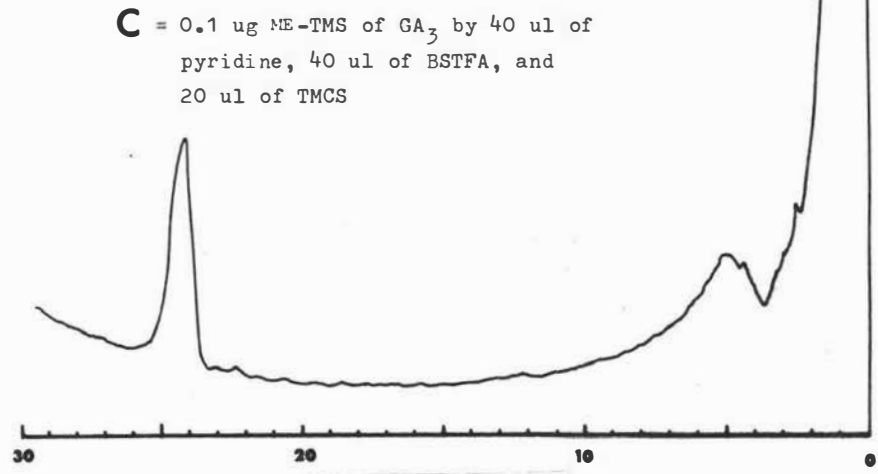
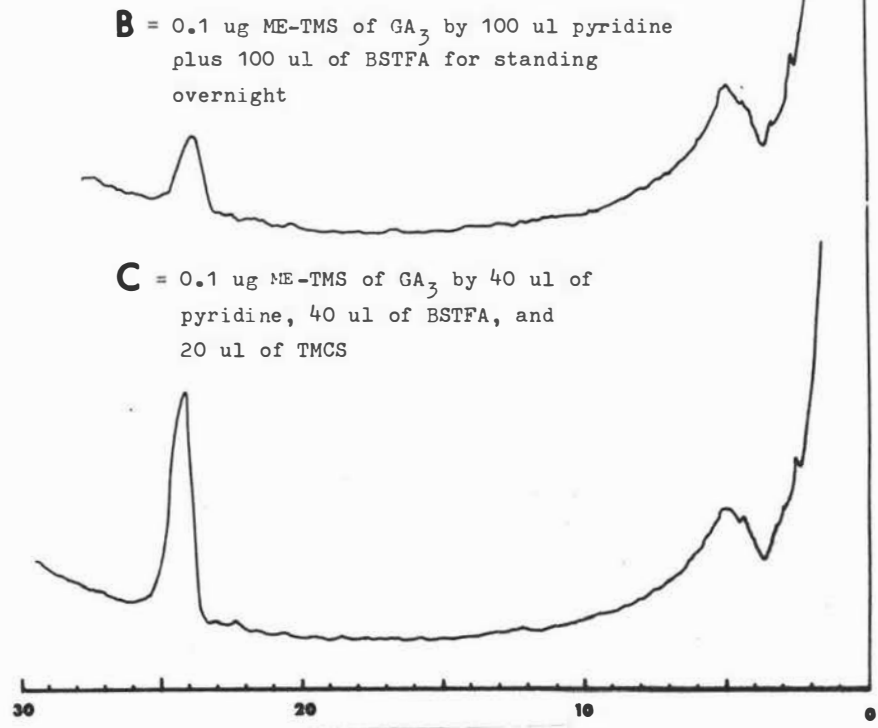
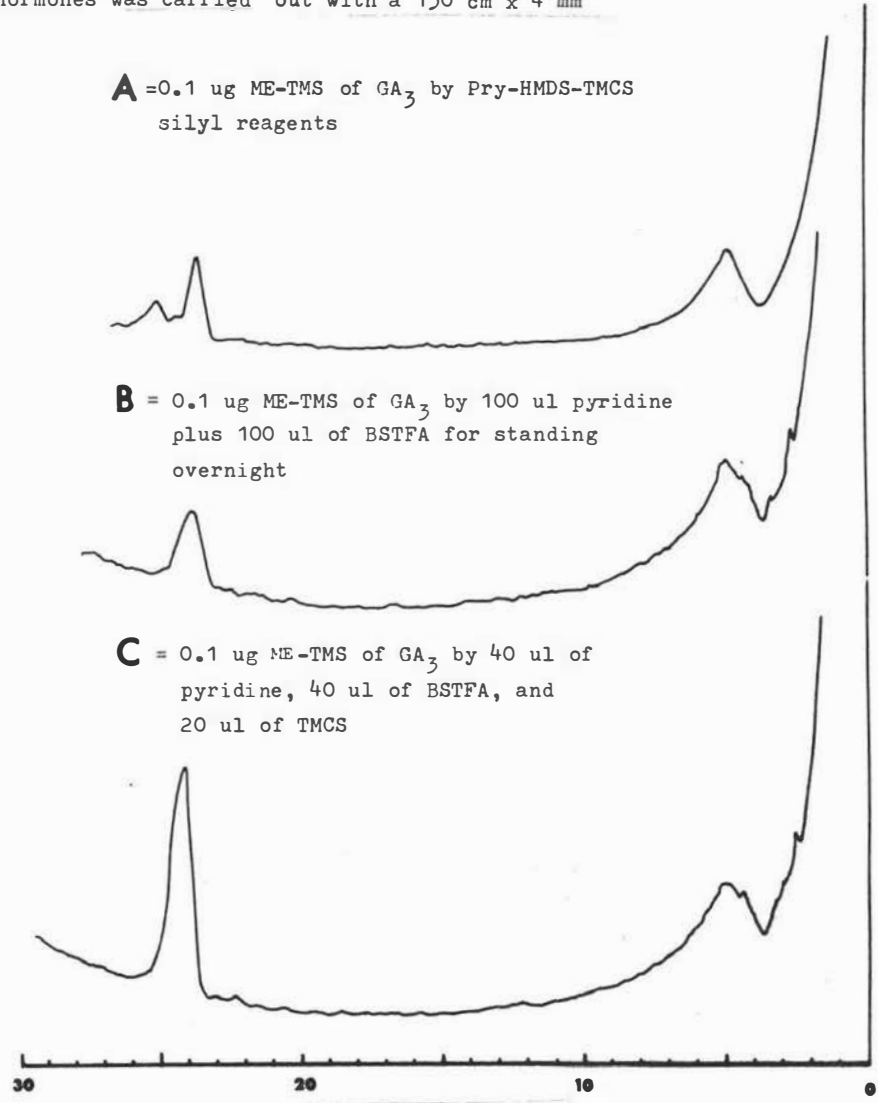
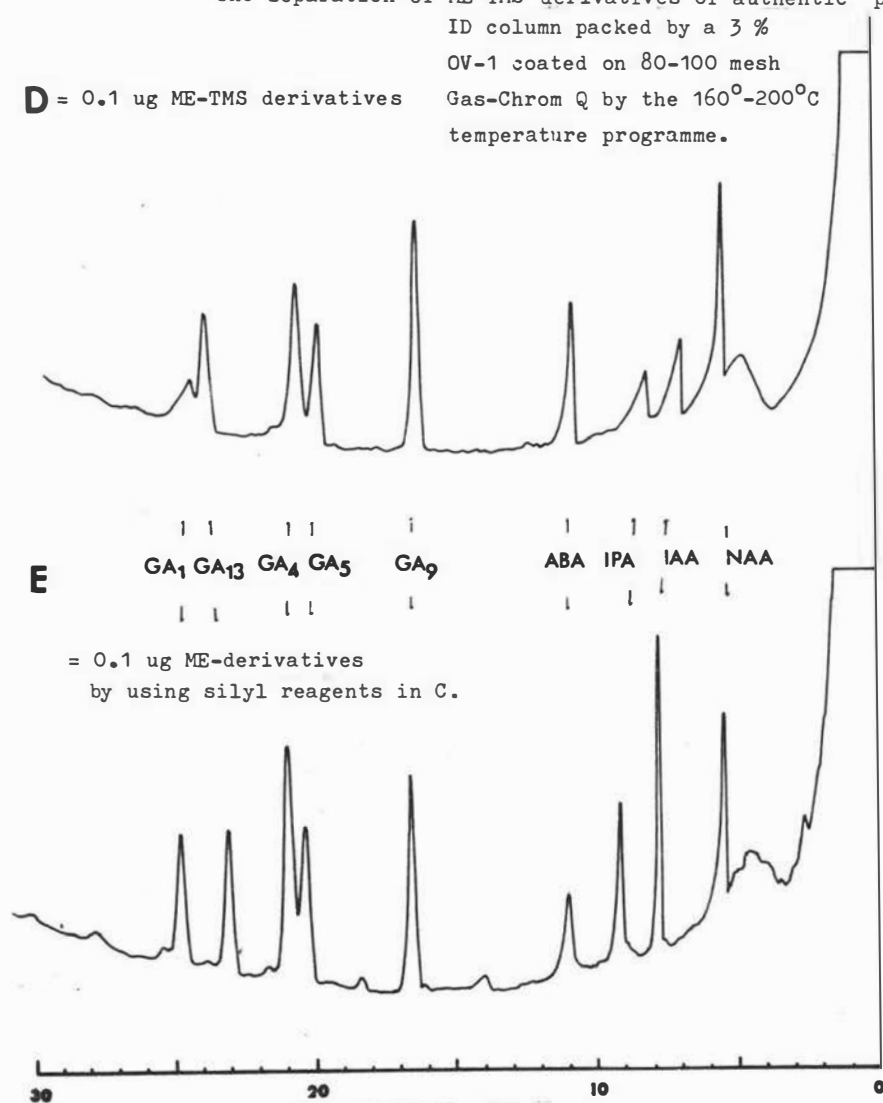
B = 0.1 ug ME-TMS of GA₃ by 100 ul pyridine plus 100 ul of BSTFA for standing overnight

C = 0.1 ug ME-TMS of GA₃ by 40 ul of pyridine, 40 ul of BSTFA, and 20 ul of TMCS

E

GA ₁	GA ₁₃	GA ₄	GA ₅	GA ₉	ABA	IPA	IAA	NAA

= 0.1 ug ME-derivatives by using silyl reagents in C.



Peak height

30 20 10 0
2 x 10²X attenuation

30 20 10 0
1 x 10²X attenuation

Retention time (min)

The initial temperature hold was at 160°C for 4 minutes and then the dial was set to 200°C for 1½ minutes by hand, before starting a 3°C/min. temperature programme from 200°C to 300°C (160-200°C temperature programme).

Table 2.1. GLC retention times and height of peaks of authentic plant hormones with the different methyl esters and methyl esters trimethylsilyl ether derivatives.

Compounds	Methyl esters (Me)			Methyl esters trimethylsilyl ether (Me-TMS)			Comparison of height of Me-TMS derivative with methyl esters
	Retention time (mm)	Height of peaks (mm)	Theoretical plates	Retention time (mm)	Height of peaks (mm)	Theoretical plates	$\frac{\text{Me-TMS}}{\text{Me}} \times 100$
NAA	54.47 \pm 0.63	75.13 \pm 3.0	3,536	56.03 \pm 0.5	67.47 \pm 5.64	4,624	84.5
IAA	71.17 \pm 1.10	29.5 \pm 2.10	4,142	78.63 \pm 0.12	107.20 \pm 12.72	9,100	363.4
IPA	80.60 \pm 1.04	17.47 \pm 1.94	5,683	92.17 \pm 0.57	66.03 \pm 11.92	11,639	378.0
ABA	110.73 \pm 5.01	52.4 \pm 1.116	12,314	109.97 \pm 0.29	28.30 \pm 1.49	14,091	54.0
GA ₉	161.90 \pm 1.15	84.87 \pm 2.77	16,507	165.43 \pm 1.50	76.23 \pm 7.23	17,466	89.8
GA ₅	195.63 \pm 1.47	45.27 \pm 1.36	20,972	202.73 \pm 0.74	55.17 \pm 5.61	23,367	121.9
GA ₄	203.23 \pm 1.86	55.10 \pm 3.36	10,302	208.60 \pm 1.66	81.73 \pm 8.50	16,290	148.3
GA ₁₃	235.17 \pm 2.20	43.13 \pm 0.51	20,031	229.53 \pm 0.12	50.23 \pm 4.50	29,836	116.5
GA ₁	241.30 \pm 1.56	16.77 \pm 1.99	8,084	247.03 \pm 1.35	45.03 \pm 4.06	32,269	268.5

Generally the retention time increases in the ME-TMS derivatives compared to the methylated compounds. The comparison between the ME- and ME-TMS derivatives was made by the percentage increase over the methyl derivative as shown in Table 21. The most marked increase was that given by IAA, IPA, and GA₁, nearly 4 times for the indole compounds and 3 times for the GA₁. Slightly increased peak heights were given by GA₅, GA₄, and GA₁₃, but the ABA peak height was reduced by half and a slightly reduced peak height occurred for NAA and GA₉. Therefore BSTFA with TMCS could be used indirectly to identify IAA and ABA, observing change of their peak height before and after using BSTFA and TMCS.

One of the prominent effect was good resolution between GA₁₃ and GA₁ by using ME-TMS derivatives.

Chapter 10

Discussion

10.1. Loss of ^{14}C -IAA during extraction and purification procedures

10.1.1. Effect of pH in the aqueous phases on the loss of ^{14}C -IAA

In order to fractionate four series of plant hormones such as auxins, gibberellins, cytokinins, and abscisic acids from plant extracts, the appropriate pH can be selected according to the dissociation constant (pK) of the hormone and the partition coefficient will then depend on the selectivity and polarity of the solvent used. The solvent partitioning at different pHs using various solvent has already been reviewed in section 7, IAA is easily partitioned between pH 5.5-2.5 with diethyl ether (Larson, 1955) whereas GA_3 is not appreciably partitioned into diethyl ether (Durley and Pharis, 1972; Badr, et al., 1971). However the pKs of GA_3 and GA_8 are 4.1 and 4.0 respectively and can be appreciably partitioned under acid conditions with ethyl acetate (Durley and Pharis, 1972). Therefore, diethyl ether is not suitable for GA_3 and in the present studies ethyl acetate was chosen for solvent partitioning. The aqueous phase at pH 2.5 and 8.0 was used to test ^{14}C -IAA with plant materials, because ^{14}C -IAA was the most labile plant hormone during the plant extraction procedures (Mann and Jaworski, 1971). Photooxidation of ^{14}C -IAA during extraction procedures was minimised by carrying out experiments under low light intensities.

The most surprising results found in the solvent partitioning of apple stem tissues was the great loss of ^{14}C -IAA at two steps in the procedures shown in Table 7-A. A 66 % loss occurred at the change from the aqueous phase at pH 4.5 after removal of MeOH to pH 8.0 with 1.0 M phosphate buffer solution and KOH solution and 78 % loss during 8 x ethyl acetate partitioning after adjusting to pH 8.0. No crucial loss of ^{14}C -IAA occurred during the subsequent steps of the extraction procedures. On the other hand, with pH 2.5 aqueous phase, the final recovery over the whole extraction was 80 %. The results in Table 7-A and B show that the crucial loss of ^{14}C -IAA occurred when the aqueous phase was at pH 8.0.

The extraction procedures for testing recovery of IAA reviewed in section 7.6 vary. Accordingly, percentage of final recovery varies as follows : 25 - 30 % (Hamilton, et al., 1961), 60 - 85 % (Galun, et al., 1965), 65 - 79 % (Ueda and Bandurski, 1969), 88 - 89 % (Goldschmidt, et al., 1971).

The variable loss of IAA in the recovery test with plant materials might be associated with the use of extracted materials from

different tissues and plant. There was a great loss of IAA from plant materials when ethanolic or ethereal extraction was used with an extended period of extraction (Vlitos and Meudt, 1954) and oxidation during grinding (Mann and Jaworski, 1970). Mann and Jaworski (1970) assumed that the loss with plant tissues could be due to peroxidation catalyzed by unsaturated fatty acids. If this assumption is correct, then the peroxidation catalyzed by unsaturated fatty acids could be stimulated by alkaline condition.

An alternative explanation might be that it is due to enzymatic activity. Ueda and Bandurski (1969) conducted an experiment on the loss of ^{14}C -IAA to find out if it is due to destruction or adsorption of ^{14}C -IAA to complex water soluble components. They added 400 μg of IAA in the supernatant of ethyl acetate extract after removal of acetone. They concluded that there was considerable destruction of IAA particularly in ground germinating pea seedlings, although there was a small possibility that it was adsorbed to the water soluble components. Acetone extraction may have contained some IAA destroying enzyme, because acetone solvent did not prevent polyphenoloxidase action (Nitsch, 1956).

Absolute alcohol is assumed to inactivate enzymes by the dehydrating effect of the alcohol (Thimann and Skoog, 1940; Kefford, 1955 a). Nitsch (1956) also reported on the activity of polyphenoloxidase systems during solvent extraction with several different solvents. An absolute MeOH system had no polyphenoloxidase activity after 18 hour extraction, but an ethyl alcohol system exhibited a slight browning. 50 % of ether plus 50 % MeOH also induced enzyme activity. The extraction procedures employed in the present studies used 80 % MeOH (Phillips and Wareing, 1959; Thurman and Street, 1960; Elliott and Stowe, 1970). If the 80% MeOH is used, some enzymes, generally protein, could not be denaturated with alcohol, in the present studies, loss of ^{14}C -IAA at pH 8.0 may be due to an enzyme system. That is the activation of an enzyme system or cofactor under alkaline conditions. The present results are supported by Janssen's experiment (1970a). Janssen found three kinds of enzyme activity from extracts of seedling Pisum sativum root tips using sodium diethyldithiocarbamate purified with Sephadex G-10 column. He found that the peroxidase activity was low at pH 5.0, and increased at pH 7.0, whether sodium diethyldithiocarbamate was present or not. However, IAA-oxidase and polyphenoloxidase were inhibited by the addition of sodium diethyldithiocarbamate. In the present studies 0.02% of sodium diethyldithiocarbamate in MeOH was used at the beginning of extraction. The sodium diethyldithiocarbamate remained in aqueous

solution after removal of MeOH. A concentration of 10^{-4} g/ml of sodium diethyldithiocarbamate can prevent the activity of IAA-oxidase and polyphenoloxidase (Janssen, 1970 a). There is evidence that oxidase activity is dependant on pH. The optimal pH at which IAA is destroyed by IAA oxidase varies according to the presence of different natural cofactors. The optimum pH in the presence of p-hydroxybenzoic acid is pH 6.0, with resorcinol at pH 5.5 in Lens root extract (Gaspar, 1966); with the crude extract at pH 6.5 but after purification and addition of 2,4-dichlorophenol at pH 5.0 (Stutz, 1957); and with a boiled crude cucumber extract at pH 3-5 in a purified IAA-oxidase from pea roots or cucumber roots, but a boiled crude pea extract at pH 6-7 in the same media mentioned above (Janssen, 1969 b). Janssen (1970 b) suggested that the inhibition mechanism in IAA-oxidase activity by phenolic substances is due to the production of quinones by the oxidation of phenolic substances. On the other hand, some phenolic compounds which do not form quinones are cofactors of IAA-oxidase activity (Janssen, 1970 b). The optimum pH varies between 4-6, depending on the concentration of the different cofactors (Janssen, 1969 c). From the characteristics of IAA-oxidase mentioned above, degradation of ^{14}C -IAA in the alkaline aqueous phase in the present studies might not be due to an IAA-oxidase.

After removal of MeOH from MeOH extract, the pH of the aqueous phase was about 4.5-5.0, depending on the tissue used. When the aqueous phase was adjusted to pH 8.0, the colour changed to purple brown from greenish yellow. This was probably not due to polyphenoloxidase, because Janssen demonstrated that a mixture of proline and catechol became purple at pH 6.5 (Janssen, 1969 a).

Mann and Jaworski (1970) gave % recovery from 4 steps in their methods, using acidic aqueous extract with ether partitioning. Main loss of ^{14}C -IAA occurred when ether was evaporated with the evaporator during the final stage, although there was considerable loss at each step. They concluded that ^{14}C -IAA loss was due to sublimation in ether. This loss was eliminated by first testing with 50 ml ether with the ^{14}C -IAA. A small quantity of a distilled water was added before evaporation of ether. In fact there was no loss of ^{14}C -IAA whether distilled water was added or not when only 50 ml of ether was dried. However, to avoid any loss of ^{14}C -IAA by a complete dryness of organic solvents when using large quantities of solvent to evaporate, drying over anhydrous sodium sulphate was not used but material was freeze dried. In Fig. 31 there was no loss of ^{14}C -IAA during vacuum drying when solvents were added to

the vials as soon as the vacuum oven was opened. The 30 % loss of ^{14}C -IAA might be due to either oxidation or sublimation. Therefore, a 20 % loss of ^{14}C -IAA in the whole extraction procedures at pH 2.5 aqueous phase could be due to losses occurring at the various extraction steps, by means of oxidation, sublimation, handling of glassware and incomplete partitioning into ether.

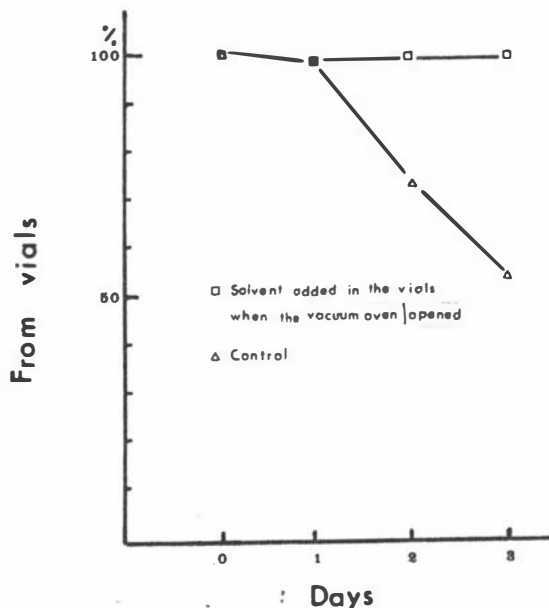


Fig.31. Loss of ^{14}C -IAA in vacuum oven

10.1.2. Column chromatography of ^{14}C -IAA

Sephadex G-10 column chromatography has been used for purification of gibberellins by Crozier, Aoki, and Pharis (1969). Crozier (personal comm.) recommended that it is useful to purify acidic ethyl acetate fractions through a Sephadex G-10 column. In the recovery of ^{14}C -IAA from apple stem tissues, there was no loss of ^{14}C -IAA from the Sephadex G-10 column, eluted with 1,000 ml of 0.1 M phosphate buffer adjusted to pH 2.5 and subsequently partitioned 8 x with ethyl acetate. IAA is stable under alkaline conditions (Mann and Jaworski, 1970). There was no risk of IAA formation under alkaline conditions because tryptophane remains in the aqueous phase after initial solvent partitioning (Dannenburg and Liverman, 1957). After eluting 1,000 ml of 0.1 M phosphate buffer from the column, a further 500 ml was collected, but only 4 % of the ^{14}C -IAA was present, an acceptable degree of tailing in column chromatography.

Silica gel-celite column chromatography was studied by Khalifah, Lewis, and Coggins (1965) as a method for separating several gibberellins and citrus IAA (Lewis, Khalifah, and Coggins, 1965). They used two solvent systems in gradient elution. Of the two systems, the chloroform - ethyl acetate - MeOH solvent system was selected, because GA_1 , GA_7 , GA_9 , GA_4 , and GA_5 were eluted within 350 ml and GA_3 and GA_8 were eluted within 250 ml after addition of 100 ml MeOH. It was

considered useful to separate the two large pooled 350 ml and 250 ml fractions and use each to resolve different components by GLC. In the preliminary tests, 10 ug of authentic plant hormones was applied on silica gel-celite column and 350 ml collected and tested for its recovery by GLC. Most of the compounds were well recovered and therefore the silica gel-celite column with chloroform - ethyl acetate - MeOH was used to obtain more detailed information concerning ^{14}C -IAA.

60 % recovery of ^{14}C -IAA was obtained as a single 15 ml peak and 90 % as a 50 ml peak. A slight tailing problem was present in the gradient elution method. However it is clear that silica gel-celite adsorption chromatography gives a far better recovery than the 77 % obtained by silica gel partition chromatography (Hamilton, Bandurski, and Grigsby, 1961). Recovery of ^{14}C -IAA from silica gel-celite column is comparable to the 93 % recovery of IAA from polyvinylpyrrolidone (PVP) column eluted by pH 8.0 buffer solution (Glenn, Kuo, Durley, and Pharis, 1972). Durley, Crozier, Pharis, and McLaughlin (1972) pointed out that serious problems exist when silica gel from different sources, and various particle sizes and hydration are employed.

10.1.3. Recovery of ^{14}C -IAA from cellulose thin layer chromatography
(See section 9.1.3.)

30 % loss of ^{14}C -IAA using the solvent system, methyl acetate, acetonitrile, ammonium, and water occurred, but no further loss was noticed when plates were stored for 3 days after development. The loss of ^{14}C -IAA could be due to oxidation of ^{14}C -IAA during chromatographic procedures because considerable loss of IAA results from exposure to light and air (Kefford, 1955 a). Mann and Jaworski (1970) obtained a 44 % of loss of IAA in the spotted origin of non-woven silica gel impregnated sheet stored overnight before development by solvent. They obtained a 19 % loss of IAA by using antioxidant, santolquin sulphate on the origin before IAA was spotted. However, after developing silica gel thin layer sheets, IAA was lost before storage overnight. That is a large loss of IAA occurred on silica gel compared cellulose.

10.2. Behaviour of ^3H -zeatin in chromatography

10.2.1. Solvent partition chromatography

10.2.1. Effect of pH in aqueous phase on partition of ^3H -zeatin with ethyl acetate and butanol

While the investigation of loss of ^{14}C -IAA were being studied, Dr. Letham kindly supplied ^3H -zeatin to the author. Although there were

several empirical extraction procedures reported for extracting cytokinin-like substances, ^3H -zeatin provided several benefits for testing extraction procedures. Therefore, a series of experiments was carried out with ^3H -zeatin with physiological level of ^3H -zeatin used in the extraction procedures.

The main solvent partitioning in the extraction procedures was based on ethyl acetate and butanol at pH 2.5 and pH 8.3. The partition coefficients were tested for these pHs.

^3H -zeatin can be partitioned into butanol phase at pH 8.3 and partially partitioned into butanol at pH 2.5. This agrees with Letham (1974b) who obtained partition coefficient at pH 7.0 and 3.0.

^3H -zeatin significantly partitioned into the ethyl acetate at pH 8.3 and not at pH 2.5. These results are similar to those obtained by Woolley (1971), Hemberg (1974), and Letham (1974b). However, considerable loss of zeatin would occur with ethyl acetate partitioning at pH 3.0 if the partitioning is carried out several times (e.g. 8 x ethyl acetate partitioning should be employed for gibberellins as Crozier, Durley, and Pharis (1969) recommended). According to Hemberg's data each partition can remove approximately 2.33% zeatin to the ethyl acetate. That means 8 x ethyl acetate partition will remove about 18.6% of zeatin from the acidified aqueous phase. However, in the present studies at pH 2.5 it could remove only 4.15% and 4.96% of ^3H -zeatin by 8 x ethyl acetate partitions, saturated with 0.5 M HCOOH and non-saturated with 0.5 M HCOOH respectively. The slight difference in the partition coefficient is possibly due to the protonation of the purine ring (pK ca 4.0) under more acidic conditions (Letham, 1974b). However, van Staden (1973) exhibited almost completely free base of cytokinin in petroleum ether and ethyl acetate, this disagreement requires further investigation.

10.2.1.2 Back washing

Housley and Bentley (1956) and Housley and Tayler (1958) employed a back washing technique, whereby they partitioned ether (5 x 800) from sodium bicarbonate and the combined ether extract was back washed with 200 ml distilled water. However, most extraction procedures in the literature do not use back washing techniques. In the recovery test of ^3H -zeatin 4-5% of ^3H -zeatin partitioned 8 times with ethyl acetate remained in the ethyl acetate, therefore it could be necessary to wash ethyl acetate by distilled water (pH 2.5) with a small quantity of water. In fact, the solubility of water in ethyl acetate is 8.6% (Tautvydas, 1965) and therefore considerable amounts of water and water soluble compounds could be retained in ethyl acetate. 80% of ^3H -zeatin

was recovered from the ethyl acetate phase by one back washing. Further back washing did not improve the recovery.

The solubility of water in n-butanol is greater than in ethyl acetate, and only 2.46% recovery of ^3H -zeatin was obtained for each back wash.

10.2.2. ^3H -zeatin in column chromatography

A Sephadex G-10 column chromatography was used successfully to recovery ^{14}C -IAA in a 1,000 ml fraction (section 9.1.2.1.). Recovery of ABA in the preliminary studies using wheat coleoptile bioassay is shown in Fig. 32. Crozier, Aoki and Pharis (1969) have reported that GA-like compounds can be recovered in 1,000 ml fraction from Sephadex G-10. The molecular weights of zeatin and the other plant hormones such as IAA, GAs, and ABA are similar. Therefore zeatin can be fractionated from Sephadex G-10. The elution behaviour of zeatin in Sephadex G-10 was attempted.

Dowex 50 W x 8 resin has been used by many research workers as reviewed in section 7.4.3.1.1. An eluting pattern of cytokinin in Dowex 50 W x 8 column could be useful for purification of cytokinin-like substances. The Dowex 50 W x 8 resin was tested with a known sample of ^3H -zeatin.

Armstrong, Burrows, Evans and Skoog (1969) first reported useful techniques for the separation of cytokinins by Sephadex LH-20 using 35% ethanol. It takes several hours to elute cytokinins from Sephadex LH-20 with 35% ethanol. In order to shorten the eluting period, 95% EtOH acidified to 0.001 M HCl was used for the fractionation of cytokinins. In fact, Eliasson (1969) used 96% EtOH with 0.001 M HCl in Sephadex LH-20 for the purification and separation of IAA and ABA from Populus tremula.

Even though application of samples to top of the column was cautious in order to minimize the tail problem, Sephadex G-10, Sephadex LH-20, and Dowex 50 W x 8, exhibited a tailing problem, possibly because of the radioisotope used, but even a sensitive UV recorder might not pick up such very low concentrations. In Sephadex LH-20 300 μg of 6(α,β -dimethylallylamino)purine, zeatin, dihydrozeatin and zeatin-riboside was spread over only a few fractions. 80% recovery was obtained in the first 1,000 ml with Sephadex G-10 and Dowex 50.

On the other hand, 80% of ^3H -zeatin was recovered in the first 15 ml from Sephadex LH-20 and about 90% in the first 20 ml. There is also reasonable resolution between zeatin riboside and the following cytokinins, i.e., zeatin, dihydrozeatin and 6(α,β -dimethylallylamino)purine. But the resolution between 6(α,β -dimethylallylamino)purine,

zeatin and dihydrozeatin is not well resolved. Both zeatin-riboside and zeatin are well resolved with either 95 % or 25 - 35 % ethyl alcohol but the order of elution patterns changed in the two systems. Using 95 % ethyl alcohol with 0.001 M HCl, 6(*s,r*-dimethylallylamino) purine eluted slightly earlier and zeatin-riboside was retarded, whereas using 25 - 35 % ethyl alcohol in the same column (Hewett and Wareing, 1973 a,b,c), zeatin-riboside was eluted before zeatin. The retardation of elution behaviour in some compounds e.g., glycerides varies with different solvent systems such as ethyl alcohol and chloroform (Determann, 1969). In the present studies, an acidic condition together with increased % of alcohol could effect the retardation forces of zeatin-riboside in gel phase. It is also of interest to notice that in Sephadex LH-20 column the elution behaviour does not follow the molecular weight. Adsorption and partition effects are much more important, zeatin-riboside eluting before zeatin and ms-IPA eluting after IPA (Hewett and Wareing, 1973 b).

10.2.3. ³H-zeatin in paper and thin layer chromatography

On cellulose TLC ³H-zeatin shows activity Rf 0.0 - 0.1 and Rf 0.23 - 0.37. When silica gel is used with distilled water saturated in n-butanol as solvent, ³H-zeatin was spread over all Rfs, in contrast to ³H-zeatin on DEAE cellulose which exhibited a sharp resolution.

10.3. Bioassay techniques for IAA, ABA and GAs

10.3.1. The combination of 5 mm and 10 mm wheat coleoptile section

The Avena straight test (Bonner, 1933; Thimann and Schneider, 1938; Thimann and Bonner, 1948) was re-evaluated by Bentley (1950). Subsequently Bentley and Housley (1954) used a micro bioassay with a 3.2 mm Avena coleoptile mounted on a glass capillary which contained 0.25 ml of culture solution in the specimen tube (0.7 x 7.5 cm). The assay was linear from 0.1 µg to 10 µg / ml. Eliasson (1969) also obtained a standard calibration from 0.02 µg, 0.06 µg, 0.2 µg and 2.0 µg/ml of IAA using a 10 ml beaker and a reciprocal shaker. In the present work the standard calibration of

IAA using oat coleoptile sections gave a linear plot of log concentrations similar to those obtained by Bentley and Housley (1954) and Eliasson (1969). With the Avena coleoptile section the lowest concentration detected was 5×10^7 M IAA in both single or combination oat coleoptile bioassay. The wheat coleoptile bioassay (Hancock, Barlow, and Lacey, 1964) is similar. The sensitivity of oat and wheat coleoptile sections to IAA is low compared to the oat internode bioassay (Nitsch and Nitsch, 1956), which can detect levels of 5×10^{-8} M IAA.

The oat coleoptile bioassay for ABA is less sensitive than the wheat coleoptile bioassay, as shown in Table 18-B, which is why Bentley and Housley (1954) recommended using wheat coleoptiles in the straight test for inhibitors. The characteristic lower sensitivity of oat coleoptiles to inhibitors, allowed the use of the combination of wheat and oat coleoptile sections in the same test tube. Data from the combined bioassays using oat and wheat coleoptile sections for IAA and ABA are given in Fig. 18. There is no significant interaction between the single and combined bioassays, although there is a tendency for slight inhibition of oat coleoptile sections with the presence of wheat coleoptile sections in the same test tube, whereas the wheat coleoptile sections in ABA were inhibited less without the oat coleoptile sections. There are several papers discussing the volume of culture solution (Nitsch and Nitsch, 1956; Walker, Hendershott, and Snedecor, 1958; Cockshull and Heath, 1963; Hancock, Barlow, and Lacey, 1964; Sirois, 1966) and a number of sections to be used in the test tube. The test variability can be reduced by using small specimen vials with hole in the lid, varying the relative numbers of oat and wheat coleoptile sections in the specimen vials and the time of incubation.

10.3.2. The dwarf rice seedlings and lettuce hypocotyl bioassay for gibberellins

10.3.2.1. Tan-ginbozu dwarf rice seedling in the glass tubes and agar culture medium

The present data for Tan-ginbozu dwarf rice bioassay were similar to results obtained by Martin (1971), although slightly different environmental conditions and methods were used. Test tubes are suitable for small scale experiment. However for larger experiments it is more convenient if the seedlings are transplanted

directly into agar medium without using test tubes but this is less sensitive. Growth conditions of seedlings and micro environmentals have to be optimised in order to detect at levels of $0.005 \mu\text{g GA}_3/\text{ml}$. Seedling factors also modify the response to gibberellin, e.g., coleoptile size and length of primary roots.

10.3.2.2. Comparison of different varieties for use in the lettuce hypocotyl bioassays for gibberellins

Before applying to plant materials, a standard calibration was obtained. However, to use the lettuce bioassay the plant extract must be more purified than for the dwarf rice bioassay.

Arctic was the most sensitive variety (Crozier et al., 1970). Results were similar to Crozier et al., (1970), but the greatest difference was noticed in the growth of control, i.e.: 2.64 ± 0.08 mm of hypocotyl length in the petri dish (Crozier, et al., 1970) and 9.1 mm in the specimen vial sealed with thin plastic 'Glad Wrap'. The promoted growth of the control was possibly due to the sealed condition of specimen and low light intensity, although there is well established a straight linearity in log concentrations. Further studies may enable the size of the response to be increased.

10.4 Endogenous plant hormones in apple leaves

10.4.1 IAA- and ABA-like substances in apple leaves

In discussion and interpretation of chromatographic results in the present data for plant hormone analysis, one should keep in mind that no conclusive identification can be drawn from a crude extraction with a limited number of purification steps, unless chemical-physical properties of plant hormones are analysed by GC-MS or infrared spectroscopy. The present results, therefore, provide one possible approach to solving the problem of undertaking a complete analysis of small quantities of plant hormones. Recently Hopping and Buckovac (1973) noted that one dimensional chromatographic results can easily mislead the interpretation of the pattern of plant hormones which exist. Klingstrom (1967) also pointed out that generally 50 or more substances can be suspected of having similar chromatographic properties.

There have been many studies seeking to determine the presence of auxin-like substances in apple tissues (Luckwill, 1952; Hancock and Barlow, 1953; Hatcher, 1959; Kato and Ito, 1962; Neumann, 1962; von Bargaen, 1960; Martin and Stahly, 1968; Hopping, 1968; Mel'nikov and Lebedev, 1971; IL'M, 1971).

Luckwill and Powell (1956) and Luckwill (1957) produced

evidence that IAA was absent from various tissues in apple trees e.g., leaves, seeds, apples. However Kenworthy and Harris (1961) characterized physiochemically the presence of IAA in apple tissues of Golden Delicious, but not other samples and varieties of apples. Tustin (1975) obtained a high content of IAA-like substances at Rf 0.2-0.4 chromatograms in apple stems of MM 106 rootstocks, representing 1.0 µg equivalent to IAA per dry weight of stems taken in early autumn. It must be emphasised, however, that the method of extraction and purification used should be considered when evaluating data about IAA-like substances reported in the literature.

After the extraction procedures were established as shown in Table 6, the apple leaves taken on the 12th of November were tested accordingly. The presence of auxin-like substances in the fractions of silica gel-celite column where ^{14}C -IAA peaked to 15 ml was clearly shown; 60% of ^{14}C -IAA was recovered at the same Rf as auxin-like substances from leaf extracts. However, the activities of auxin-like substances were not proportional to the amount of leaf extract assayed. In other words, the highest activities of auxin-like substances were demonstrated with 2.0 and 0.2 gram dry weight leaf samples, while 1.0 gram dry weight leaf samples gave little activity in both silica gel-celite columns and cellulose thin layer plates. Possibly interactions occurred between promoters and inhibitors or competition occurred between wheat and oat coleoptiles for the utilization of active components. In fact, Letham and Davison (personal communication) emphasised that 1 or 2 logarithmic dilutions of the extract should be bioassayed. It is technically difficult to fully replicate experiments concerned with hormone analysis but it is wise to make at least 1 or 2 dilutions of each sample which can then be bioassayed together with the full strength sample.

In column chromatography, two groups of auxin-like substances in the aliquot equivalent to 2.0 gram dry weight were found as shown in Fig. 21-C and possibly three groups in the 0.2 gram sample (Fig. 21-A). However this auxin activity could not be detected when the silica gel eluate was subjected to further cellulose thin layer chromatography, although activity could be found after TLC when the sample had not first been passed through a silica gel-celite column (see Fig. 26). The different kinds of auxin-like substances in apple leaves require further detailed experiments. Luckwill (1957) indicated there are at least three auxins in apple leaves and seeds. Hancock and Barlow (1953) also demonstrated that auxin-like substances in apple leaves varies according to leaf age, the apex and leaf primordia having high activity only at the Rf of IAA, the leaves at the 1st and 2nd nodes from the tip had slightly

less activity at this Rf while the leaves at the 7th and 8th nodes showed auxin activity only at Rf 0.8-1.0, and the leaves at the 15th and 16th nodes had auxin-like substances at Rf 0.0.-0.3 and Rf 0.8-1.0 but not at any other Rf. In the present studies the leaf samples were taken on the 12th of November and included several leaf ages.

It must be emphasised with the present studies that the oat coleoptile sections were able to detect an auxin-like substance in fractions directly from the silica gel-celite column, even though interaction or inhibition by other hormones may have been occurring. The growth of oat and wheat coleoptile sections at the 13th fraction is of interest because oat coleoptiles were stimulated while wheat coleoptiles were inhibited. In fact, the degree of interaction between IAA and ABA (inhibitor- β) was dependent on the concentrations of inhibitors (see Housley and Taylor, 1958; van Stenveninck, 1958; Robinson and Wareing, 1964). At the 13th fraction the oat coleoptile growth was significantly increased, representing 0.0875 μg equivalent to IAA, whereas the wheat coleoptile sections in the same tube indicated 0.01 μg of ABA equivalent per two gram dry weight. At least 0.1 μg of ABA per ml is required to significantly reduce the growth of oat coleoptile sections as shown in the ABA standard calibration (see Fig. 18-B). Although the oat coleoptile sections are not particularly sensitive to inhibitors, the inhibitors should be eliminated from the fractions to be bioassayed. Also it is surprising that in the 9th and 10th fractions the wheat coleoptile sections were stimulated but the oat coleoptile sections were not, possibly because of the presence of ABA-like substances. The phenomena suggest some mechanism involving competition of utilization of the active component at the low level. This may be due to comparative rates of elongation of oat and wheat coleoptile sections, wheat is faster than oats (Burström, 1973). It should be noted that slight interaction between different types of coleoptile section, although no significant difference was found (see Fig. 18).

There is a possibility of synergistic effects of IAA with GAs and antagonistic effects with inhibitors, as determined with the oat and wheat coleoptile sections, when the fractions directly collected from the silica gel-celite column were used. However, the oat and wheat coleoptile sections are only slightly sensitive to gibberellins as shown also by Thomas, Wareing and Robinson (1965) and Sirois (1966 and 1968). The activity of auxin-like substances at the 13th, 14th and 15th fractions are unlikely to be influenced by gibberellins, since firstly the bioassay is insensitive to gibberellins as mentioned above and secondly, gibberellins could not be detected using the dwarf rice and barley endosperm

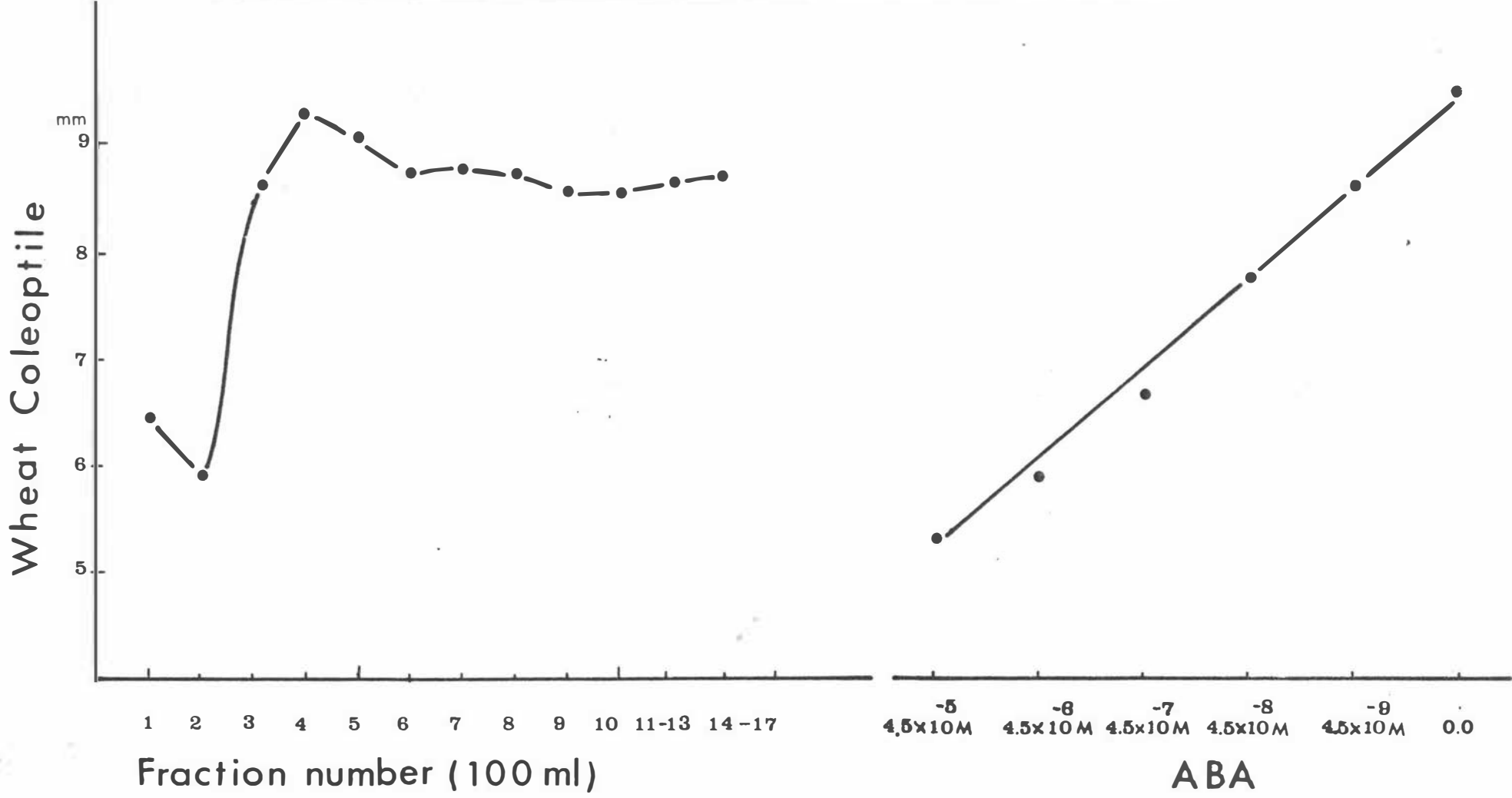


Fig.32. Fractionation of ABA in Sephadex G-10 column

bioassays (see Fig. 20-B₁ and 2). In the preliminary test for the fractionation of different gibberellins from the silica gel-celite column by GLC, the data was not clear due to interfering substances. However, in the results of Khalifah, Lewis, and Coggins (1965), GA₇ was eluted between GA₁ and GA₄, but no activity was found at that position in the present work using apple leaf extracts.

In the above section discussing the chromatography and bioassay of auxin-like substances, ABA was involved to a certain extent, therefore ABA-like substances are discussed briefly below.

In apple trees, ABA (inhibitor- β) has been demonstrated in various tissues, e.g., leaves (Luckwill, 1952, 1957; Luckwill and Powell, 1956; Pieniazek and Rudnicki, 1967; Penezek, 1971); shoots (Neumann, 1962); Seed (Rudnicki, Sinska, and Lewak, 1972); xylem sap (Davison, 1965); bark of EM IX, and EM XVI (Martin and Stahly, 1968); shoot tips (Hoad, Monselise, and Sparks, 1973). The ABA contents in the shoots, leaves and roots were used as the criteria for the degree of dwarfing of apple rootstocks (Yadava and Dayton, 1972).

The use of the pooled 1,000 ml fraction from Sephadex G-10 for the determination of IAA and GAs was decided by experimenting with ¹⁴C-IAA and by using the data of Crozier et al., (1968). However, there were no data available for fractionation of ABA from Sephadex G-10. Therefore the elution behaviour of ABA from the column was preliminarily tested and the results are shown in Fig. 32. Most of the ABA was eluted in the first 300 ml fraction, Therefore 1,000 ml which was eluted for auxin and GA-like substances would contain nearly 100% of ABA from the Sephadex G-10 column. Consequently the 1,000 ml fraction from Sephadex G-10 was used for the determination of ABA together with IAA and GA-like substances.

The presence of an ABA-like substance was clearly demonstrated by cellulose thin layer chromatography of the 11th, 12th and 13th fractions using the silica gel-celite column. The fractionation of ABA, as detected by GLC, was inconclusive and the elution behaviour of ABA from the column should be re-examined, if the ABA is eluted from the column near IAA as indicated by the co-existence of IAA and ABA at the 13th fraction in Fig. 21-C, then the first 230 ml fraction could be subjected to Sephadex LH-20 chromatography so that IAA and ABA can be clearly separated from each other (Eliasson, 1969). The separation of IAA and ABA from a Sephadex LH-20 column in a preliminary experiment was achieved, two main peaks occurring separated by 60 ml. In the preliminary test, the acidic ethyl acetate fraction from leaf samples

was subjected to Sephadex LH-20 chromatography followed by cellulose thin layer chromatography. Using the wheat coleoptile straight test, auxin-like substances at the IAA position and ABA-like substances at the Rf of ABA were clearly detected. Therefore it was intended that Sephadex LH-20 be used in the procedures listed in Table 6, but time was not available to do so.

10.4.2. Gibberellin-like substances

Because of difficulties in purification due to interfering substances in apple leaves, or the large quantity of sample required, examples of identification of GA-like substances in leaves are rare in the literature. In contrast the identification and activity of gibberellins such as GA₄, GA₇ and GA₉ from apple seeds is well documented (Tautvydas, 1965; Dennis and Nitsch, 1966; Luckwill and Whyte, 1968; Luckwill, Weaver and MacMillan, 1969; Sinska and Lewak, 1970; Smolenska and Lewak, 1971; Sinska, Lewak, Gaskin and MacMillan, 1973). The presence of gibberellins has been reported in the diffusate of apple shoots (Grauslund, 1971, 1972); xylem exudate (Jones and Lacey, 1968); the exudate of rootstocks (Ibrahim and Dana, 1971); while GA₄ and GA₇ activity in apple shoots has been determined by Kato and Ito (1962) and Muravev, Tyurina and Metlitskii (1972, 1973), while studying gravimorphic effects.

Tautvydas (1965) obtained a 36% increase in lettuce hypocotyl length over the controls (equivalent to 0.003 µg GA₃) from the acidic aqueous phase of 80 mg fresh weight of apple leaf samples chromatographed with ethyl acetate: n-hexane (75 : 25). He could not fully evaluate his results because of impurities, as more than 80 mg of leaf sample inhibited lettuce hypocotyl growth. Hopping (1968) found some differences in gibberellin activity in apple shoot tips between Alar treatment and control using the barley endosperm test and using the extraction procedure of Kende and Lang (1964) and silica gel thin layer chromatography. He obtained high reducing sugar levels in response to gibberellin activity from the Rf 0.0-0.1 in silica gel thin layer in spite of problems of interfering substances. Experiments on endogenous plant hormones in apple leaves were reported to be in progress (Hoad, Weaver and Biddington, 1968), but as yet there are no reports from them. Most recently no difference in gibberellin contents was found in spur leaves between fruiting and non-fruiting trees, on extracting auxins and gibberellins with dichloromethane with buffer solution (pH 4.0), and using twice-run paper chromatography in order to remove phlorizin (Lacey and Fulford, 1974).

In the present studies, at least 4-5 gibberellin-like substances were detected by Tan-ginbozu dwarf rice seedlings after silica gel-celite column chromatography and two kinds of gibberellin-like substances from thin layer chromatography before subjecting to the column chromatography. Unfortunately the gibberellin-like substances in cellulose thin layer chromatography of the material eluted from the column showed only one kind of gibberellin, possibly due to the fungal contamination observed during bioassay. In the preliminary test with cellulose thin layer chromatography, some gibberellin activities were found. Therefore the disappearance of some gibberellin activity in cellulose thin layer chromatography should be assessed in further work.

One remarkable aspect was the high gibberellin activity exhibited in the fractions after adding MeOH to the column in two gradient solvent systems, that is at the 38th to 40th fractions in the solvent system A, chloroform, ethyl acetate and MeOH, and at the 67th to 69th in the solvent system B, n-hexane, diethyl ether, ethyl acetate and Methanol. The gibberellins fractionated by the column were not intensively studied, although one preliminary experiment using GLC was unsuccessful, due to high levels of contaminants. Regarding the activity of individual fractions one can refer to the work of Khalifah, Lewis and Coggins (1965), keeping a few things in mind. Although the techniques were somewhat different it would seem that one of the GA-like substances was GA_3 when the results were compared to those of Khalifah et al. and also because of its behaviour in cellular TLC. After TLC gibberellin activity was found at R_f 0.2-0.3 (Fig. 22-C 1-2), but also at 0.0-0.1 (Fig. 22-E), possibly due to over-loading. Alternatively the activity is not due to GA_3 but to other kinds of GA, on the basis of the survey by Murakami (1970) of 86 species using the microdrop method for the dwarf rice bioassay, although data for Malus sp. were not reported. Murakami (loc. cit.) believed that much of the activity found that most of the high activities by the Tan-ginbozu dwarf assay at the R_f of GA_3 is not due to GA_3 but possibly due to GA_{19} in many species. However, in the present studies, the fractions had high activity in the barley endosperm test which is insensitive to GA_{19} (Crozier, Kuo, Durley and Pharis, 1970). Activity detected by the barley endosperm test could possibly be attributed to GA_1 , GA_2 , GA_{22} or GA_{23} . Of the gibberellins mentioned above, GA_1 might be one which showed activity in the fraction, if it is not due to GA_3 . Khalifah et al., (1965) demonstrated that GA_1 was eluted in the 50-90 ml fraction from the silica gel-celite column but in a preliminary test in the present work, using GLC after column chromatography, GA_9 seemed to be

eluted in the position of the GA₁ fractions obtained by Khalifah et al., (1965), although the peak of GA₉ was contaminated. Therefore, the fractionation of GAs from the column must be re-examined.

The activities of gibberellin-like substances at the fractions mentioned above coincided with a region of intense coloured components which severely inhibited the dwarf rice seedlings. When test tubes were used in the dwarf rice bioassay, the coloured components were observed along the inside surface of the tubes. The upward movement of coloured components might help to reduce the amount of inhibitors so that the dwarf rice seedlings could be promoted by gibberellin-like substances. In spite of no roots, the first leaf sheath of the dwarf rice seedlings was stimulated by fraction 41 after chromatography of 5 gram dry weight leaf samples, whereas the seedlings were all dead at the 42nd and 43rd fractions. This also occurred with the 1 gram dry weight leaf samples in the solvent system A. The dwarf rice seedlings had a stiffness in their tissues, possibly being due to a change of carbohydrate metabolism which could indicate the presence of gibberellins (Vlitos, 1974). The phenomena have been observed in peduncle of grape and cherry trees when dipped and drenched with gibberellins (Park, 1961, 1965). Because of inhibitors present, accurate quantitative analysis could not be obtained in the present studies. It is clear that the inhibitor must be removed before bioassay but, on the other hand, is there some synergistic factor involved in the same fractions as well as inhibitors? Even phlorizin which is known to be inhibitory (Grochowska, 1963), can, at low concentrations, be synergistic with IAA in the Avena coleoptile bioassay (Grochowska, 1966). This phenomenon was also found with gibberellins in the lettuce hypocotyl bioassay (Tautvydas, 1965). Jones, Hatfield, Patterson and Hopgood (1972) studying the interaction of sap factors with IAA in oat reported a similar synergistic response of isolated shoots to phloroglucinol. With the dwarf rice bioassay, this question is now open for further investigation, although it is unlikely that the fractions with which dwarf rice seedlings were promoted would contain phlorizin, because most phenolic compounds remained in the ethyl acetate from which carboxylic moieties were removed by sodium bicarbonate (Powell, 1964; Reid and Carr, 1967; Bayer, 1969). Stimulation of dwarf rice seedlings after column chromatography was also observed at the 28th-35th fractions using solvent system A. The activity may possibly be the gibberellin-like substances at the 44th and 48th fractions in the solvent system B (see Fig. 20-A₃ and B₂).

The promoting activities of those fractions might be due to GA₅, on the basis of the work of Khalifah et al., (1965). In co-chromatography by GLC (see Fig. 25) there are three small peaks near the

retention time of GA_4 , the third small peak from the retention time of GA_4 might be a peak of GA_5 in consideration of the retention time of GA_5 in the preliminary test using GLC. There is slight activity of gibberellin in the Rf 0.5-0.6 in cellulose thin layer chromatography (see Fig. 22-E₁). The activity could be both GA_4 and GA_5 or just GA_4 . GA_4 and GA_5 might be separated from each other in the cellulose thin layer as shown in Fig. 28-A or not separated as shown in Fig. 28-B. In the barley endosperm test, the activity was low suggesting that the gibberellin may be GA_5 . The reason is that when the concentration of GA_5 is very high, then the response of barley endosperm test is high, but the response falls off very rapidly at lower concentrations (Crozier, Kuo, Durley and Pharis, 1970).

The activities in the 18th and 20th fractions in the solvent system A, and the 27th and 29th fractions in the solvent system B might be due to GA_4 (Khalifah et al., 1965). In fact the small peak in GLC (see Fig. 25-A, B, C; GA_4) was at the retention time of GA_4 . In cellulose thin layer the GA_4 or GA_5 -like substances occurred at Rf 0.5-0.6 in Fig. 22-E.

Activity was also detected at the fraction from the 5th-10th in the solvent system A and at the 14th-15th fractions in the solvent system B (see Fig. 20-A₃, B₂). In those fractions, there are several things to be considered concerning the activity shown by dwarf rice seedlings, if we consider 10% growth over controls to be due to gibberellins (Murakami, 1968). Firstly, GA_1 might be responsible for the activity (see Khalifah et al., 1965) but then one would expect activity in the barley endosperm test; no activity was found (see Fig. 23-A₁ and B₁). GA_1 was eluted in nearly the same fractions as GA_3 , using partition chromatography (Dennis and Nitsch, 1966; Powell and Tautvydas, 1967). Secondly, the polar gibberellin GA_9 might be responsible for the activity, considering the elution behaviour in the partition column chromatography as mentioned above, possible detection by GLC in the present preliminary test, no activity in the barley endosperm test, and the behaviour in cellulose thin layer chromatography using the solvent system MAAW (see Fig. 28-A and B Rf 0.87-0.92 and 0.8-0.9 respectively). However, it should be remembered that GA_9 might be partitioned slightly into petroleum ether under acidic conditions (see Durley et al., 1972).

Small peaks appeared at the 50th fraction in solvent system A and at the 90th and 91st fractions in solvent system B. The gibberellin-like substance in these fractions was eluted by 80% MeOH in ethyl acetate in both the solvent systems (see Fig. 13 and 15). As indicated

in the available references on GAs in the silica gel-celite column chromatography (Khalifah et al., 1965), it may be a GA₈-like substance.

Unfortunately cellulose thin layer chromatography had been unsuccessful for some reason or other. It should not be concluded that cellulose thin layer chromatography is not suitable.

Nevertheless, in spite of the high content of inhibitors in the extract, the technique clearly shows the existence of gibberellin-like substances, although proportional activities could not be obtained with a diluted concentration of plant extract.

The aliquots of each fraction were tested simultaneously using both the α -amylase and glucose test and using separate test tubes in the system A silica gel-celite (see 8.4.1.4.2. and 9.4.2.1.). It was surprising to see high activities of α -amylase in all fractions, although changes in intensity were similar to the glucose test. At one stage of experimentation with α -amylase test, a different source of potato powder brought about the same problem. No attempt was made to find out the reason and thereafter use of the α -amylase test was abandoned. One thing to mention here about the barley endosperm test is that in the system B silica gel-celite column the activity in the glucose test was low as compared with the activity of fractions from the system A silica gel-celite column (see also Crozier, Kuo, Durley and Pharis, 1970).

10.4.3. Cytokinin-like substances

A rich source of cytokinin-like substances in apple fruit-lets was investigated by several workers (Goldacre and Bottomley, 1959; Bottomley, Goldacre and Kefford, 1960; Bottomley, Kefford, Zwar and Goldacre, 1963; Zwar, Bottomley and Kefford, 1963; Zwar, Bruce, Bottomley and Kefford, 1964; Letham, 1963; Letham, 1969; Letham and William, 1969; Zwar and Bruce, 1970). Zeatin has been found in apple shoots (Letham and William, 1969) and cytokinin activity in apple xylem sap (Jones, 1965; Luckwill and Whyte, 1968), was mainly zeatin-riboside (Jones, 1973).

However, the presence of cytokinins in apple leaves is rarely reported. Probably it is difficult to purify leaf samples in order to obtain cytokinin activity, as was the case with gibberellins (see 10.4.2).

In the present studies, however, it is clearly demonstrated, using Sephadex LH-20, cellulose TLC and the radish cotyledon bioassay, that there are several groups of cytokinin-like substances in the apple leaves.

In studying the activity of cytokinins in apple leaves, there are several problems if column and DEAE cellulose thin layer chromatography are used. For example, zeatin-like substance in the fractions

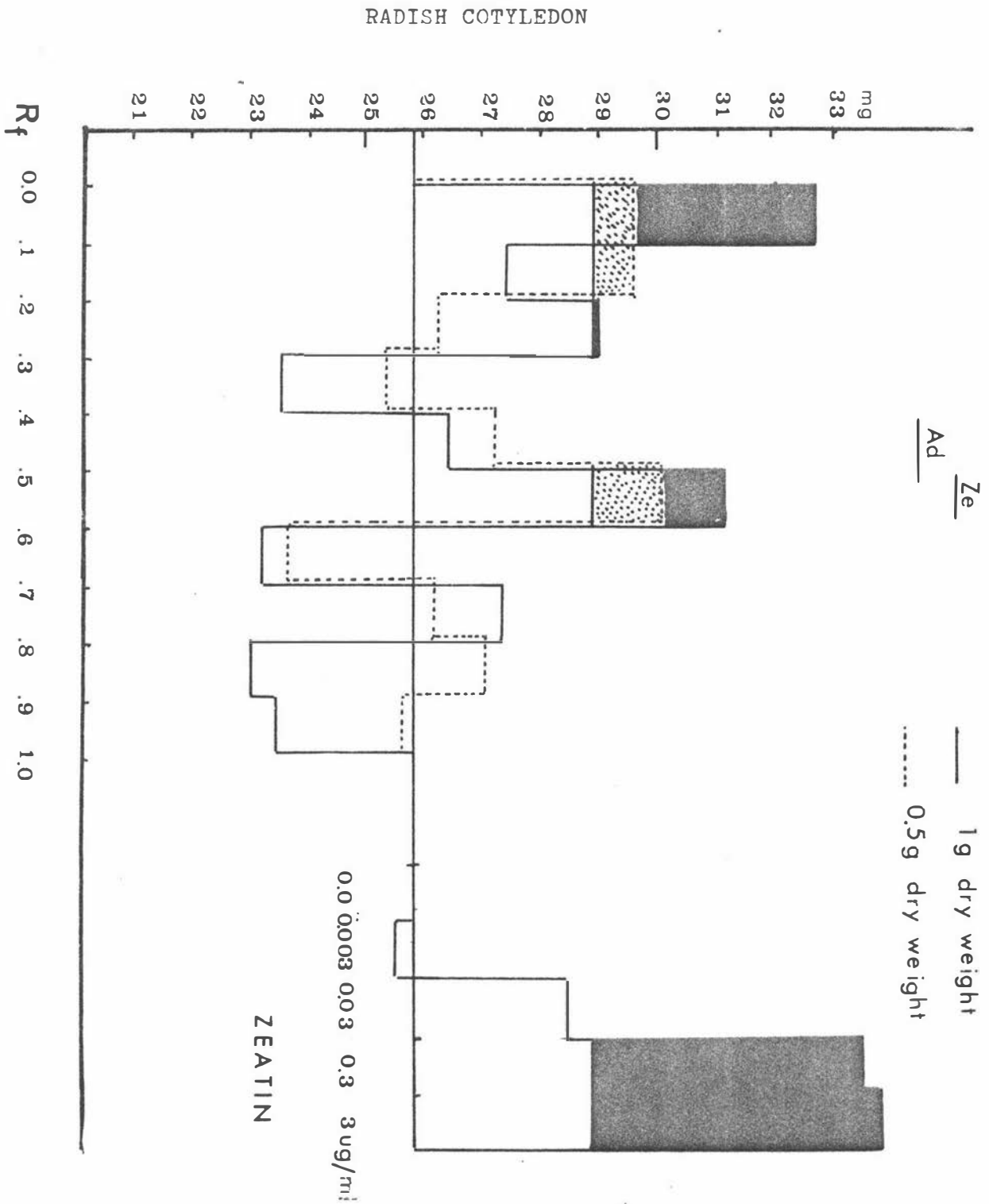


Fig.33. Cytokinin-like activity in the shoot tips of apple variety Sturmer Pippin (Silica gel TLC developed by water saturated butanol with two replicates)

where ^3H -zeatin was eluted were not detected, whether zeatin was present or not, because of severe inhibition in the fractions, whereas some evidence of the presence of zeatin could be seen in DEAE cellulose thin layer chromatography. However it is not possible to clearly distinguish between zeatin and zeatin-riboside because both slightly overlapped each other. In addition, with 5 gram dry weight of leaf sample the plate may be overloaded so that one could not detect any activity of zeatin and zeatin-riboside, although some evidence of their presence is shown with 1.0 and 0.5 gram dry weight of leaf sample. This overloading problem also could be seen in the activities of Group VI which were developed on DEAE cellulose thin layer. The activities were spread over almost all Rfs with the 5 gram dry weight sample, whereas only one zone of activity at Rf 0.2-0.3 was exhibited with the one gram dry weight sample. It is of interest to note that with Group V cytokinins inhibition of radish cotyledons did not occur in the 5 ml fractions eluted from the column with even 5 gram dry weight of leaf samples, whereas there was severe inhibition with the 5 gram dry weight samples in DEAE cellulose thin layer chromatography, although it is clearly demonstrated that there are three groups of cytokinin activities with 0.5 gram dry weight of leaf sample.

In the present studies cytokinin activity was only detected by radish cotyledon bioassays of a single chromatogram. According to general observation through all the experiments with the radish cotyledon bioassay, the bioassay is very rapid, easy to handle, and requires only a short period to obtain the results. However, in order to confirm activity another bioassay, such as soybean bioassay should be used and also a different solvent system in paper or thin layer chromatography. In addition, cytokinins in the fractions can be detected by GLC and GC-MS techniques.

In the preliminary experiment with the shoot tips of apple (See Fig. 33), variety Sturmer Pippin (5 cm length), a positive response of radish cotyledons occurred at three different Rfs in the butanol soluble fractions purified on a Sephadex G-10 column and on silica gel GF-254 thin layer chromatography. In the apple shoot tips a cytokinin active substance appeared at the Rf position of zeatin. When zeatin-riboside was available, the Rf of zeatin-riboside was tested on silica gel GF-254 thin layer developed by water saturated in n-butanol. Zeatin-riboside was found at Rf 0.5-0.58, zeatin at Rf 0.58-0.65, and 6(α , γ -dimethylallylamino)purine at Rf 0.75-0.84. Therefore cytokinin activity at Rf zeatin position is unlikely to be zeatin-riboside. However it must be confirmed whether the activity is due to a single substance or a complex (Hewett and Wareing, 1973b). In fact, zeatin was detected in

stems by Letham and William (1969). In apple tips, Friedrich, Heijmova and Chvojka (1972) also found two compounds with cytokinin activity in their bioassay using thin layer chromatography with three different solvent systems. The other highly active cytokinin at Rf 0.0-0.1 is rather more non-polar than zeatin. It is very difficult to compare with a known cytokinin. Similarly, the cytokinins detected by radish cotyledons from the apple leaves cannot be compared with a known cytokinin, because a limited number of known cytokinins were fractionated from the column. According to the reference on the elution behaviour of cytokinins (see Table 13), it is possible to assume the removal of 6(δ , γ -dimethylallylamino)purine, zeatin, and zeatin-riboside from the Sephadex LH-20 column in the Group 1 and 2. Beyond this, for other groups such as III, IV, V, and VI, the cytokinin activities have not yet been identified. However, Hewett and Wareing (1973b and a) provided some evidence that there are different kinds of cytokinins present in the different aged leaves of Populus x robusta Schneid. They obtained at least 6 kinds of cytokinin activity in expanding leaves, probably only two kinds of cytokinin in apices, and at least 4 kinds of cytokinin activity in fully expanded upper leaves. The phenomena with auxin-like substances are already been discussed for auxin-like substance in apple leaves (Hancock and Barlow, 1953) (see 10.4.1.). In the present studies, the time of sampling the spur leaves on the 12th of November covered various stages of apple leaf development so that there is a possibility that they could exhibit many different kinds of cytokinins. The present data are therefore, supported by the result of Hewett and Wareing (1973d).

It must be emphasised, however, that the method of extraction and purification used should be considered when evaluating data about cytokinin-activity reported in the literature. A complicated extraction procedure may cause some results to be artefacts of the procedure used. The strong acids during some extraction steps may have changed the chemical identity of cytokinins without destroying their activity in bioassay (Zwar, Bruce, Bottomley and Kefford, 1964). Cytokinin activities have been increased by acidic hydrolysis of certain plant extracts (Kende, 1965; Gazit and Blumenfeld, 1970). A free form of cytokinin may occur during passage through cation exchange column (Tegley, Witham, Krasnuk, 1971). Despite the occurrence of zeatin and zeatin-riboside in maize milk, plum and apple fruitlet (Letham, 1966b, 1968; Miller, 1965, 1967a; Letham and Miller, 1965), Zwar and Bruce (1970) reported that zeatin in apple fruitlets was derived from the acidic treatment in the procedure. Furthermore they could not obtain

zeatin-riboside or zeatin ribotide by treating with lanthanum chloride giving some evidence that it was a free base.

It must be mentioned here that only the 16th to 86th fractions eluted from the Sephadex LH-20 column were tested. Further fractions may have cytokinin activity as mentioned by Hewett and Wareing (1973b).

10.5. GLC of authentic plant hormones and plant materials

As already reviewed in the literature for the determination of plant hormones by GLC, recently there have been a number of reports with promising data on the use of GLC to evaluate plant hormones. The idea was to use GLC to reduce the number of purification steps. But after experience with GLC, it was realized that it was necessary to purify plant materials with much more before injection for GLC. There are a number of successful instances of GLC for the determination and identification of several plant hormones from non-photosynthetic materials such as immature seeds, young stage of fruits, cotton ovules, and xylem saps. However, it is not so easy to eliminate interfering substances from photosynthetic materials. One of the biggest problems would be the presence of an interfering substance near the retention time of known hormones because plant hormones exist at exceedingly low concentration in the plant tissue. In the present studies of plant materials in GLC, it was not possible to definitely identify plant hormones by their retention time of GA₄, GA₅, and ABA. In the preliminary test with the 160-200°C temperature programme, using the same methylated plant materials used in Fig. 25, there seemed to be an IAA-like substance very close to the retention time of IAA, but it could not definitely be concluded because there were many interfering peaks present.

In the standard calibration of several authentic plant hormones (see 9.5.2.) it was possible to detect most gibberellins at a concentration of 2.5 ng/ul using low attenuation. However one of the problems would be the presence of interfering component, when plant extracts are used. That is at least 50 µl of silylating reagent is required to make ME-TMS derivatives. If 5 µl are required to be injected onto the GLC column, it means that this volume would contain proportionally more of other plant components which could interfere with the detection of the small quantities of plant hormones present.

In the present studies, cellulose thin layer chromatography was used for further purification after column chromatography. It was also thought to be necessary to further purify the plant extract when it was subjected to GLC. Therefore authentic plant hormones, after

cellulose thin layer chromatography, were tested by GLC. Rfs of authentic plant hormones varied from one plate to the other. The solvent system MAAW was good to use for cellulose thin layer but the question of reproducibility, together with overloading the sample in the cellulose thin layer, needs further investigation. For example, when cellulose powder was washed with 2% EDTA and other organic solvents, the Rf of IAA was moved upward and one lost the compact spot.

Bis-(trimethylsilyl)trifluoroacetamide (BSTFA) has been intensively evaluated for making protein amino acid trimethylsilyl derivatives (Gehrke, Nakamoto and Zumwalt, 1969) and for purine and pyrimidine bases (Gehrke and Lakings, 1971). In the present studies, an excellent improved peak was obtained for GA₃, IAA, and IPA when this silylating agent was used. There was also a slightly improved peak with GA₄ and GA₁. Especially IAA and IPA exhibited 4 times greater peak height than that of ME-derivatives. It means that it was possible to detect down to 2.5 ng GAs (see Fig. 27). Possibly ME-TMS IAA using BSTFA and TMSCl silyl reagents could be comparable to the heptafluorobutyl derivative of IAA (Powell, 1972), if the GLC conditions are optimized. It was now possible to get a single peak for GA₃ so that now the retention times of all the available gibberellins could be studied simultaneously. Unfortunately the ABA peak was reduced by half, probably due to decomposition during GLC, since a small peak occurred with a retention time of 14.3. Also the GA₉ peak was reduced by 20% and a small peak appeared with a retention time of 18.5.

10.6 General discussion in conjunction with further work and conclusion

There is a vast literature dealing with the identification and determination of plant hormones. However, no literature is available which outlines a reasonably quick method of analysing plant hormones as a whole for interpretation of physiological and biochemical phenomena in terms of $\frac{\text{Cks}}{\text{Axs}} \text{ (Ibs)}$, $\frac{\text{GAs}}{\text{Axs}} \text{ (Cks)}$, or $\frac{\text{Cks}}{\text{Axs}} \text{ (Ibs)}$, as proposed in Fig. 1 and 2.

Most published papers deal with one or possibly two hormonal groups in an attempt to obtain at least a partial answer to a specific question. Furthermore photosynthetic tissue is extremely difficult to purify in order to remove interfering substance before bioassays or GLC. Powell (1972) discussed the evolution of plant hormone analysis and listed various new techniques, but as yet many problems remain to be solved.

In the present studies, potentially useful methods were selected in an attempt to analyze four series of plant hormones. The

selection was based mainly on reports in the literature.

80% MeOH appears to be one of the best solvents for initial extraction from lyophilized plant materials. However, further investigations are required to determine whether most plant materials could be completely extracted at low temperature such as -20°C (Ellicott and Stowe, 1970) without any artefacts or liberation from bound form, especially for IAA-like substances. If IAA is not released from bound compounds then extraction periods could be extended to obtain complete extraction. The addition of dimethyldithiocarbamate in MeOH may be essential to prevent the activity of certain enzyme systems, such as IAA-oxidase and polyphenoloxidase, either during the grinding or extraction process. A low pH may be considered when MeOH is used for the initial extraction in order to prevent possible enzymic degradation (Coombe and Tate, 1972) or to prevent the conversion of tryptophan to IAA (Gordon and Paleg, 1961). When petroleum ether is used after centrifugation the pH should be 6.5 or higher so that GA_9 is not partitioned into the petroleum ether (Durley and Pharis, 1972). Petroleum ether may also partition some cytokinin activity so that it must be used with caution (van Staden, 1973) and it should be checked whether some cytokinin activity can be transferred back into the water phase when back washing is employed. It may also be useful to bioassay the petroleum ether fraction to obtain an estimate of the inhibitors present.

^{14}C -IAA in the aqueous phase after removal of MeOH was not destroyed when stored overnight at $1-2^{\circ}\text{C}$ in the refrigerator so that possibly phlorizin can be precipitated in the aqueous solution (Tautvydas, 1965). However, the degree of precipitation of phlorizin was not determined and further investigations are required to find out the degree of precipitation of phlorizin, compared with the precipitation of phlorizin using cold water after ethyl acetate extraction from the aqueous phase at pH 2.5 (Tautvydas, 1965).

After the aqueous phase is partitioned to ethyl acetate (see M-8 ethyl acetate extract in Table 6), aliquots of the aqueous phase could be partitioned with n-butanol so that butanol-soluble gibberellins could be obtained from the aqueous phase (Coombe and Tate, 1972; Naito, Inoue and Bukovac, 1972). The reason why aliquots of the aqueous phase at pH 2.5 should be used is that three times of butanol partition at pH 2.5 could remove at least 20% of cytokinin-like substances from the acidic aqueous phase. (See Table 9).

Polyvinylpyrrolidone (PVP) column chromatography would seem to be a useful purification step, because not only are phenolic compounds removed and possibly other inhibiting components, but also separation of different plant hormones can be achieved (Glenn, Kuo, Durley

and Pharis, 1972). That is the first large fraction eluted from PVP at pH 8.0, can be partitioned for gibberellins and ABA and the second large fraction partitioned for IAA. Thus no interference from ABA should occur when the residue eluted from the PVP column is subjected to silica gel-celite column and possibly inhibition of coleoptile sections will not occur at the 13th fraction (see Fig. 21-C).

For further purification of auxin-containing fractions from silica gel-celite eluted with solvent system A, the residue could be chromatographed on Sephadex LH-20 column without using thin layer chromatography, because only 50% ^{14}C -IAA recovery was obtained (see Fig. 16). The residue can be subjected to GLC or bioassays. In bioassay the combined oat and wheat coleoptile sections could be used for each fraction. Furthermore, oat internode sections which are particularly sensitive to IAA could be used together with the combined oat and wheat bioassay in the same specimen vials. Because oat internode sections can detect 5×10^{-8} M IAA and are possibly free from gibberellins, much information and accurate determination of IAA could be obtained by this trio-combined bioassay.

In the case of gibberellin in the first fraction eluted from the PVP column, the residue could be chromatographed on a silica gel-celite column and then determined by the Tan-ginbozu dwarf rice bioassays using glass tubes or the micro drop method (Murakami, 1968). The aliquots could be tested with the barley endosperm bioassay (Coombe, 1971) using the glucose test and each fraction could also be subjected to GLC, using BSTFA and TMCS silyl derivatives.

For GLC, further investigations are required to select a stationary phase such as OV-17 which can provide much more discrimination of interfering substance than QF-1 or SE-30 (Coombe and Tate, 1972). There is a number of purification methods reported which must be tested to establish their suitability for use with apple tissues. If further purification is required paper or thin layer chromatography could be employed.

For the determination of cytokinin activities, after ethyl acetate partitioning of the acidic aqueous phase, the aqueous phase should be partitioned against n-butanol at pH 8.0-8.3 so that some purple coloured compounds will remain in the aqueous phase which will also contain zeatin-ribotide. If ethyl acetate is used however, van Staden (1973) found that much zeatin-like activity would enter the ethyl acetate phase. In fact 4.5% ^3H -zeatin partitioned into the ethyl acetate at the pH 2.5 in the present studies, and there are several reports that zeatin (Woolley, 1971; Hemberg, 1973; Letham, 1974b) are transferred into the ethyl acetate phase under acidic conditions.

The fraction of zeatin in the ethyl acetate phase could be removed by back washing into the aqueous phase. However, ethyl acetate can remove 6(α , β -dimethylallylamino)purine at pH 2.5 and therefore caution must be exercised if ethyl acetate is used (Hemberg, 1974; Letham, 1974b) The slight cytokinin activity at the fractions where 6(α , β -dimethylallylamino)purine would elute cannot conclusively give evidence, but if the activity is due to 6(α , β -dimethylallylamino) purine it could be transferred back to the aqueous phase in some degree by the back washing, together with zeatin-like substances which remained in the ethyl acetate phase (see M-9 in Table 6 and 9.2.1.2.), although the presence and behaviour of 6(α , β -dimethylallylamino)purine should be confirmed.

With the apple shoot tips high cytokinin activity was obtained after elution from a Sephadex G-10 column, however there is inhibition at three Rf's from TLC using the radish cotyledon bioassay, which in particular would interfere with the detection of 6(α , β -dimethylallylamino)purine at Rf 0.75-0.84. There are still problems with some inhibitors even after purification on Sephadex G-10 followed by silica gel TLC, of apple shoot extracts as mentioned above, or with Sephadex LH-20 followed by DEAE cellulose TLC for apple leaf extracts. Therefore purification with polyvinylpyrrolidone column may be useful to eliminate some components which might inhibit bioassays (Glenn, Kuo, Durley and Pharis, 1972), after or before subjecting to Sephadex G-10 or Sephadex LH-20. Sephadex LH-20 chromatography separated cytokinin activity into several groups and could be used in conjunction with thin layer chromatography. GLC of the fractions should also be possible using BSFA followed by TMGS.

The purpose of the present studies was not to study methodology but without the appropriate techniques and careful purification of plant materials the results cannot be conclusive. Therefore it was attempted to establish the purification of apple leaves and determine the ratio of plant hormones under various experimental conditions. In spite of severe inhibition in some fractions, positive results were obtained and it may be that meaningful ratios can be calculated from the data.

In most cases leaf extracts were diluted to give two or three different concentrations. Therefore the data were assessed by taking the average of the aliquots. There were many problems in evaluating data in the present studies because of inhibitors present and unexpected obstacles.

Ratio		B $\mu\text{g}/\text{kg}$ Dry Weight				
$\frac{B}{A}$		Auxins (Axs)	Inhibitors (Ibs)	Gibberellins (GAs)	Cytokinins (Cks)	Total Relative Plant Hormone Index (TRPHI)
		373.0	100.4	179.0	473.6	
A	Auxins 373.0		0.269	0.48	0.788	
	Inhibitors 100.4	3.72	-	1.78	4.72	
	Gibberellins 179.0	2.08	0.56	-	2.65	
	Cytokinins 473.6	0.788	0.21	0.38	-	
Total ratio (RPHI)		6.59 (RAAI)	1.039 (RIAI)	2.64 (RGAI)	8.158 (RCAI)	18.43 (TRPHI)

Table 22. An example of the use of Relative Plant Hormone Indices (RPHI) to represent the balance of plant hormones; in this case apple leaves taken on the 12th of November.

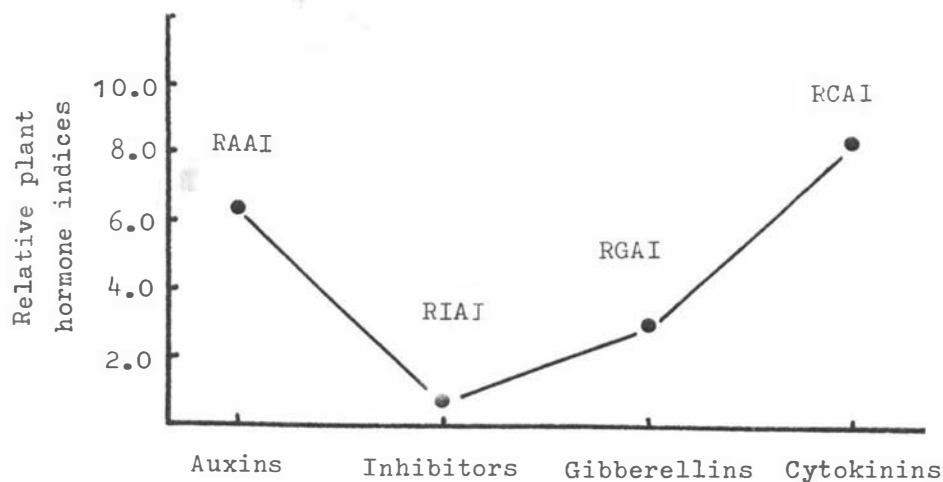


Fig. 34. A Relative Plant Hormone Indices in the apple leaves taken on the 12th of November.

Therefore, values obtained by bioassays from only thin layer chromatography were utilized to construct a tentative scheme based on hormonal ratios as shown in Fig. 1 and 2. It should be remembered that considerable loss of plant hormones occurs during purification and that inhibitory substances were not clearly separated from promoters.

Four series of plant hormones from apple leaf samples were calculated in terms of IAA, ABA, GA₃ and zeatin equivalents.

The values are from thin layer chromatography results except for gibberellins, where the values originate from the aliquots equivalent to 3 gram dry weight leaf sample using the system B silica gel-celite column (see Table 19) and are shown in Table 22.

Each ratio in Table 22 (e.g., the ratio $\frac{Axs}{Ibs} = \frac{373.0}{100.4} = 3.72$) can aid the understanding of some physiological phenomena. However, plant growth and development phenomena are probably controlled by complex interaction between many plant hormones and therefore the term Relative Plant Hormone Index (RPHI) is used in Table 23. Relative Auxin Activity Index (RAAI) represents a total ratio of $\frac{Axs}{ABA} + \frac{Axs}{GAs} + \frac{Axs}{CKs}$ for the auxin index column in Table 22. A Relative Inhibitor Activity Index (RIAI), giving a total ratio of $\frac{Ibs}{GAs} + \frac{Ibs}{GAs} + \frac{Ibs}{CKs}$ for inhibitors in the Ibs column, a Relative Gibberellin Activity Index (RGAI) for GAs in the GAs column, and a Relative Cytokinin Activity Index (RCAI) for cytokinins in the Cks column in Table 22. The values obtained are Relative Auxin Activity Index, 6.59; Relative Inhibitor Activity Index, 1.04; Relative Gibberellin Activity Index, 2.64; and Relative Cks Activity Index, 8.16. A high ratio indicates a predominant content of that plant hormone against the other plant hormone in the tissue.

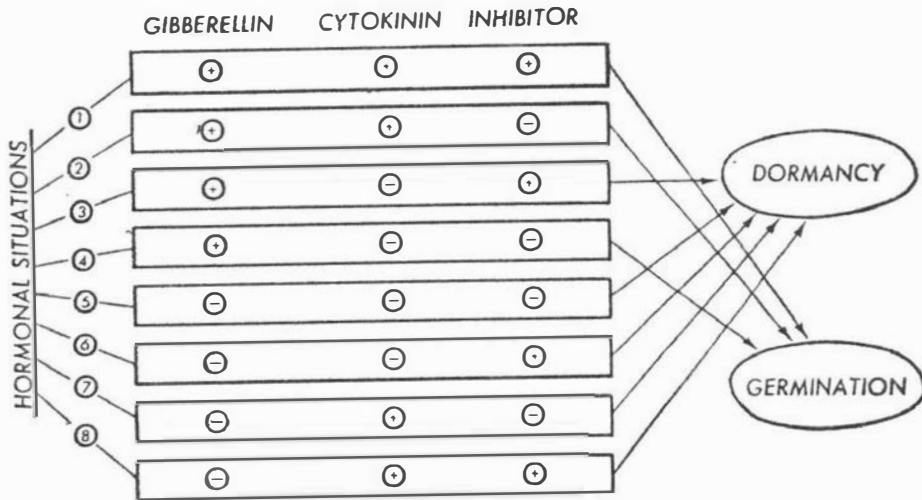
In the interpretation of Relative Plant Hormone Activity Index, as shown in Fig. 34, one would conclude that probably the most dominant growth substance in the leaf sample at the 12th of November was cytokinin, then auxin-like substance and thirdly gibberellins. The inhibitory activity was relatively low at that time.

A Relative Plant Hormone Index is proposed to represent the postulate that: "Complex growth and development response are rarely due to one type of hormone but to interactions between two or more growth substances. In many cases growth processes are probably controlled by all three growth promoters and growth inhibitors". The Relative Plant Hormone Index is derived from the total of each ratio of one plant hormone to the other three groups of plant hormones and is thought of as representing the interactions of that hormone in the tissue at a given time (see Table 22 and Fig. 34). Therefore this index possibly gives an interpretation of the background level of hormones as described by Shein and Jackson (1971) and Woolley and Wareing (1972a & b) in apical dominance (see Part I, 2.6. apical dominance). Other physiological phenomena are interpreted by a balance hormone theory in the speculative flow chart of Fig. 1 and 2, e.g. bud burst, shoot growth and flowering (see part I. section 5.4. conclusion in summary of discussion).

Because of difficulties of analysis of plant hormones, most hormone balance theories are only speculative. However, many physiological phenomena are studied by exogenous application of plant hormones. Thimman (1966) summarised several physiological phenomena in the integration of plant growth hormones e.g. geotropism and phototropism, the formation of parthenocarpic fruits, abscission, the initiation of lateral roots, germination of lettuce or tobacco seed, bud development in tissue culture, apical dominance and xylem and phloem production. However, caution should be used in interpreting data obtained by exogenous plant hormones as follows: (1) Presence or absence of endogenous plant hormones may affect the response to the exogenous hormone (e.g. supra-optimal responses). (2) The hormone may not reach the target area, either not penetrating or not being transported. (3) The concentration of exogenous hormones may be sub or supra-optimal. (4) The response may vary depending on species, type and age of tissues.

Little data are available concerning the balance of endogenous plant hormones. However, a number of examples are available, based on the use of exogenous plant hormones, which can be used to examine the usefulness of the Relative Plant Hormone Index concept. For example, Khan (1971) has investigated the interaction between ABA, GA₃ and kinetin in controlling lettuce seed germination.

The data of Khan (1971) relate only to the presence or absence of the hormone rather than to a continuously variable gradient, but the data can be represented by RPHI concept which may also indicate the predominant hormonal effect, keeping in mind that a Relative Plant Hormone Activity Index is not based simply on the presence or absence of



C. A model for the hormonal mechanisms of seed dormancy and germination using gibberellin, cytokinin, and inhibitor. It shows eight hormonal or physiological situations likely to be found in seeds. Presence of any one type of hormone at physiologically active concentrations is designated by the plus sign and its absence by the minus sign. See text for details. Adapted from table 3 in Khan and Waters (61). (After Khan, 1971)

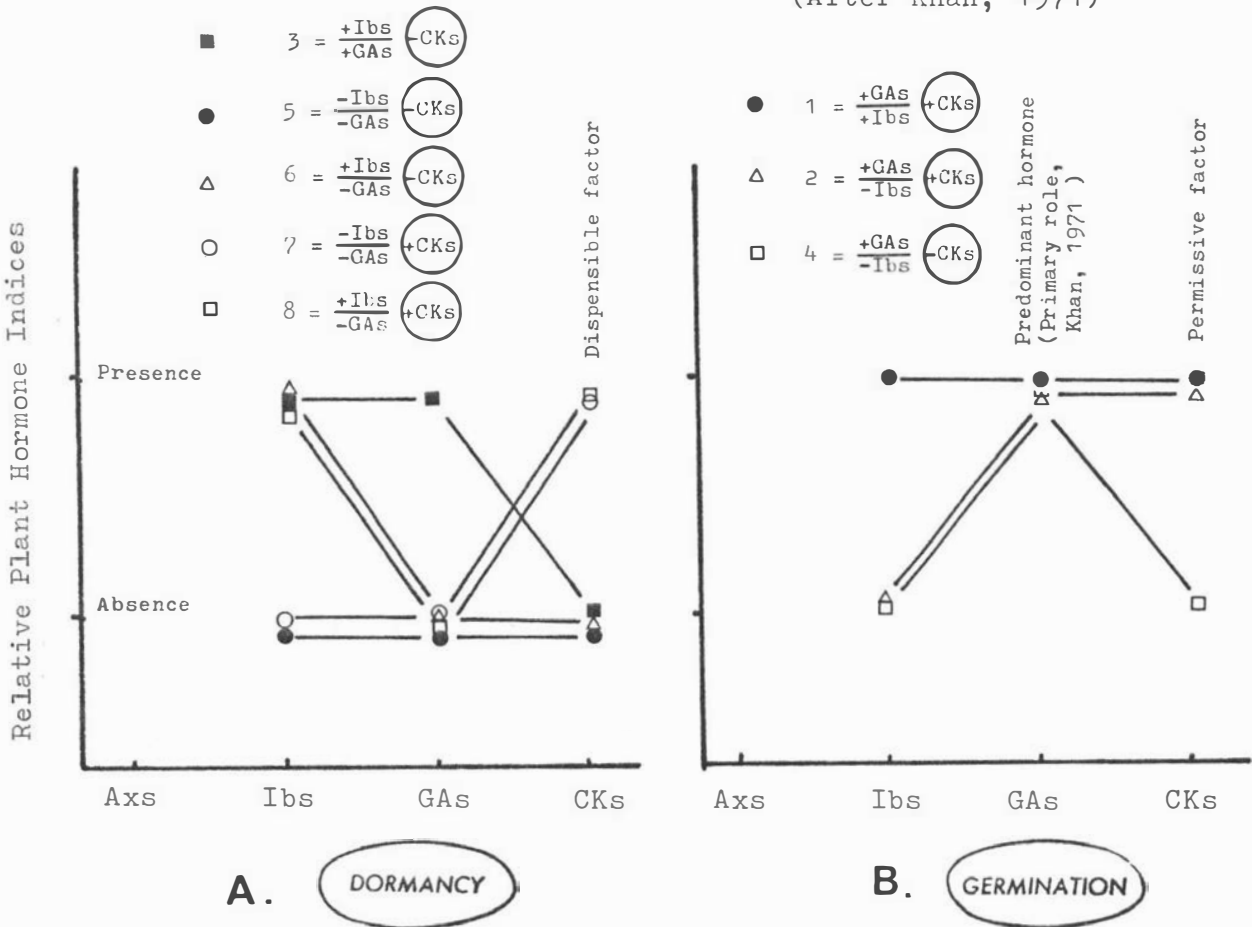


Fig. 35. An interpretation of Khan's hypothetical model in seed dormancy and germination in terms of Relative Plant Hormone Index.

a plant hormone. Khan (loc. cit) studied the germination of lettuce seed subjected to either different hormonal combinations and found that: "the seed is dormant in the absence of gibberellin whether cytokinin or inhibitor is present or not; or in its presence when the inhibitor is also present but cytokinin is absent. Germination occurs in the presence of gibberellin and absence of inhibitor whether cytokinin is present or not; or in the presence of an inhibitor with cytokinin to oppose its effect". This statement is clearly demonstrated in Fig. 35-A for seed dormancy and Fig. 35-B for seed germination using the Relative Plant Hormone index scheme.

Although no attempt is made here to explain any mechanism of dormancy, however Khan's hypothetical model can be interpreted in terms of RPHI as follows:

- (1) The determination of predominant hormones is based on a high or low Index; for example Gibberellin Activity Index is low or high in Fig. 35-A or B respectively, suggesting that gibberellin has a primary role (predominant hormone) in seed dormancy.
- (2) Dispensable or permissive factors will have a low or high index; for example, the index of cytokinin is high in Fig. 35-A. The index is also high in Fig. 35-B but could be low if the inhibitor was absent.
- (3) An inhibitor of the predominant hormone will always have a high ratio when the predominant hormone is present but no response is obtained.

In Fig. 1 and 2 there are symbols such as $\frac{GAs}{Ibs} \text{ (Cks)}$ which would denote the interaction of cytokinins with the gibberellin/inhibitor ratio. For example in the hypothetical scheme of Khan (1971) GA_3 could be used as the numerator and Ibs as the denominator while the permissive factor Cks is represented in the side circle (in interacting with the GAs/inhibitor ratio). Thus in Khan's hypothetical model seed germination would be represented as follows:

$$(1) \quad \frac{+GAs}{+Ibs} \text{ (} +Cks \text{)} \quad (2) \quad \frac{+GAs}{-Ibs} \text{ (} +CKs \text{)} \quad (4) \quad \frac{+GAs}{-Ibs} \text{ (} -CKs \text{)}$$

In seed dormancy, the numerator could be Ibs while GAs could be the denominator, and CKs as a dispensable factor. The ratios would then be:

$$(3) \quad \frac{+Ibs}{+GAs} \text{ (} -CKs \text{)} \quad (5) \quad \frac{-Ibs}{-GAs} \text{ (} -CKs \text{)} \quad (6) \quad \frac{+Ibs}{-GAs} \text{ (} -CKs \text{)}$$

$$(7) \frac{-Ibs}{-GAs} \textcircled{+CKs}$$

$$(8) \frac{+Ibs}{-GAs} \textcircled{+CKs}$$

where the numbers in brackets represent Khan's eight hormonal situations as illustrated in Fig. 35-C.

In conclusion, as a new terminology, the Relative Plant Hormone Indices such as RAAI, RIAI, RGAI, and RCAI are now replaced with the terms $\frac{CKs}{Axs} \textcircled{Ibs}$, $\frac{GAs}{Axs} \textcircled{Cks}$, and $\frac{CKs}{Axs} \textcircled{Ibs}$ in the speculative flow chart representing the integration between biochemical and physiological phenomena in the whole plant as depicted in Fig. 1.

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Part II

(Plant hormone studies)

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