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THE PARTITION COEFFICIENTS OF THE GROWTH REGULATORS OF APPLE, THEIR ISOLATION FROM APPLE SEED, AND THE INVOLVEMENT OF GIBBERELLINS IN THE BIENNIAL BEARING OF 'EARLY MCINTOSH'

A Dissertation Presented

By

FRANCIS MARINO

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ABSTRACT

The Partition Coefficients
of the Growth Regulators of Apple,
their Isolation from Apple Seed, and the
Involvement of Gibberellins in the Biennial Bearing
of 'Early McIntosh'

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Partition coefficients were determined for those growth regulators known or thought to be found in apple cv. Early McIntosh (Malus domestica Borkh.): abscisic acid, indoleacetic acid, indoleacetamide, indoleacetonitrile, indoleacetaldehyde, gibberellin A_3, gibberellin A_{4-7}, isopentyl adenine, isopentyl adenosine, zeatin, and zeatin riboside. Organic solvents normally used for extraction and/or clean up of plant extracts were selected: petroleum ether (b.p. 30-60 C), hexanes, ethyl ether, ethyl acetate, and n-butanol. The growth regulators were partitioned against phosphate-citrate buffer solutions at pH 3, 4, 5, 6, 7, 8, 9, and 5% sodium bicarbonate. The growth regulators were quantified by the appropriate bioassay: Avena coleoptile, lettuce hypocotyl, or tobacco callus.

A method to separate the growth regulators was devised and evaluated with the optimum conditions of separation, percent recovery, and minimum detection determined for pure compounds. The method was tested by determining the percent recovery from an apple seed

extract. Abscisic acid, gibberellin A_{4-7}, and zeatin were detected and quantified by gas chromatography or bioassay; no endogenous auxins were found although physiological levels could be detected.

The involvement of gibberellins in the biennial bearing of apple was investigated by determining the effect of gibberellin treatments on the flowering and growth of spurs and one-year-old wood. Both gibberellin A_3 and A_{4-7} reduced the flowering of spurs and one-year-old wood without increasing growth. The time of flower bud initiation, determined by periodically removing fruit, was found to be later than 45 days after full bloom (FB).

Peak levels of gibberellin A_{4-7} were found in seed at FB+ 5, 15, and 45-60 days. The peak levels of gibberellin in fruit diffusate were found at FB (ng/g FW) and FB+ 45 (pg/seed) basis. Significantly higher levels of gibberellin A_{4-7} were present in fruited spurs as compared to vegetative spurs. The time course profile of the fruited spurs revealed no peak of gibberellin A_{4-7} corresponding to a peak in the fruit diffusate.

It was concluded that the inhibition of flowering was not determined by the time or levels of gibberellin from the fruit diffusate, but by the time of the sensitivity of the spur to the influxing gibberellin.

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INTRODUCTION

In order to ascertain the involvement of a plant hormone in some physiological process its identity and quantity must be correlated with that process. There are numerous instances of isolation of plant growth regulators from many plant species. However, no standard procedure has been adopted since each group of workers has its own method although partitioning and chromatography are standard techniques. After purification, bioassays were employed to quantify the hormone although currently gas chromatography and high pressure liquid chromatography are being used more frequently. For the latter two techniques to be feasible a large preliminary clean up is necessary. A complete procedure including quantification is necessary where the latter techniques are too expensive or not available to the researcher.

In general, after the tissues were extracted and suction filtered, the extract was reduced in volume in vacuo. Partitioning, the first phase of cleanup, removed chlorophyll, inhibitors, and lipid-like materials (51, 87, 113, 146, 170). Chromatography was a second phase of cleanup and separation of hormonal classes. A third phase was chromatographic separation within a class of growth regulators followed by the assay system and, if necessary, gas chromatography—mass spectrometry to establish exact identity. The growth regulators of interest were those known or thought to be found in apple.

Partitioning can provide the first phase of isolation and purification of the growth regulators. Most plant growth regulators have an acidic or basic functional group; hence, they can be extracted as a function of pH (41, 88, 93, 134). They also differ in polarity and, therefore, will dissolve variably in organic solvents. Thus, a wide range of partition coefficients can be obtained for a particular growth regulator ranging from a high to a low value (41). Within the spread of pH values and organic solvents a combination can be obtained to extract or leave in the aqueous phase a particular growth regulator. A logical partitioning system can sufficiently purify the extract to allow the use of chromatography for further purification.

Many workers have employed ion-exchange chromatography (90, 116) for the isolation of cytokinins, and thin layer chromatography (29, 36, 58, 101, 113, 153) for separation and purification of cytokinins, and the other growth regulators. When separated and purified, bioassays (36, 76, 99, 113), gas chromatography-mass spectrometry (30, 50, 99, 102, 153, 183), and high pressure liquid chromatography (22, 25, 37, 38, 141, 159, 160, 163) were employed for quantification of the growth regulators.

Isolation of purified growth regulators allows the physiological processes which they regulate to be more easily understood by determining the appearance and time course levels of the growth regulators in the tissue.

Biennial bearing is considered by many growers to be the

major cultural problem in apple production. A consistent annual apple tree provides higher quality fruit and more stable fruit production which facilitates marketing and stabilizes prices. A tree in the 'on' year has a greater fruit set which increases competition for photosynthate and nutrients. This decreases growth and the ultimate size of the remaining fruit. In the 'off' year the yield is lower and fruit is usually larger (179).

The variety chosen for investigation was 'Early McIntosh'. This variety is strongly biennial in its flowering character and this extreme nature of 'Early McIntosh' makes it a good model system for studying biennial bearing. Williams and Edgerton (179) have stated the basic cause of bienniality is common to all cultivars.

Chan and Cain (26) demonstrated the effect of seed on flower initiation. The inhibiting effect of the seed on the bud development in the spur implied a hormonal messenger. Fulford (48, 49) suggested that flower formation was more likely caused by the removal of inhibiting factors than synthesis of a specific flower inducing substance. There is much evidence in the literature of the inhibiting effect on flower initiation by gibberellins (34, 43, 54, 55, 64, 107, 108,161, 175). Grochowska (59, 60, 61) provided evidence of the diffusion of exogenously applied growth regulators from the fruit. Grochowska and Karaszewska (62) demonstrated that two biennial cultivars diffused a greater content of auxins and gibberellins at a greater rate than two annual cultivars. This

with the quantitative evidence of Chan and Cain (26) comparing percent return bloom versus number of seed per spur, is ample evidence for the hypothesis of the inhibiting influence of seed on flower initiation working through a hormonal messenger. Therefore, gibberellin treatments were sprayed on field grown trees of cv. 'Early McIntosh' to determine the physiological effect on flower initiation. A fruit removal experiment was undertaken to determine the time interval of flower initiation. Finally, the gibberellin analysis of those tissues involved in flower initiation—the seed, fruit diffusate, fruit bearing, and vegetative spurs—was measured in an attempt to relate their gibberellin levels with the flower bud formation.

REVIEW OF LITERATURE

Isolation of Endogenous Growth Regulators

Any critical assessment of the significance of a putative hormone in the life of a plant must take many factors into consideration; not the least of these is the unequivocal demonstration of the presence of that compound in the plant (111). When purified compounds are isolated and identified a quantified correlation to a physiological process is possible. Determining the levels of growth regulators in plants is difficult because hormonal levels are in the part per million to part per billion range making large sample size and/or sensitive assay systems necessary. There are many isolation procedures in the literature though the actual techniques are few: solvent partitioning, paper and thin layer chromatography, column chromatography, gas chromatography, and high pressure liquid chromatography. Since it is becoming recognized that it is the relation between hormonal classes that is usually involved in a physiological process (2, 10, 11, 28, 53, 85, 95), methods of isolation which are inclusive of each hormonal class are needed.

Of all the methods in the literature for preliminary cleanup certain techniques have received the greatest emphasis: solvent partitioning and thin layer chromatography. The traditional assay system was the bioassay, although gas chromatography (connected to mass spectrometry) is now common and use of high pressure

liquid chromatography is increasing (22, 25, 37, 38, 141, 159, 160, 163).

Partition Coefficients

Partitioning as a means of extraction or as a step in purification has not received the quantitative attention in proportion to its use. Indoleacetic acid was partitioned and reported as a percent recovery by Larsen (88). He found it to be 95% extracted by ethyl ether when partitioned between pH 5 and 2.6. Knegt and Bruinsma (87) found it to be 95% extracted at pH 3 with the same solvent. Park (129) extracted indoleacetic acid with ethyl acetate at pH 2.5 and reported 81% recovery. Atsumi et al. (6) extracted it with ethyl ether at pH 3.5 and reported 81% recovery. Durley and Pharis (41) partitioned 27 gibberellins at 5 pH values revealing large differences between gibberellins at the same conditions. Hemberg and Westlin (72) partitioned kinetin between various organic solvents and reported the percent recovered. Hemberg (73) partitioned kinetin, isopentyl adenine, zeatin, and their ribosides with ethyl acetate at pH 3 reporting their values as partition coefficients. Hahn (66) partitioned isopentyl adenine, zeatin, and their ribosides with ethyl acetate at pH 7.7. Letham (93) partitioned zeatin, kinetin, isopentyl adenine, and zeatin riboside with petroleum ether, ethyl ether, ethyl acetate, and n-butanol at pH 3 and 7. Purse (136) partitioned isopentyl adenine, zeatin, kinetin, and their ribosides with a glucoside and sulfur containing cytokinins at pH 2.5 and 8.2 with petroleum ether, diethyl ether, ethyl acetate, and n-butanol and reported the values as partition

coefficients. Although petroleum ether is routinely used in partitioning to remove lipids and chlorophyll then discarded, Van Staden (170) found cytokinin activity in it.

Chromatography

Thin layer and column chromatography have been used as intermediary techniques for isolation and purification of the growth regulators. Thin layer chromatography on silica gel with several solvent systems was employed by Milborrow (113) to separate abscisic acid from other organic acids. Gibberellins were separated on thin layers of silica gel and kieselguhr by MacMillan and Suter (101) and in columns by Khalifah et al. (84) and Powell and Tautvydas (135). Pitel et al. (131) used silicic acid and Sephadex columns to separate gibberellins from Gibberella fujikuroi cultures. Reeve and Crozier (144) reported separation of gibberellins A_3, A_4, A_9, A_{13}, abscisic acid, indoleacetic acid, zeatin, and zeatin riboside with gel permeation chromatography. Cytokinins were separated by Miller (117) and Crafts and Miller (29) using paper and thin layer chromatography. Hahn (66) used silica gel in column chromatography to separate cytokinins. Raj and Hutzinger separated naturally occurring indoles by ion exchange (139), by cellulose thin layer, and Sephadex column chromatography (140). Grochowska (58) identified phloridzin in apple by thin layer and paper chromatography.

Instabilities of the Growth Regulators

In the isolation of a growth regulator it is important that the compound isolated is that which exists in vivo. Instabilities

of the growth regulators are due to an enzymatic, physical, or chemical nature. Nitsch and Nitsch (126) noted that indoleacetonitrile and indoleethylacetate could migrate to another plate in the same chromatographic chamber. Kefford (81) reported there were less losses of indole compounds when the procedure was performed in the dark and chromatograms were not stored in air. Mann and Jaworski (106) showed that indoleacetic acid was unstable toward oxygen and sublimes when flash evaporated. They added an antioxidant and suggested that sublimation could be decreased if flash reduction is performed at a higher pH. Milborrow and Mallaby (114) found methyl abscisate to be an artefact of extraction when methanol was used to extract tissue under neutral or basic conditions. The extraction of cytokinin nucleotides can be hampered by nonspecific phosphatases which may not be completely inactivated by methanol at 0 C. Bieleski (12) has developed a mixture to extract the nucleotides intact. Sachs et al. (147) have shown nonmetabolic catalytic degradation of kinetin on plant surfaces and glass and porcelain ware.

The use of cation exchange resins is standard for the concentration and purification of cytokinins, having been emphasized by Miller (116) and Letham (90). However, even then Kefford (82) mentioned the possible hydrolysis of cytokinins by a strong cation exchange resin. Miller (116) was able to hydrolyze cytokinins by boiling them with Dowex 50. Gazit and Blumenfeld (51) were able to release bound cytokinins by acid hydrolysis or passage through Dowex 50. Tegley et al. (162) noted losses of zeatin riboside when hydrolyzed

by acid or passage through Dowex 50. Dekhuijzen and Gevers (33) also noted the hydrolysis of zeatin riboside and reported the absence of hydrolysis when cellulose phosphate was used as the exchange resin. Vreman and Corse (172) advocated use of a weak exchange resin. Dyson and Hall (42) in an extraction method suggested the heat of neutralization in the column can hydrolyze the cytokinins; therefore, they placed the resin in a beaker of cold water and slowly neutralized it before elution in the column. Miller (118) devised two methods for cytokinin extraction which stood away from pH extremes and strong cation exchange resins. He used KH_2PO_4 and K_2HPO_4 for pH regulation and polyvinylpyrrolidone in column chromatography. However, contrary to these reports, Van Onckelen and Verbeek (169) obtained 100% recovery of zeatin and zeatin riboside from Dowex; and Van Standen (171) obtained similar amounts of zeatin and zeatin riboside by separation on a Dowex 50 and a Sephadex column which they interpreted as no cytokinin hydrolysis with resin use.

In their use of Dowex 50 as the exchange resin Vreman and Corse (172) reported that the cytokinin was tightly bound to the resin and that the inclusion of an organic solvent in the eluant increased the recovery of the cytokinins.

Quantification Techniques

The classical quantifying procedure for growth regulators was the bioassay. There were many bioassay systems developed for each hormonal class. For auxin measurement the Avena curvature test (173),

Avena coleoptile straight growth test (125), Avena first-internode test (125), split pea test (174), and mung bean root formation test (74) were developed. For abscisic acid measurement the cotton explant abscission test (1) and cress seed germination test (94) were developed. For gibberellin measurement the dwarf corn (124), dwarf rice (127), dwarf pea (18), cucumber hypocotyl (19), and Rumex (176) test were developed. For cytokinin measurement the radish cotyledon expansion test (91) and Xanthium test (128) were developed. Though there were many bioassay systems, few have been routinely chosen. Nitsch and Nitsch (125) devised the Avena straight growth test for auxins using either coleoptile or mesocotyl sections. Franklin and Wareing (46) determined gibberellin concentration by lettuce seedling hypocotyl growth. Jones and Varner (79) followed starch degradation by the enzyme α-amylase in barley. Milborrow (112) used the oat mesocotyl as a test for the inhibitor abscisic acid. Cytokinins were bioassayed by the callus growth of tobacco (Linsmaier and Skoog, 95) or soybean (Miller, 115).

Gas chromatography has been frequently employed to separate and quantify the growth regulators from tissue extracts. Except for the neutral auxins, derivatives must be made (Powell, 134). Auxins have been analysed by gas chromatography as the methyl ester by Powell (134) and Grundwald et al. (63) and as halogenated derivatives for the electron capture detector by Brook et al. (20), Seely and Powell (151), and Bittner and Even-Chen (14).

Allen et al. (4) determined indoleacetic acid by mass fragmentography. Cytokinins have been analysed by gas chromatography as trimethylsilyl derivatives by Most (122) and Dauphin et al. (30) and as permethylated derivatives by Young (183). Gibberellins have been analysed by gas chromatography as trimethylsilyl derivatives by Cavell et al. (23), as the methyl esters and trimethylsilyl ethers by Binks et al. (13) and as fluorinated derivatives by Seely and Powell (151).
Abscisic acid has been analysed as the methyl ester by Gaskin and MacMillan (50) and as the trimethylsilyl derivatives by Davis et al. (32). Seely and Powell (150) reported the strong electron capturing property of methylated abscisic acid and the use of the electron capture detector for its analysis in the picogram range. Swartz and Powell (158) determined picogram quantities of indoles with an alkali flame ionization detector.

The use of high performance liquid chromatography has been growing recently for each class of growth regulator. It is a rapid and highly efficient technique which operates at ambient temperatures, needs no derivatization, and has easy sample recovery. Sweetser and Vatvars (160), Rapp et al. (141), During and Bachmann (37), Quebedeaux et al. (138), and During (38) employed high performance liquid chromatography for analysis of abscisic acid. Cytokinins were analysed by Carnes et al. (22), Morris et al. (120), Thomas et al. (163), Pool and Powell (132), and Challice (25). Indoleacetic acid was analysed by Sweetser and Swartzfager (159) and During (38) and gibberellins by Reeve et al. (145).

Endogenous Growth Regulators of Apple

Apple has been shown to contain representatives of each class of growth regulators. Raussendorff-Bargen (143) cited evidence that along with 3-indoleacetic acid apple contains 3-indoleacetamide, 3-indoleacetaldehyde, 3-indoleacetonitrile, and 3-indoleethylacetate. Durkee and Poapst (39, 40) gave chromatographic evidence that indoleacetic acid can be produced under certain storage conditions. Pieniazek and Rudnicki (130) and Rudnicki (146) found the presence of abscisic acid in non-stratified apple seed. Goldacre and Bottomly (52) and Letham and Bollard (89) found cytokinin activity in apple fruitlets. Letham and Williams (92) offered chromatographic evidence for the presence of zeatin, zeatin riboside, and zeatin ribotide in apple seed. Subsequently, Zwar and Bruce (184) reported that zeatin was not present but a derivative. Dennis and Nitsch (35) have demonstrated the presence of gibberellin A_4 and A_7 in immature apple seed, a finding later confirmed by Luckwill et al. (99) and MacMillan (102) using gas chromatography-mass spectrometry. Though not as prevalent as the reports for gibberellin A_{4-7}, apple has been reported to contain gibberellin A_9 in trace amounts by Sinska et al. (153) and Halinska and Lewak (67) and gibberellin A_3 by Hayashi et al. (71). Dennis (36) reported gibberellin activity at column chromatographic elution volumes similar to those of gibberellin A_5 and A_6. Phloridzin, the main phenolic glycoside in apple tissue and a growth inhibitor, was observed by Grochowska (57) and its presence later confirmed by her (58).

Biennial Bearing

Many apple growers consider biennial bearing to be the number one problem in apple production (Williams and Edgerton, 179) and one of the most serious hindrances to profitable orcharding (Wicks,177; Wilcox, 178).

Biennial bearing is the cyclic process where heavy cropping occurs every second year. A heavy bloom generally results in heavy fruit set that year. This is referred to as the 'on' year. Fruit present on a spur inhibits flower formation for the crop the following year. The second year characterized by few flowers is called the 'off' year. Flower bud formation on vigorous spurs lacking fruit is generally extensive leading to a high percentage of flower buds. The biennial habit will continue until a break in the cycle is effected. The self-pollinating cultivars, which contain more fertile seed per fruit and more fruit per spur, tend to bear biennially more than self-sterile types (179). It was stated by Williams and Edgerton (179) that no two cultivars react the same to a given treatment, but the basic cause of bienniality is common to all. To overcome the problem much work centered around balancing tree vigor, reducing crop loads, spur vigor, and increasing flower initiation. The problem was given much attention and took form from techniques of culture (3, 7, 110, 180, 181) genetics (100), and chemical application (9, 15, 47, 70, 103, 104).

The possible causes of biennial bearing are many and varied. Carne (21) suggested that an infestation of thrips disturbed the

tree balance by preventing normal setting in one year. The following year a bumper crop was set because nitrogen supply went into vegetative growth and increased the carbohydrate:nitrogen ratio. Potter and Phillips (133) reported the condition most consistently associated with blossom formation was insoluble nitrogen, whereas, reducing sugars depressed fruit bud formation. Feucht (45) found reproductive spurs to contain less reducing sugars. Hamilton (68) and Aldrich and Fletcher (3) suggested practices to get more uniform leaf area and Fulford (47) used copper sulfate to defoliate trees and obtained regular bloom. To obtain annual production of biennial varieties Blinov (15) rotated cropping within a tree and Childs and Brown (27) spot sprayed branches with tar oil to destroy blossoms on specific branches of the tree. Magness and Batjer (103) sprayed tar distillate or dinitroorthocyclohexylphenol to prevent the set of a large proportion of fruit in apple trees. Wilcox (178) suggested meteorological conditions rather than tree vigor to be associated with biennial bearing. Ljones (96), Emmert (44), and Mason (109) found the leaf analysis of some minerals to differ when comparing the 'on' with the 'off' year.

Nitrogen fertilization can be ineffective in offsetting biennial bearing (Auchter and Schrader, 7) or increase the tendency (McCormick, 110). Later the time of application in relation to the growth phase of the tree was shown to be an important factor of nitrogen fertilization (Williams and Rennison, 181; Williams, 180). Nitrogen fertilizers applied in the spring promoted vegetative growth

but had little immediate effect on fruit bud differentiation; but, when applied in late summer, when extension ceased, a marked stimulation of fruit bud formation occurred.

Hormonal compounds were used to improve regular bearing either by directly increasing flower initiation (70) or by fruit thinning which allows spurs to initiate flowers. Magness et al. (104) did not increase flower initiation with naphthaleneacetic acid or the acetamide although Murneek (123), Southwick and Weeks (155), Wurgler et al. (182), and Bowman (16) reported lessening biennial tendencies.

Singh (152) suggested that previous studies of single aspects as tree vigour, spur growth, or the chemical composition of spurs failed to give a clear picture of the whole phenomenon. In the attempt to control biennial bearing, many treatments gave conflicting results; whereas, a comprehensive study of all the growth characteristics under the same environmental conditions was needed.

Time of Flower Bud Initiation

Rasmussen (142) reported that different localities vary in the time and period of fruit bud differentiation. Barnard and Read (8) found that initiation begins about five or six weeks after spur leaves are full grown. Celjniker (24) determined the isoelectric point of the meristematic tissue of apple buds and concluded the two kinds of buds could be detected three to six weeks before morphological differentiation occurred, as the isoelectric point of the flower buds was of a lower pH level. Gyuro (65) reported Jonathan flower bud differentiation started about four to five weeks after the end of shoot growth. By periodically removing

fruit, Harley et al. (69), Chan and Cain (26), and Luckwill (100) obtained a return bloom relation between presence of fruit and time of their removal. Luckwill's data demonstrated that early fruit removal was promotive to flowering. It then decreased with time reaching the 50% return six weeks after bloom and leveled at 10% return approximately eight weeks after. Harley et al. (69) presented data that 'Yellow Newton' had a return bloom of 50% thirty days after bloom and decreased to zero return 70 days after bloom. Chan and Cain (26) demonstrated 'Spencer Seedless' and 'Ohio 3' to have a return bloom of 50% thirty days after bloom and a slow decrease thereafter.

Relation of Growth and Flowering

The inverse relation of growth on flowering has long been known though there is evidence to the contrary that it is a causal relation. Tromp (164) argued for a separation of the two effects from his data of gibberellin, SADH (succinic acid dimethylhydrazide), and shoot growth. Luckwill (100) reported data of 500 ppm B.9 (succinic acid dimethylhydrazide) for optimum flower bud production but growth decreased up to 2000 ppm. Batjer et al. (9) sprayed the lower third of apple trees with B.9 and increased flower production over the whole tree while observing growth reduction only where sprayed. Davis (31) was of the opinion that the carbohydrate: nitrogen ratio was a consequence rather than a cause of flowering.

Gibberellin and Flower Inhibition

It is known that gibberellins inhibit flowering in several

species: pear (77), (166), (167); stone fruits (17), (43), (78); citrus (75), (119), (121); grapes (5); black currants (149), and cranberries (105). There are several reports of gibberellin being a flower inhibitor in apple. Marcelle and Sironval (107) and Marcelle (108) inhibited flowering in 'Golden Delicious' with 300 ppm gibberellic acid. Guttridge (64) inhibited flowering in 'Bendiction', 'Delicious', 'McIntosh', 'Lord Derby', and 'Laxton's Superb' with weekly applications of 10 and 50 ppm gibberellin A_3. Dennis and Edgerton (34) inhibited flowering with concentrations of 100 to 1000 ppm of potassium gibberellate, butyl cellosolve ester of gibberellin A_3, and gibberellin A_{4-7} applied at full bloom and petal fall to 'Wealthy' and 'McIntosh'. Greenhalgh and Edgerton (55) also inhibited flowering in 'McIntosh' with 100, 200, 400 ppm potassium gibberellate applied at 2 and 25 days after bloom. Taylor (161) reduced the return bloom of 'Golden Delicious' with concentrations of 10 to 200 ppm gibberellin A_{4-7} applied at petal fall, 11 days after, and 3 weeks after petal fall. Contrary to other reports, Luckwill (100) found lower concentrations of gibberellin A_3 (50 ppm) more effective than higher concentrations (500 ppm) in reducing flower initiation. Unrath (168) sprayed trees with a 100 and 200 ppm gibberellin A_{4-7}/benzyladenine mixture at full bloom and petal fall and substantially reduced flowering in 'Starkrimson Delicious'. Wertheim (175) significantly reduced flowering in 'Cox's Orange Pippin' with a mixture of gibberellin A_{4-7} applied between 1 and 40 days after bloom.

Greene and Williams (54) inhibited flowering in spur type 'Delicious' with 300 ppm gibberellin A_{4-7} applied 25 days after full bloom.

Effect of Fruit on Flowering

Tumanov (165) showed that spurs in the immediate proximity of the fruit did not produce flower buds. Fulford (48) concluded that the failure to form flowers on apple trees was not due to a competitive effect of fruit for nutrients and suggested (49) that flower formation was more likely due to the removal of inhibiting factors than the synthesis of a specific flower inducing substance.

The inhibiting influence of seed on flowering was demonstrated by Chan and Cain (26) using two apetalous apple varieties, 'Spencer Seedless' and 'Ohio 3'. These varieties normally bear annual crops of seedless fruit but, if hand pollinated, carry seeded fruit and become biennial. With 'Spencer Seedless', 95.3% of the spurs bearing seedless fruit flowered again the following year though 13.1% flowered for spurs bearing seeded fruit. With 'Ohio 3' the return flowering for spurs of seedless fruit was 98% and 31.4% for spurs of seeded fruit. They concluded the inhibition resided in the seed and was not caused by nutrient competition. They suggested a hormonal messenger diffusing out of the fruit. They also showed the number of seed per spur was negatively correlated with flowering.

Diffusion of Growth Regulators from Seed

Grochowska (60) removed the core of apple fruitlets and

inserted cotton wetted with auxins and gibberellins; naphthalene-acetic acid increased flower bud formation, whereas gibberellin decreased it. With the addition of labeled indoleacetic acid to seed of five week old apples she demonstrated that it was translocated to the peduncle, bourse, and adjacent leaves (59). Indoleacetic acid labeled at C^1 and C^2 and labeled γ-aminobutyric acid were applied to the seed surface, into the seed, and on the surface of the pericarp. Both the labeled auxins when applied into or on the seed appeared in spur tissue; although, when placed on the pericarp, the radioactive auxins and γ-aminobutyric acid were found only in fruit tissue with very small quantities in the peduncle or spur tissue (61). Grochowska and Karaszewska (62) compared annual bearing 'Jonathan' and 'Oberlander Himberapfel' with biennial bearing 'Landsberger Reinette' and 'Wealthy'. The auxin and gibberellin amount actively diffused from seed was 60% greater and the rate of translocation was 2-10 times greater in the biennial varieties. They suggested that besides the number of fertile seeds per fruit the natural capacity of the fruit to produce and diffuse growth substances determine flower bud initiation. Hoad (76) collected and analysed the gibberellin content in fruit diffusate and seed from 'Laxton's Superb' and 'Cox's Orange Pippin'. He found higher levels in the diffusate of the biennially bearing 'Laxton's Superb' though seed levels were similar.

Seasonal Levels of Gibberellin in Seed and Spurs

Luckwill et al. (99) measured the gibberellin levels of seed

from three apple varieties: 'Cox's Orange Pippin', 'Emneth Early', and 'Laxton's Superb'. In each case gibberellin appeared approximately five weeks after bloom, peaked at nine weeks, and decreased to disappearance at eighteen weeks. Dennis (36), extracting seed from 'Golden Delicious', followed the gibberellin levels which appeared ~10 days after bloom, peaked ~60 days after bloom, and slowly decreased approaching zero ~150 days after bloom. Luckwill (100) extracted bourses from which fruit was removed two weeks after bloom and compared levels with bourses bearing fruit. For the defruited bourses the growth promoters, as measured by the Avena mesocotyl test, remained at a low constant level throughout the season. However, where fruit was present, strong flushes of growth promoting activity appeared, part of which was gibberellin activity as indicated by the lettuce hypocotyl test. Hoad (76) found gibberellin levels to increase with time in 'Laxton's Superb' and to increase then decrease in 'Cox's Orange Pippin'.

Hypotheses of Biennial Bearing

Various hypotheses have been proposed to explain the process of biennial bearing in apple. With the demonstration of the effect of seed on subsequent flowering by Chan and Cain (26) the carbohydrate:nitrogen ratio, assimilate competition, and nutritional effects were given less attention vis-á-vis hormonal involvement.

Luckwill (100) suggested that when a long shoot ceases extension growth, the supply of gibberellin from young leaves ceases and buds are potentially able to initiate flowers. If cytokinin is

still present in the xylem sap a release from dormancy can be effected and flowers initiated.

A negative correlation often exists between internode growth and flower bud initiation. Batjer et al. (9) demonstrated that certain inhibitors reduce growth and promote flowering in apple.

Sachs (148) suggested a nutrient diversion to account for promotion of flowering by diverse phenomena as leaf removal, dwarfing rootstocks, inhibitors of stem elongation, girdling, high light flux, and salt or water stress. The notion is that a critical part of the shoot apical meristem is relatively deprived of nutrients during vegetative development or must receive a higher level of assimilates for gene expression than required for vegetative development.

MATERIALS AND METHODS

The growth regulators used were obtained from the following sources Sigma: 3-indoleacetic acid, 3-indoleacetamide, 3-indoleacetaldehyde (bisulfite complex), 3-indoleacetonitrile, 6-(3-methylbut-2-enylamino) purine (isopentyl adenine), and 6-(3-methylbut-2-enylamino) purine riboside (isopentyl adenosine); Calbiochem: gibberellin A_3 (K-salt 80%), 6-(4-hydroxy-3-methyl-butylamino) purine (zeatin), and 6-(4-hydroxy-3-methylbutylamino) purine riboside (zeatin riboside); Imperial Chemical Industries: gibberellin A_{4-7} (a mixture of unknown proportions) and from Burdick and Jackson Laboratories: abscisic acid (95%).

The organic solvents (AR grade), used after double distillation from glass were obtained from Fisher: methanol and ethyl acetate and Mallinckrodt: petroleum ether (bp 30-60 C), hexanes, and n-butanol. The solvents were water saturated before use.

Buffer solutions were prepared by mixing 0.01 M citric acid (AR grade) with 0.02 M potassium phosphate dibasic (AR grade) for the appropriate values as measured on a pH meter.

Chromar $7GF_{254}$ 250μm silicic acid thin layer plates were obtained from Mallinckrodt.

Dowex 50-H^+8-X (20-50 mesh) was the cation exchange resin.

A Varian aerograph series 2700 was used for gas chromatography.

Determination of the Partition Coefficients of the Growth Regulators

Time Course

The growth regulators were added to 50 ml of petroleum ether in a separatory funnel and partitioned with 50 ml of buffer pH 9. The partitioning times chosen were 0, 15, 45, 90, and 120 seconds. A 5 ml aliquot of the organic layer was evaporated in the draft of a hood. An equal volume (5 ml) of medium of the appropriate bioassay was reintroduced. Each growth regulator was partitioned in triplicate and bioassayed in duplicate.

Partitioning Procedure

The growth regulators were added to 50 ml of buffer, followed by 50 ml of the organic solvent in a separatory funnel, and partitioned for approximately one minute. A 5 ml aliquot of the organic layer was transferred to the vessel used for bioassay and evaporated in the draft of a hood. A 5 ml volume of medium was reintroduced. The quantity of the growth regulator partitioned into the organic layer was determined by the appropriate bioassay. When the partition coefficient became large, the aliquot size was reduced to 2.5 ml to increase the accuracy of the log-linear response. Each growth regulator was partitioned in triplicate and bioassayed in duplicate.

Quantification of the Partitioning

Bioassay of Abscisic Acid. These adjustments were made to the method of Milborrow (112), other conditions were followed. Seed of 'Victory' oats (South Dakota State, Brookings) was soaked in water

for 24 hours in the presence of room light and sown in vermiculite. The seed was germinated in the dark at 25 C and coleoptiles were selected for uniformity when ~2 cm long (4th day). If necessary, water was added during the germination. A 10 mm section was cut 2 mm below the tip and the sections, used immediately, were transferred to 5 cm petri dishes containing standard and test solutions. Growth was measured with a metric rule 24 hours later. Since the growth response was small, the length of the coleoptiles was read to a millimeter and estimated within as mm +.3, mm +.5, mm +.8.

Bioassay of Auxins. These adjustments were made to the method of Nitsch and Nitsch (125), other conditions were followed. Seed of 'Victory' oats was grown as above. For the determination of indoleacetic acid, 10 mm sections were harvested 4 mm below the tip and placed in a solution of 1 ppm $MnSO_4$ in the dark for 3 hours. For the neutral auxins the sections were placed in distilled water for 3 hours, then transferred to the test and standard solutions. The coleoptiles were measured as above.

Bioassay of Gibberellins. These adjustments were made to the method of Franklin and Wareing (46), other conditions were followed. Lettuce seed ('Paris Island Cos', Harris) was germinated on filter paper in the dark at 25 C. Seedlings were selected for uniformity when the radicals were 3-3.5 mm long (~1 day). Approximately 10 seedlings were transferred to 5 cm petri dishes containing a filter paper and the test and standard solutions. The petri dishes were placed 35 cm below four 40 watt fluorescent bulbs (3 cool white, 1 gro

lux; 420 ft-c). The hypocotyls were measured to the nearest millimeter after three additional days growth.

Bioassay of Cytokinins. Callus was formed from the tobacco variety 'Sansum N' and cultured on the medium of Linsmaier and Skoog (95). The stock callus was no longer used for bioassay after six subculturings. This variety responded linearly to two and one-half orders of magnitude and a sensitivity of 0.4 part per billion.

Method for Separation of the Growth Regulators

Conditions and Percent Recovery

Two titer solutions were prepared containing all the growth regulators of this investigation: one for the percent recovery and one for the minimum detection. Each was separated in triplicate and contained the following amount of hormones.

Growth Regulator	% Recovery (µg Added)	Minimum Detection (ng Added)
Abscisic acid	50	50
Indoleacetic acid	500	500
Indoleacetamide	500	5000
Indoleacetonitrile	500	1500
Gibberellin A_3	200	80
Gibberellin A_{4-7}	88	35
Isopentyl adenine	60	500
Isopentyl adenosine	60	500
Zeatin	30	20
Zeatin riboside	30	20

A 1.0 ml aliquot of the titer solution was diluted to 50 ml with buffer at pH 3 (figure 2) and partitioned 3x against petroleum ether. The petroleum ether was discarded and the aqueous layer was partitioned 3x against ethyl acetate, then adjusted to pH 9 with sodium hydroxide, and partitioned 3x against n-butanol. The organic layers of the ethyl acetate and n-butanol

were combined and reduced in a flash evaporator at 50 C and taken up in a minimum of 95% ethanol. This was passed through a 50 ml burette containing Dowex 50-H^+ of bed volume 25-30 ml. The column was washed with 250 ml of 10% ethanol collected at a rate of ~20 ml/minute. Further washing of the column was discarded. To prevent heating during elution, ~50 ml of a cold dilute solution of ammonium hydroxide was passed through the column and collected with the eluant. The column was eluted with 600 ml of 8% v/v ammonium hydroxide: 10% v/v 95% ethanol at a rate of 20 ml/minute. The 250 ml wash fraction was made alkaline by the addition of 0.5 g solid potassium phosphate dibasic. The wash and eluant fractions were reduced in vacuo at 60 C to a volume of ~50 ml. The wash fraction was adjusted to pH 10 with sodium hydroxide and partitioned 3x against an equal volume of ethyl acetate. The ethyl acetate layer was reduced to dryness in vacuo at 50 C, taken up in 95% ethanol, transferred to a thin layer plate, and chromatographed 2-dimensionally, first with water and then with water saturated ethyl acetate. The respective areas (table 1) for the neutral auxins were eluted and then quantified by bioassay and/or gas chromatography.
The aqueous fraction was adjusted to pH 3 with dilute hydrochloric acid and partitioned 3x against ethyl acetate. The organic layer was reduced to dryness in vacuo, taken up in a minimum of 95% ethanol, and transferred to a thin layer plate. The application was separated 2-dimensionally with benzene (*): ethyl acetate: acetic acid (10:1:1) followed by water. The respective areas of

the acidic hormones (table 1) were scraped from the plate and eluted with ethyl acetate. The gibberellins were bioassayed directly; indoleacetic acid was bioassayed and methylated for quantification by gas chromatography with a flame ionization detector. Abscisic acid was methylated for quantification by gas chromatography with the electron capture detector (150). The methylation was carried out according to the method of Powell (134) but had ethyl ether substituted for methylene chloride.

The eluant was reduced to ~50 ml and adjusted to pH 9 with sodium hydroxide and partitioned 3x against an equal volume of n-butanol. The n-butanol layers were reduced to dryness and taken up in 95% ethanol for transfer to a thin layer plate. The cytokinins were separated 2-dimensionally with water saturated ethyl acetate followed by water. The respective areas of the cytokinins (table 1) were scraped and bioassayed directly.

Minimum Detection

The above procedure was similarly used for the minimum detection of the growth regulators.

Test of the Method Using Apple Seed

Apple seed extracts were prepared by removing seed from 'Early McIntosh' harvested 60 days after full bloom. Seed was weighed and freeze-thawed. Absolute ethanol was introduced and the vials were capped under nitrogen and stored at -10 C until extracted. The seed was ground in acid washed sand (figure 1) (35-40 mesh) with a mortar and pestle. The ground seed was extracted 3x with 100 ml

of 80% methanol held at 0 C for 10 hours each extraction. The combined methanolic fractions were suction filtered, adjusted to pH 8 with sodium hydroxide, and reduced in vacuo at 50 C to a volume of ~75 ml. The reduced extract was diluted to 100 ml with distilled water and divided for a non-spiked and spiked extract. A parallel level of a known amount of each growth regulator was added to yield a spiked extract. Separation was performed in quintuplicate and the percent recovery calculated for each endogenous growth regulator.

Stability of Auxins and Cytokinins to Isolation Techniques

Indoleacetonitrile and indoleacetamide (5 µg each) were added to 500 ml of 10% ethanol and flash reduced at 60 C to the point of dryness. They were transferred and made to a volume of 0.1 ml with ethyl acetate and injected into a gas chromatograph. Standards were prepared and the percent recoveries calculated.

Indoleacetic acid (50 µg) was added to 500 ml of 10% ethanol buffered at pH 8.5 and pH 3 and then flash reduced at 60 C to the point of dryness. Each was transferred for methylation with diazomethane (Powell, 134) and made to a volume of 0.1 ml with ethyl acetate. The derivative was quantified on a gas chromatograph and compared to derivatized standards.

To determine the extent of hydrolysis of the cytokinin ribosides by the cation exchange resin, 400 µg of isopentyl adenosine and zeatin riboside were exchanged on a Dowex 50-H^+ column. They were eluted without the generation of heat by passing ~50 ml

of a cold dilute solution of ammonium hydroxide and following with 600 ml of 8% v/v ammonium hydroxide:10% v/v 95% ethanol. The eluant was reduced in vacuo at 60 C, transferred for silylation with N, O-bis-(trimethylsilyl)-acetamide (BSA, Pierce), and injected into a gas chromatograph for quantification. In addition to the standards for each riboside, standards were prepared for the hydrolysis products: isopentyl adenine and zeatin.

Each instability determination was performed in triplicate.

Involvement of Gibberellins in Biennial Bearing

Application of Gibberellins to 'Early McIntosh'

Gibberellin A_3 and A_{4-7} were each made to a concentration of 30 and 300 ppm by the dissolution of 30 and 300 mg in 5 ml of 95% ethanol and dilution to a liter with distilled water. The surfactant, Tween-80, was added to the mixture to yield a solution of 0.005%. A control spray was similarly prepared but without gibberellin. Six trees ('Early McIntosh'/M7, planted 1963) each with nine uniform limbs were randomly selected at the Horticultural Research Center in Belchertown, Massachusetts. Each limb contained between 25-50 vegetative spurs with any flowering spurs having been removed. Spray treatments were applied (to drip) on full bloom +10 days (May 17, 1977) with one limb selected for the control and two limbs selected for each of the four gibberellin treatments. A repeat (R) application of each treatment was sprayed 10 days later (FB +20) on the control and one of the two previously treated limbs.

Determination of the Time Interval of Flower Initiation

Flowers or fruit were removed from spurs at dates of full bloom (FB), FB+5, FB+10, FB+15, FB+20, FB+30, FB+45, FB+60, and FB+105 (harvest). Five tree replicates with 8 spurs/tree/day were randomly selected.

Harvest and Preparation of Tissues for Time Course Gibberellin Extraction

Materials for gibberellin extraction were randomly harvested from four tree replicates at dates of FB, FB+5, FB+10, FB+15, FB+20, FB+30, FB+45, and FB+60.

1. Seed. A quantity of seed was removed from fruit, weighed, and freeze-thawed. Absolute ethanol was introduced and the vials were capped under nitrogen and stored at -10 C until extracted.

2. Diffusate. Flowers or fruit were removed from trees with the pedicel placed in scintillation vials containing 20 ml of a solution of buffer pH 8, 2% sucrose (Griggs et al., 56), and Tween-80 added to yield a 0.005% solution. The diffusate was collected for 24 hours, capped under nitrogen, and frozen at -10 C until extracted.

3. Spurs. Fruit bearing and vegetative spurs were cut from trees containing ~1.5 cm of the preceding years growth and all of the current years growth. The spurs were freeze-thawed then, freeze-dried and stored at -10 C until extracted.

4. Extraction of Tissues. To prepare extracts, seed was ground in a mortar and pestle with sand; spurs were cut into small pieces;

then, each was extracted with methanol and the preparation continued as shown in figure 1. The diffusate entered the separation procedure directly (figure 3).

The isolation procedure (figure 3) was shortened for tissue extraction to include only the pathway necessary for gibberellin isolation (34, 64, 107). The extract was made alkaline by the addition of ~0.5 g solid potassium phosphate dibasic. Dilute sodium hydroxide was added to increase the pH to 10. The extract was then partitioned 2x against an equal volume of ethyl acetate and adjusted to pH 3 with hydrochloric acid. The aqueous layer was partitioned 3x against equal volumes of ethyl acetate which were combined and reduced to dryness at 50 C, taken up in ethanol, and transferred to a thin layer plate. The gibberellins were separated 2-dimensionally with water saturated ethyl acetate, followed by water. The respective areas of the gibberellins (table 1) were scraped from the plate and dissolved in 5 ml of 95% ethanol. Each tissue extract was divided for duplicate bioassays.

5. Percent Recovery of Tissues. Extracts were prepared in quadruplicate for diffusate and vegetative spurs (seed having been determined previously) with one-half of each extract chosen for the endogenous measurement and the other for the spiked measurement. A parallel amount of gibberellin was added to the latter. These were separated following the method for gibberellin isolation (figure 3) and the percent recovery was calculated for each tissue.

Table 1. Chromatographic Conditions.

Thin Layer Chromatographic Conditions

1. Neutral Fraction

	R_f water	R_f EA·H_2O
Indoleacetamide	0.87	0.45
Indoleacetonitrile	0.82	1.0

2. Acidic Fraction

	φ:EA:HOAc(10:1:1)	water	EA·H_2O
Abscisic acid	0.45	0.81	1.0
Gibberellin A_3	0.17	0.90	1.0
Gibberellin A_{4-7}	0.50	0.75	1.0
Indoleacetic acid	0.71	0.92	1.0

3. Cytokinins

	EA·H_2O	water
Isopentyl adenine	0.48	0.42
Isopentyl adenosine	0.48	0.32
Zeatin	0.13	0.44
Zeatin riboside	0.12	0.65

Gas Chromatography

Derivative	Detector	Column T C*
Abscisic acid-Me	Electron Capture-H^3	195
Indoleacetic acid-Me	Flame Ionization	182
Indoleacetamide	"	200
Indoleacetonitrile	"	145
Isopentyl adenine-BSA	"	215
Isopentyl adenosine-BSA	"	240
Zeatin-BSA	"	295
Zeatin riboside-BSA	"	295

* A 3% SE-30 5 ft 1/8" SS Chrom W HP 100/120 mesh.
N_2 30 ml/min.

Figure 1. Preparation of an Extract.

Plant material

|

Ground in sand,
Blended
or
Cut into pieces

|

Extracted 3x
80% methanol (10ml/g)
10 hours, 0 C

|

Suction filter

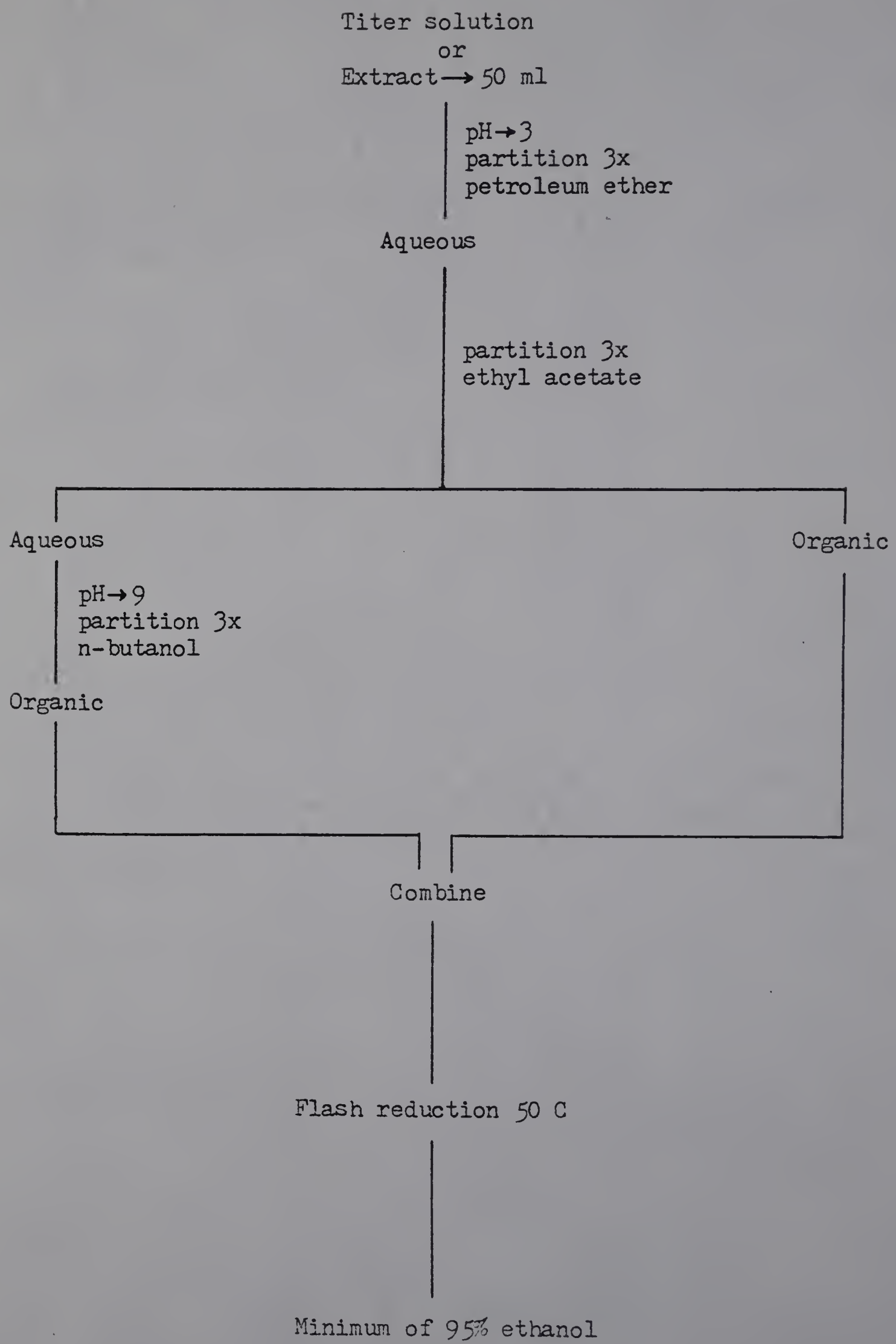
|

Flash reduction
pH 8, 50 C

|

Extract
N_2, -10 C

Figure 2. Method for the Complete Separation of the Growth Regulators.
All Fractions not Shown can be Discarded.


Titer solution
or
Extract → 50 ml
pH → 3
partition 3x
petroleum ether
Aqueous
partition 3x
ethyl acetate
Aqueous
Organic
pH → 9
partition 3x
n-butanol
Organic
Combine
Flash reduction 50 C
Minimum of 95% ethanol

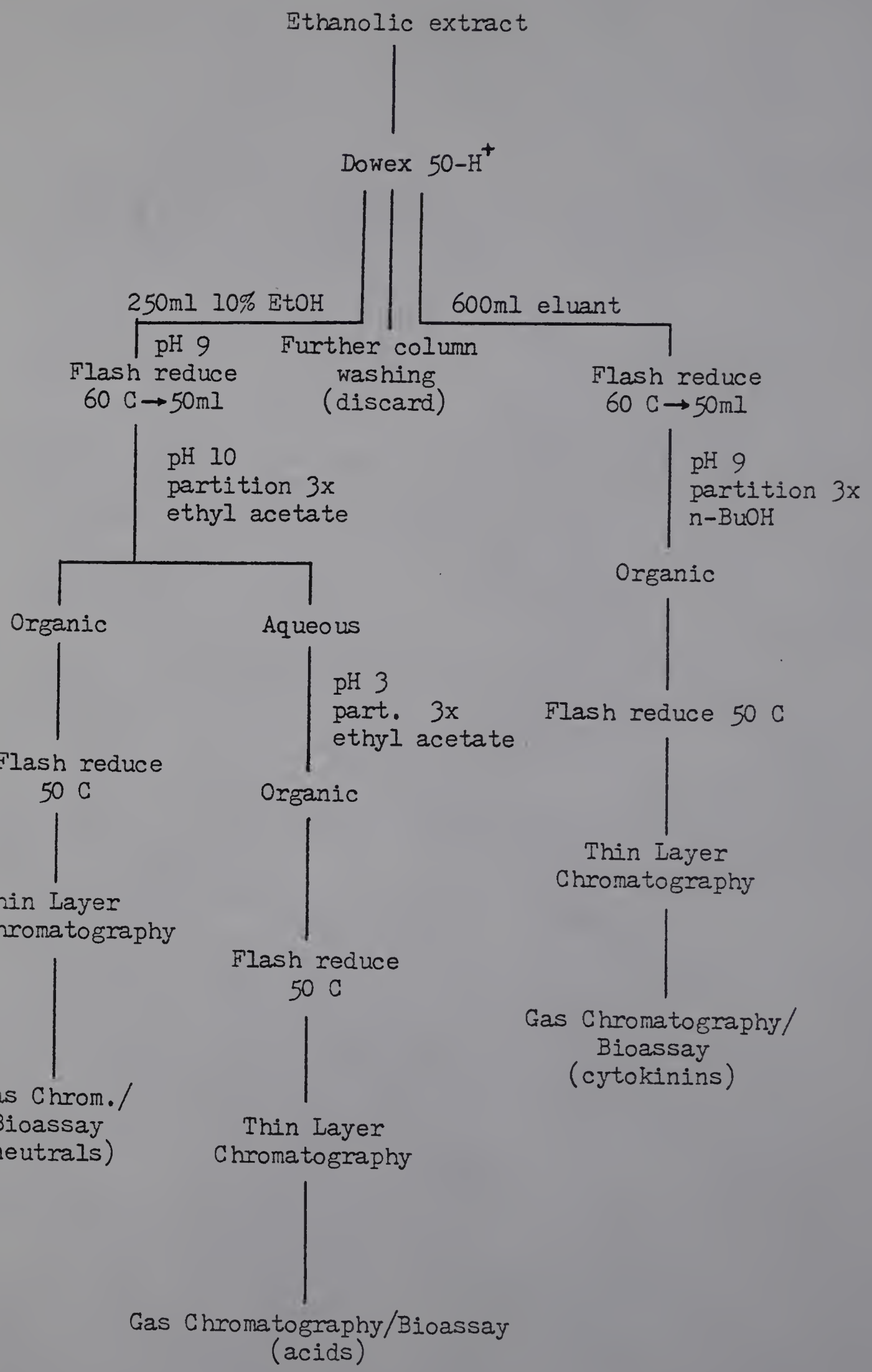
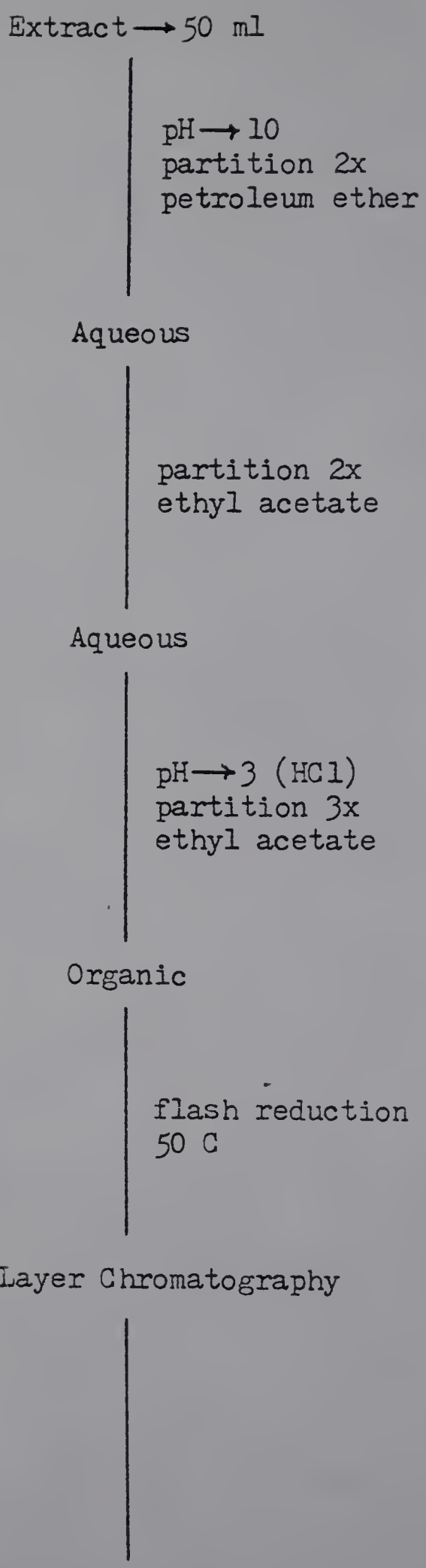

Ethanolic extract
Dowex 50-H+
250ml 10% EtOH
600ml eluant
pH 9
Flash reduce
60 C→50ml
Further column
washing
(discard)
Flash reduce
60 C→50ml
pH 10
partition 3x
ethyl acetate
pH 9
partition 3x
n-BuOH
Organic
Organic
Aqueous
Flash reduce 50 C
pH 3
part. 3x
ethyl acetate
Flash reduce
50 C
Organic
Thin Layer
Chromatography
Thin Layer
Chromatography
Flash reduce
50 C
Gas Chrom./
Bioassay
(neutrals)
Thin Layer
Chromatography
Gas Chromatography/
Bioassay
(cytokinins)
Gas Chromatography/Bioassay
(acids)

Figure 3. Gibberellin Extraction Procedure.
Fractions not shown can be discarded.


Extract → 50 ml
pH → 10
partition 2x
petroleum ether
Aqueous
partition 2x
ethyl acetate
Aqueous
pH → 3 (HCl)
partition 3x
ethyl acetate
Organic
flash reduction
50 C
Thin Layer Chromatography
Gas Chromatography/Bioassay

RESULTS

Partition Coefficients

The formula used for the calculation of the partition coefficients follows:

$$P.C. = \frac{(\text{organic})}{(\text{aqueous})}$$

The quantity of the growth regulator added for all partitioning was experimentally determined to be the maximum amount in the linear region of the dose-response curve (tables 2,3). All quantification was determined by the appropriate bioassay. The partitioning was performed in triplicate and bioassays in duplicate.

Equilibrium distribution of each growth regulator was found to take place within 15 seconds as the distribution of each growth regulator was essentially completed (from ∞ to 0) for successive time intervals (table 2).

The solvents petroleum ether and hexanes did not partition any growth regulator at any pH value (table 3). Ethyl acetate had a greater partitioning ability than ethyl ether for each growth regulator. The carboxylic acid-containing growth regulators partitioned according to acid-base equilibria except for the solvent n-butanol. The neutral auxins did not partition as a function of pH. Cytokinins were partitioned into n-butanol and being weakly basic (93) were only slightly influenced by the pH of the buffer solution.

Table 2. Time Course of Partitioning.

Growth Regulator	Amount Partitioned (μg)	Partitioning Time (sec)				
		0	15	45	90	120
Abscisic acid	50	∞	0	0	0	0
Indoleacetic acid	5	∞	0	0	0	0
Indoleacetamide	50	∞	0	0	0	0
Indoleacetonitrile	10	∞	0	0	0	0
Indoleacetaldehyde	50	∞	0	0	0	0
Gibberellin A_3	200	∞	0	0	0	0
Gibberellin A_{4-7}	88	∞	0	0	0	0
Isopentyl adenine	50	∞	0	0	0	0
Isopentyl adenosine	50	∞	0	0	0	0
Zeatin	8	∞	0	0	0	0
Zeatin riboside	8	∞	0	0	0	0

Standard deviation of all data = 0.0

Table 3. The Partition Coefficients of the Growth Regulators.

Abscisic acid								
pH	3	4	5	6	7	8	9	5% HCO_3^-
petroleum ether	0	0	0	0	0	0	0	0
hexanes	0	0	0	0	0	0	0	0
ethyl ether	1.9	.67	.29	.14	.58	.02	0	0
ethyl acetate	9.0	2.3	0.67	.22	.07	.02	0	0
n-butanol	.92	1.2	1.1	.92	.85	1.0	1.1	1.2
Indoleacetic acid								
pH	3	4	5	6	7	8	9	5% HCO_3^-
petroleum ether	0	0	0	0	0	0	0	0
hexanes	0	0	0	0	0	0	0	0
ethyl ether	2.1	.64	.32	.16	.05	.02	0	0
ethyl acetate	4.6	1.0	.56	.28	.09	.03	.01	.01
n-butanol	.43	.56	.32	.44	.39	.56	.44	.47
Indoleacetamide								
pH	3	4	5	6	7	8	9	5% HCO_3^-
petroleum ether	0	0	0	0	0	0	0	0
hexanes	0	0	0	0	0	0	0	0
ethyl ether	.39	.56	.41	.54	.43	.47	.52	.47
ethyl acetate	.79	.79	.85	.67	.79	.92	.85	.72
n-butanol	.41	.33	.25	.27	.35	.28	.33	.25

Indoleacetonitrile

pH	3	4	5	6	7	8	9	5% HCO_3^-
petroleum ether	0	0	0	0	0	0	0	0
hexanes	0	0	0	0	0	0	0	0
ethyl ether	1.9	1.8	2.1	1.5	1.6	1.9	1.8	1.5
ethyl acetate	4.0	11.5	3.4	5.3	3.2	11.5	4.0	2.6
n-butanol	3.0	4.0	3.2	6.1	11.5	4.0	2.3	5.7

Indoleacetaldehyde

pH	3	4	5	6	7	8	9	5% HCO_3^-
petroleum ether	0	0	0	0	0	0	0	0
hexanes	0	0	0	0	0	0	0	0
ethyl ether	∞	2.3	4.0	∞	5.7	2.3	2.6	4.0
ethyl acetate	5.3	4.0	9.0	3.0	4.6	2.9	6.1	2.3
n-butanol	1.5	9.0	1.1	3.2	2.3	4.0	1.5	3.6

Gibberellin A_3

pH	3	4	5	6	7	8	9	5% HCO_3^-
petroleum ether	0	0	0	0	0	0	0	0
hexanes	0	0	0	0	0	0	0	0
ethyl ether	0.34	0.09	0.02	.01	0	0	0	0
ethyl acetate	3.2	0.26	0.06	.01	0	0	0	0
n-butanol	0.2	0.23	0.24	0.21	0.21	0.20	0.17	0.23

Gibberellin A_{4-7}

pH	3	4	5	6	7	8	9	5% HCO_3^-
petroleum ether	0	0	0	0	0	0	0	0
hexanes	0	0	0	0	0	0	0	0
ethyl ether	2.9	1.2	.32	.10	.03	.01	0	0
ethyl acetate	7.3	3.2	1.5	.44	.11	.03	.01	0
n-butanol	1.1	1.1	1.1	1.0	1.0	.92	.85	.56

Isopentyl adenine

pH	3	4	5	6	7	8	9	5% HCO_3^-
petroleum ether	0	0	0	0	0	0	0	0
hexanes	0	0	0	0	0	0	0	0
ethyl ether	.54	1.7	3.0	1.7	3.0	5.7	3.0	4.0
ethyl acetate	1.9	1.4	4.6	9.0	5.7	11.5	5.7	4.0
n-butanol	∞	6.1	∞	∞	∞	∞	∞	∞

Isopentyl adenosine

pH	3	4	5	6	7	8	9	5% HCO_3^-
petroleum ether	0	0	0	0	0	0	0	0
hexanes	0	0	0	0	0	0	0	0
ethyl ether	.33	.43	.41	.60	1.0	.82	.66	1.5
ethyl acetate	.66	.79	1.0	1.5	2.0	4.0	2.3	4.0
n-butanol	1.5	2.3	4.0	2.3	3.0	4.0	9.0	5.7

Zeatin

pH	3	4	5	6	7	8	9	5% HCO_3^-
petroleum ether	0	0	0	0	0	0	0	0
hexanes	0	0	0	0	0	0	0	0
ethyl ether	.01	.01	.01	.01	.02	.02	.02	.02
ethyl acetate	.04	.04	.05	.06	.06	.09	.08	.07
n-butanol	1.4	2.1	3.0	12	∞	3.0	2.8	∞

Zeatin riboside

pH	3	4	5	6	7	8	9	5% HCO_3^-
petroleum ether	0	0	0	0	0	0	0	0
hexanes	0	0	0	0	0	0	0	0
ethyl ether	0	0	0	0	.01	.01	.01	.01
ethyl acetate	.03	.03	.04	.04	.06	.06	.06	.08
n-butanol	1.0	1.4	2.1	2.3	2.1	1.9	3.0	1.5

P.C. $<0.01 = 0$; P.C. $>15 = \infty$
pH of 5% $NaHCO_3$ = 8.7
Standard deviation usually less than 15% of the P.C.

Separation of the Growth Regulators

Conditions and Percent Recovery

One ml of an ethanolic titer solution contained the specified amounts of growth regulators which were separated as outlined in figure 2 and the percent recoveries were calculated (table 4). The percent recoveries were the averages of three 1 ml separations of the titer solution. The auxins were determined by gas chromatography and the others were determined by bioassay. The acidic and neutral growth regulators were recovered quantitatively within the first 250 ml of 10% ethanol as a second wash (250 ml) of the column contained no further recovery. The cytokinins were not eluted from the column with 750 ml of 10% ethanolic wash. A 600 ml volume of eluant (8% NH_4OH, 10% EtOH) removed the cytokinins from the exchange resin completely as a second 250 ml volume did not further remove cytokinins.

Minimum Detection of the Method

One ml of an ethanolic titer solution contained the specified amounts of the growth regulators (table 5) which were separated as outlined in figure 2 and their presence detected. The quantity added was the least amount of each growth regulator which gave a response in the assay system. Separation was performed in triplicate. The auxins were determined by gas chromatography and the others were determined by bioassay. A plus sign signifies detection of the growth regulator.

Table 4. Conditions and Recovery of the Isolation Procedure.

Growth Regulator	Amount Added (µg)	% Recovery 1st 250ml wash	% Recovery 2nd 250ml wash
Abscisic acid	50	92 ± 8.0	0 ± 0
Indoleacetic acid	500	79 ± 1.7	0 ± 0
Indoleacetamide	500	76 ± 2.1	0 ± 0
Indoleacetonitrile	500	84 ± 2.1	0 ± 0
Gibberellin A_3	200	96 ± 4.0	0 ± 0
Gibberellin A_{4-7}	88	95 ± 6.1	0 ± 0

Growth Regulator	Amount Added (µg)	% Recovery 750ml wash	% Recovery 1st 600ml eluant	% Recovery 2nd 250ml eluant
Isopentyl adenine	60	0 ± 0	80 ± 2.5	0 ± 0
Isopentyl adenosine	60	0 ± 0	84 ± 4.0	0 ± 0
Zeatin	30	0 ± 0	80 ± 10	0 ± 0
Zeatin riboside	30	0 ± 0	87 ± 13	0 ± 0

Table 5. Minimum Detection of the Method.

Growth Regulator	Amount Added (ng)	Detection*
Abscisic acid	50	+,+,+
Indoleacetic acid	500	+,+,+
Indoleacetamide	5000	+,+,+
Indoleacetonitrile	1500	+,+,+
Gibberellin A_3	80	+,+,+
Gibberellin A_{4-7}	35	+,+,+
Isopentyl adenine	500	+,+,+
Isopentyl adenosine	500	+,+,+
Zeatin	20	+,+,+
Zeatin riboside	20	+,+,+

* Performed in triplicate, + signifies detection of the growth regulator after separation of the titer solution.

Check of the Method Using Apple Seed

Seed extracts were prepared from five tree replicates harvested 60 days after bloom with one-half of each extract separated for measurement of the endogenous amount of each growth regulator and the other half spiked with the specified amount of each growth regulator to calculate the percent recovery (table 6). The levels were calculated on a ng/g fresh weight basis. No auxins were detected in the nonspiked extract though an appreciable amount (79%) of the spike was recovered. Zeatin had the greatest variability of the growth regulators between tree replicates. Abscisic acid and gibberellin A_{4-7} were detected. No other hormones were detected. The growth regulators detected were identified by chromatographic properties and/or bioassay specificity.

Stability of Auxins and Cytokinins to Isolation Techniques

Auxins were tested for sublimation during flash evaporation and cytokinins for hydrolysis on Dowex 50. The percent recoveries of the neutral auxins (indoleacetonitrile 94%, indoleacetamide 85%) preclude the possibility of significant loss by sublimation. Indoleacetic acid was flash evaporated buffered at pH 8.5 and 3.0 to compare its sublimation properties in the dissociated and non-dissociated form (table 7). The more stable salt form had a recovery of 85% whereas no indoleacetic acid was detected from the acidic form.

Isopentyl adenosine and zeatin riboside were recovered from the exchange column 90 and 95% respectively. Their small loss

Table 6. Endogenous Levels of the Growth Regulators.

Growth Regulator	Endogenous Measurement (ng/g FW)	Spike (μg)	% Recovery	Endogenous Amount (ng/g FW)
Abscisic acid	109 ± 15	4	90 ± 4.2	121 ± 15.5
Indoleacetic acid	0 ± 0*	10	78 ± 6.5	0 ± 0 (<50 ng)
Indoleacetamide	0 ± 0*	10	79 ± 8.5	0 ± 0 (<500 ng)
Indoleacetonitrile	0 ± 0*	10	79 ± 2.1	0 ± 0 (<100 ng)
Gibberellin A_{4-7}	330 ± 26	10	81 ± 2.6	407 ± 32
Zeatin	374 ± 388	10	90 ± 4.9	416 ± 431

$$\% \text{ Recovery} = 100 \times \frac{\text{spiked measurement - endogenous measurement}}{\text{amount of spike}}$$

* Not detected with avena coleoptile bioassay or gas chromatography with a flame ionization detector.

Abscisic acid identified by 2-dimensional thin layer chromatography and co-chromatography on 3% SE-30 and 1.5% OV-17 with an electron capture detector.

Gibberellin A_{4-7} identified by 2-dimensional thin layer chromatography and specificity of the lettuce hypocotyl bioassay.

Zeatin identified by 2-dimensional thin layer chromatography and specificity of the tobacco callus bioassay.

Table 7. Stability of Auxins and Cytokinin Ribosides to Isolation Techniques.

Growth Regulator	Amount Added (μg)	% Recovery *
Indoleacetonitrile	5	94 ± 1.0
Indoleacetamide	5	85 ± 4.0
Indoleacetic acid pH 8.5	50	85 ± 8.0
Indoleacetic acid pH 3	50	0 ± 0.0
Isopentyl adenosine	400	90 ± 5.0
Isopentyl adenine	0	0 ± 0.0
Zeatin riboside	400	95 ± 1.0
Zeatin	0	0 ± 0.0

* Average of 3 trials, assayed by gas chromatography.

and the lack of the hydrolysis products, isopentyl adenine and zeatin, eliminate hydrolysis of the ribosides by the exchange resin (table 7).

Involvement of Gibberellins in Biennial Bearing

Effect of Gibberellin Spray Treatment on Flower Bud Initiation

Gibberellin sprayed limbs were evaluated the spring following treatment. In April, limbs from three of the six tree replicates were forced in a greenhouse with the remainder left on the trees to await normal bloom. There was no significant difference between the groups. Data of the return bloom of spurs (table 8) showed that both gibberellin A_3 and A_{4-7} at 30 ppm were ineffective in reducing flower initiation. Each gibberellin was effective at 300 ppm with gibberellin A_{4-7} having almost twice the inhibiting effect. In each of the latter treatments there was no difference between the single and repeat application.

The length of the spurs was measured to determine the effect of the gibberellin treatment on growth (table 9). The length of the flowering spurs (1.05 cm) was significantly greater than the length of the vegetative spurs (0.99 cm). This measured difference was not because of the treatments applied, the tree variability, or their interaction (table 9, analysis of variance).

The effect of gibberellin on the bloom of one-year-old wood was calculated three ways: bloom/cm shoot length, % flowering, bloom/cm limb circumference (table 10). When calculated as bloom/cm

Table 8. Effect of Gibberellin Spray Treatments on the Flowering of Spurs.

Treatment (ppm)	% Return Bloom
Control	99 a
GA_3 30	96 a
GA_3 30 R	91 a
GA_{4-7} 30	94 a
GA_{4-7} 30 R	95 a
GA_3 300	81 b
GA_3 300 R	76 b
GA_{4-7} 300	62 c
GA_{4-7} 300 R	60 c

Mean separation by Duncan's multiple range test, 5% level.

Table 9. Effect of Gibberellin Spray Treatments on the Growth of Spurs.

Treatment (ppm)	Spur	Spur Length * (cm)
Control	Flowering	1.03
	Vegetative	0.97
GA_3 30	Flowering	1.07
	Vegetative	0.94
GA_3 30 R	Flowering	0.98
	Vegetative	0.96
GA_{4-7} 30	Flowering	1.05
	Vegetative	0.92
GA_{4-7} 30 R	Flowering	1.08
	Vegetative	1.04
GA_3 300	Flowering	1.04
	Vegetative	0.94
GA_3 300 R	Flowering	1.07
	Vegetative	0.98
GA_{4-7} 300	Flowering	1.05
	Vegetative	1.04
GA_{4-7} 300 R	Flowering	1.08
	Vegetative	1.10

* Average of approximately 6 spurs.
Average of flowering spurs = 1.05 cm.
Average of vegetative spurs = 0.99 cm.

Analysis of Variance

Source	F (5%)
Treatment	Not significant
Trees	Not significant
Spurs	Significant
Treatment-Trees	Not significant
Treatment-Spurs	Not significant
Trees-Spurs	Not significant

Table 10. Effect of Gibberellin Spray Treatments on Lateral Bloom.

Treatment (ppm)	Bloom/cm shoot length	% Flowers *	Bloom/cm limb circumference
Control	0.170 a	83 a	13.5 bc
GA_3 30	0.068 b	39 ab	20.0 a
GA_3 30 R	0.053 b	61 a	12.8 bcd
GA_{4-7} 30	0.100 ab	64 a	15.7 b
GA_{4-7} 30 R	0.100 ab	62 a	15.0 b
GA_3 300	0.072 b	46 ab	14.1 b
GA_3 300 R	0.010 b	9.5 b	12.0 bcd
GA_{4-7} 300	0.067 b	42 ab	8.9 d
GA_{4-7} 300 R	0.067 b	40 ab	9.6 cd

* Percentage of buds which broke.

Mean separation in columns by Duncan's multiple range test, 5% level.

shoot length each treatment except gibberellin A_{4-7} 30 ppm and gibberellin A_{4-7} 30 R had a significant inhibiting effect although there was no dependence on concentration or time of application. Only gibberellin A_{4-7} 300 R had an inhibiting effect when bloom was calculated as % flowering. When expressed as bloom/cm limb circumference gibberellin A_3 30 ppm had a significant effect on flower promotion whereas only gibberellin A_{4-7} 300 ppm had an inhibiting effect.

The effect of the gibberellin treatments on growth of one-year-old wood was calculated two ways: average growth (length of laterals/no. of laterals) and total growth (sum of laterals) (table 11). There was no effect of the gibberellin treatments on the average growth of the shoots and total growth was significantly increased only with gibberellin A_{4-7} 300 ppm.

Timing of Flower Bud Initiation

Spurs from which flowers or fruit were removed were counted for flowering the following spring. The percent flowering versus time of removal was plotted to determine the interval of flower bud initiation (table 12, figure 4). There was a complete return bloom of spurs to full bloom +45. Fruit removed at FB +105 (harvest) did not initiate flowers. Flowers or fruit were removed from 8 spurs/tree/day from five tree replicates.

Time Course Extraction of Gibberellins

Extracts of seed, diffusate, and vegetative spurs were prepared from four tree replicates. The diffusate had a recovery

Table 11. Effect of Gibberellin Spray Treatments on the Growth of One-Year-Old Wood.

Treatment (ppm)	Average Growth (cm)	Total Growth (cm)
Control	24.5 a	92.6 a
GA_3 30	26.9 a	100.3 a
GA_3 30 R	33.8 a	162.8 a
GA_{4-7} 30	30.6 a	75.2 a
GA_{4-7} 30 R	33.5 a	106.0 a
GA_3 300	37.3 a	129.3 a
GA_3 300 R	31.1 a	153.8 a
GA_{4-7} 300	41.5 a	262.0 b
GA_{4-7} 300 R	38.9 a	101.7 a

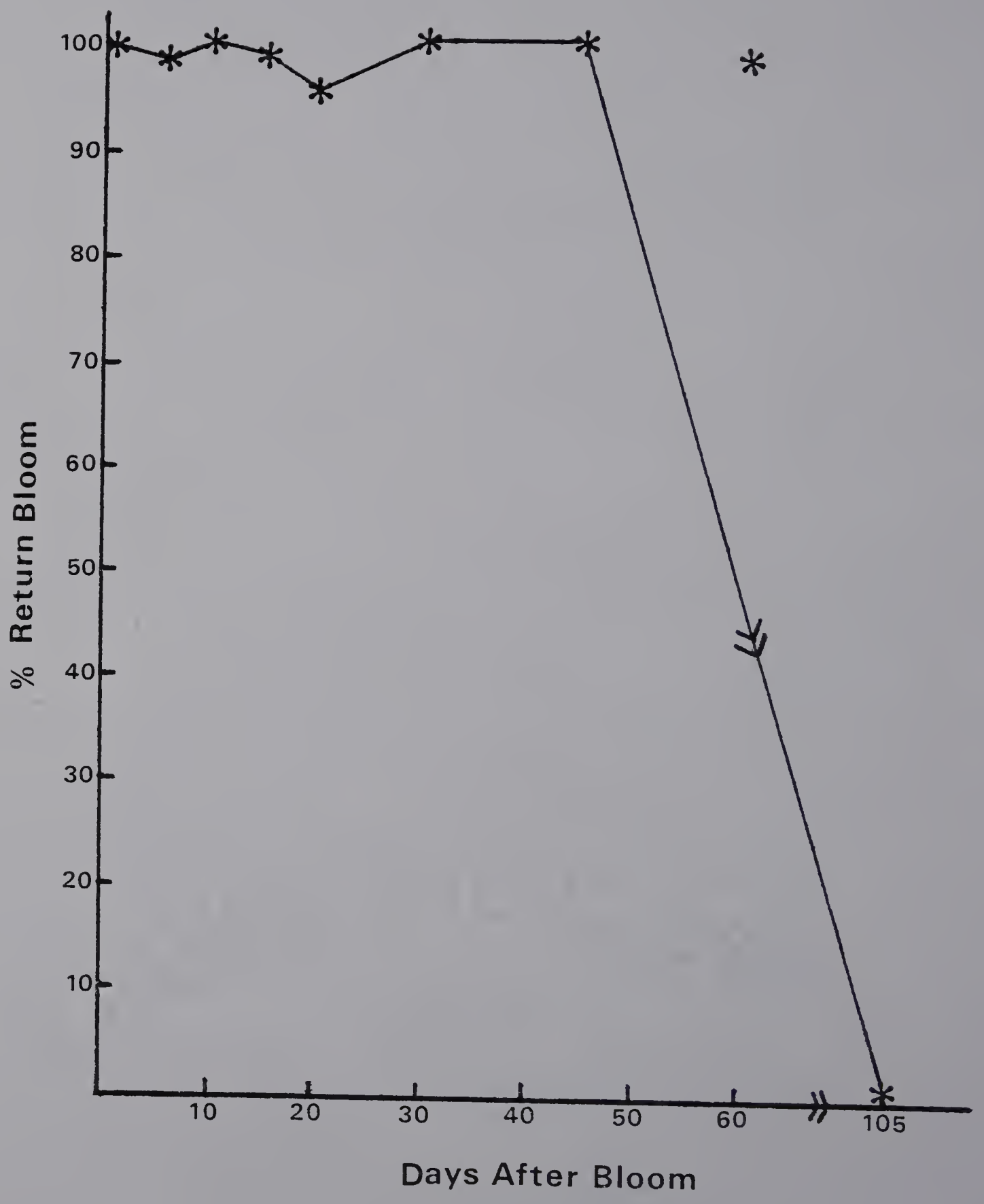
Mean separation in columns by Duncan's multiple range test, 5% level.

Table 12. Effect of Fruit Removal on the Return Bloom of Spurs.

Removal Date (FB +)	% Return Bloom
0	100 a
5	98 a
10	100 a
15	98 a
20	95 a
30	100 a
45	100 a
60	98 a
105 (harvest)	0 b

Mean separation in columns by Duncan's multiple range test, 5% level.

Figure 4. Effect of Fruit Removal on the Return Bloom of Spurs.


100
90
80
70
60
50
40
30
20
10
% Return Bloom
10
20
30
40
50
60
105
Days After Bloom

of 94%, the seed 81%, and the spurs 80% (table 13). These percent recoveries were used to calculate the actual levels of gibberellin A_{4-7} in the tissues. The lettuce hypocotyl bioassay was used for gibberellin measurement.

Seed was extracted at various dates after bloom and the gibberellin A_{4-7} content analysed (table 14). The gibberellins were present at each harvest date with the highest amount (640 ng/g FW) at FB +5 and the lowest amount (21 ng/g FW) at FB +30. A time plot of gibberellin levels (figure 5) contained three peaks of gibberellin activity: FB +5, FB +15, and FB +60.

Fruit were weighed before and cut open after diffusion and a seed count taken (table 15). The gibberellin levels have the recovery factor (94%) included. The amount of gibberellin A_{4-7} collected decreased from 39.1 ng (FB) to 6.2 ng (FB +60). The number of fruit was decreased as their size (weight) increased. The number of seed per fruit decreased from 8 seed or ovules (FB) to 4 seed (FB +60). The gibberellin levels were plotted on a pg/seed (figure 6) and ng/g fruit basis (figure 7). The gibberellin levels in the former generally had an increase in amount with time while the latter had a decrease (hyperbolic curve). The gibberellin levels at FB +5 does not reflect this trend and is lower in value because of frost damage at that harvest date.

The vegetative and fruited spurs were analysed for gibberellin content and the time course plotted (table 16, figure 8). No time related trend was evident from either spur analysis. The fruited

Table 13. Percent Recovery of Gibberellin A_{4-7} from Tissues.

Tissue	Harvest Date (FB +)	Spiked Amount (ng)	% Recovery
Seed	60	10,000	81 ± 2.6
Diffusate	10	20	94 ± 6.6
Vegetative Spurs	45	90	80 ± 3.2

Table 14. Levels of Gibberellin A_{4-7} in Seed.

Date of Harvest (FB +)	Measured Amount (ng/g FW)	Actual Amount (ng/g FW)
5	518 ± 171	640 ± 211
10	65 ± 24	80 ± 30
15	260 ± 165	321 ± 204
20	34 ± 6.4	42 ± 7.9
30	17 ± 6.0	21 ± 7.4
45	275 ± 230	340 ± 284
60	330 ± 26	407 ± 32

Figure 5. Levels of Gibberellin A_{4-7} in Seed.

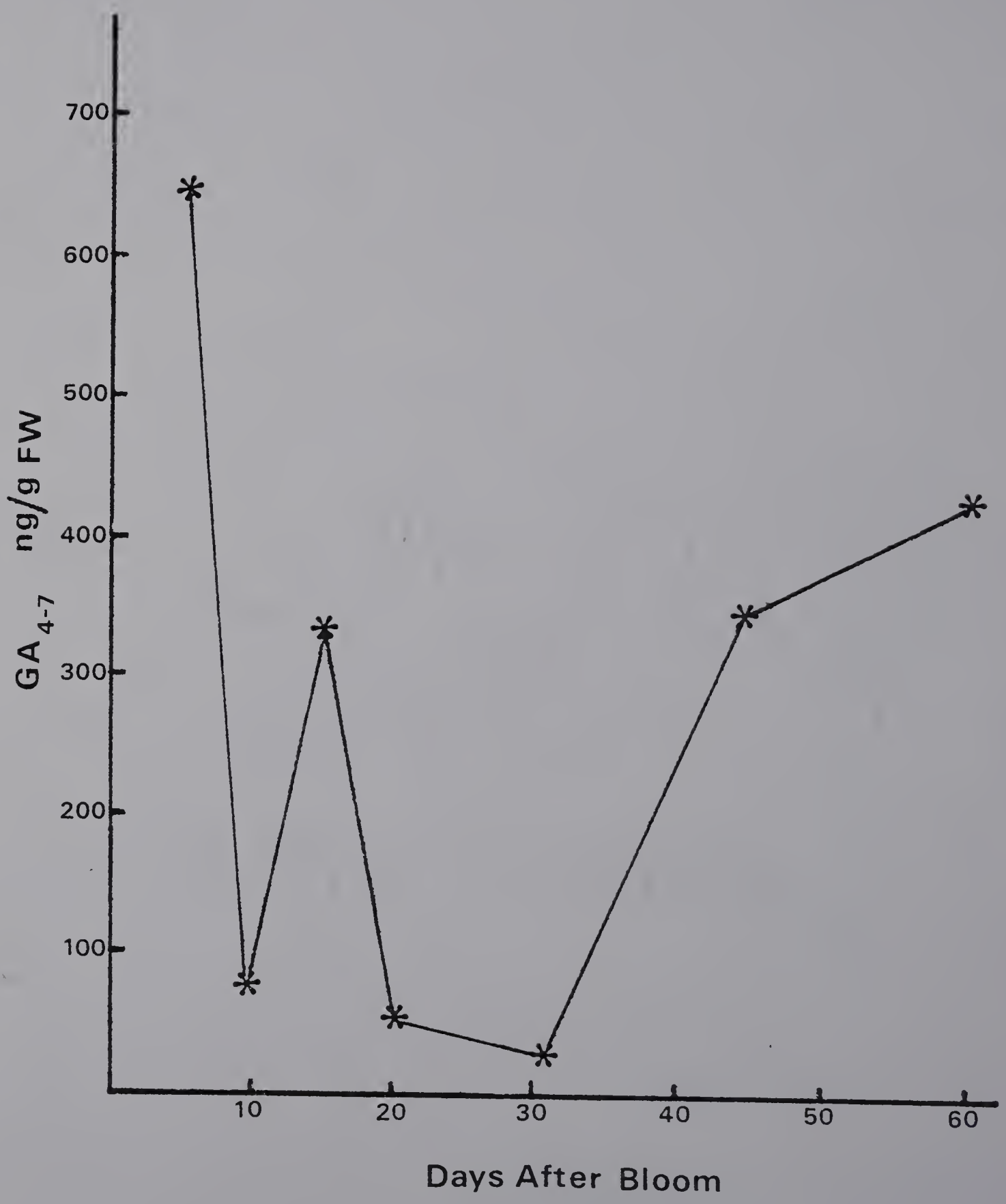

700
600
500
400
300
200
100
GA_{4-7} ng/g FW
10
20
30
40
50
60
Days After Bloom

Table 15. Levels of Gibberellin A_{4-7} in Fruit Diffusate.

Harvest (FB +)	Actual Amount (ng)	# Fruit x # seed/fruit	pg GA_{4-7}/ seed	Total Wt. Fruit (g)	ng GA_{4-7}/ g fruit
0	39.1 ± 14	15 x 8	327 ± 113	2.06	19.0 ± 6.8
5	10.0 ± 2	15 x 8	84 ± 17	2.30	4.30 ± .87
10	37.8 ± 30	10 x 9	421 ± 328	2.71	13.9 ± 11
15	19.2 ± 14	10 x 6	320 ± 237	5.92	3.20 ± 2.4
20	18.9 ± 7	5 x 5	757 ± 281	15.27	1.24 ± .46
30	8.8 ± .6	2 x 4	1106 ± 72	25.88	0.34 ± .02
45	8.1 ± 2.3	1 x 4	2016 ± 585	35.23	0.23 ± .07
60	6.2 ± .77	1 x 4	1542 ± 191	58.00	0.11 ± .01

Figure 6. Levels of Gibberellin A_{4-7} in Fruit Diffusate, Seed Number Basis.

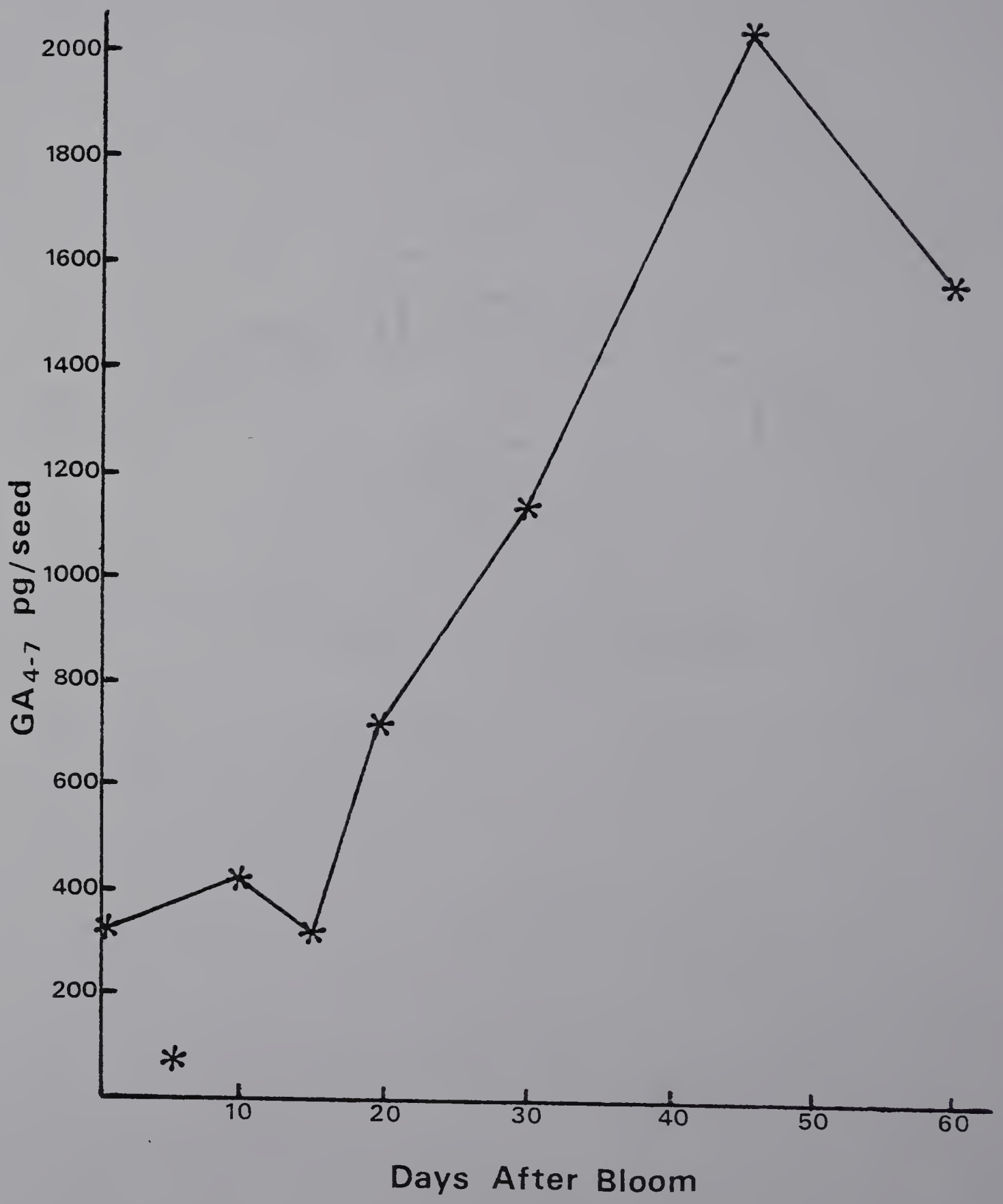
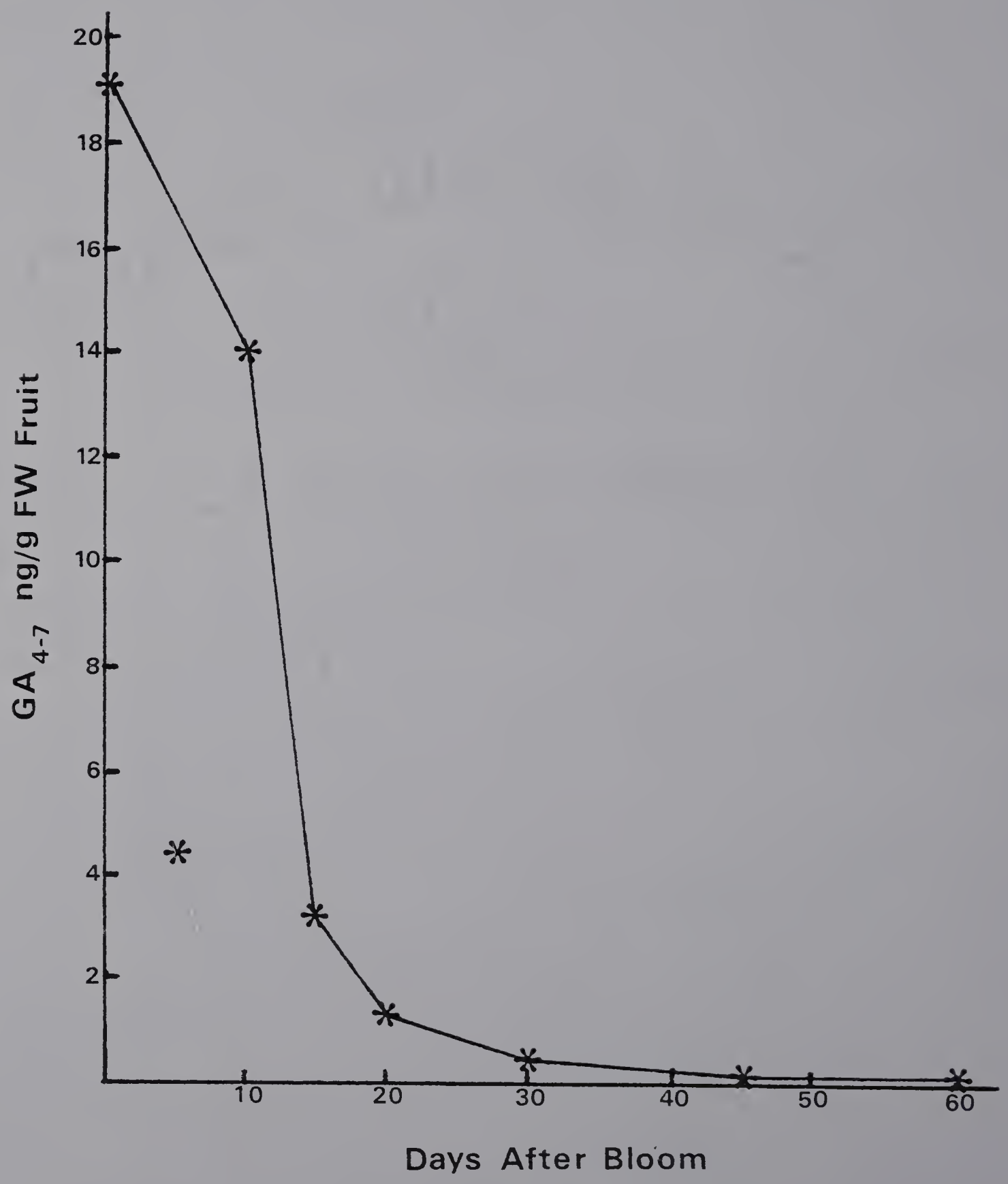

2000
1800
1600
1400
1200
1000
800
600
400
200
GA4-7 pg/seed
10
20
30
40
50
60
Days After Bloom

Figure 7. Levels of Gibberellin A_{4-7} in Fruit Diffusate, Fresh Weight Basis.


20
18
16
14
12
10
8
6
4
2
GA_{4-7} ng/g FW Fruit
10
20
30
40
50
60
Days After Bloom

spurs contained a significantly greater amount of gibberellin activity than the vegetative spurs (analysis of variance).

Statistical Analyses of Data

All means were separated by Duncan's multiple range. Means followed by the same letter are not significantly different at the 5% level. Data of extraction analyses contain the mean ± standard deviation of the mean and comparisons were made with the analysis of variance.

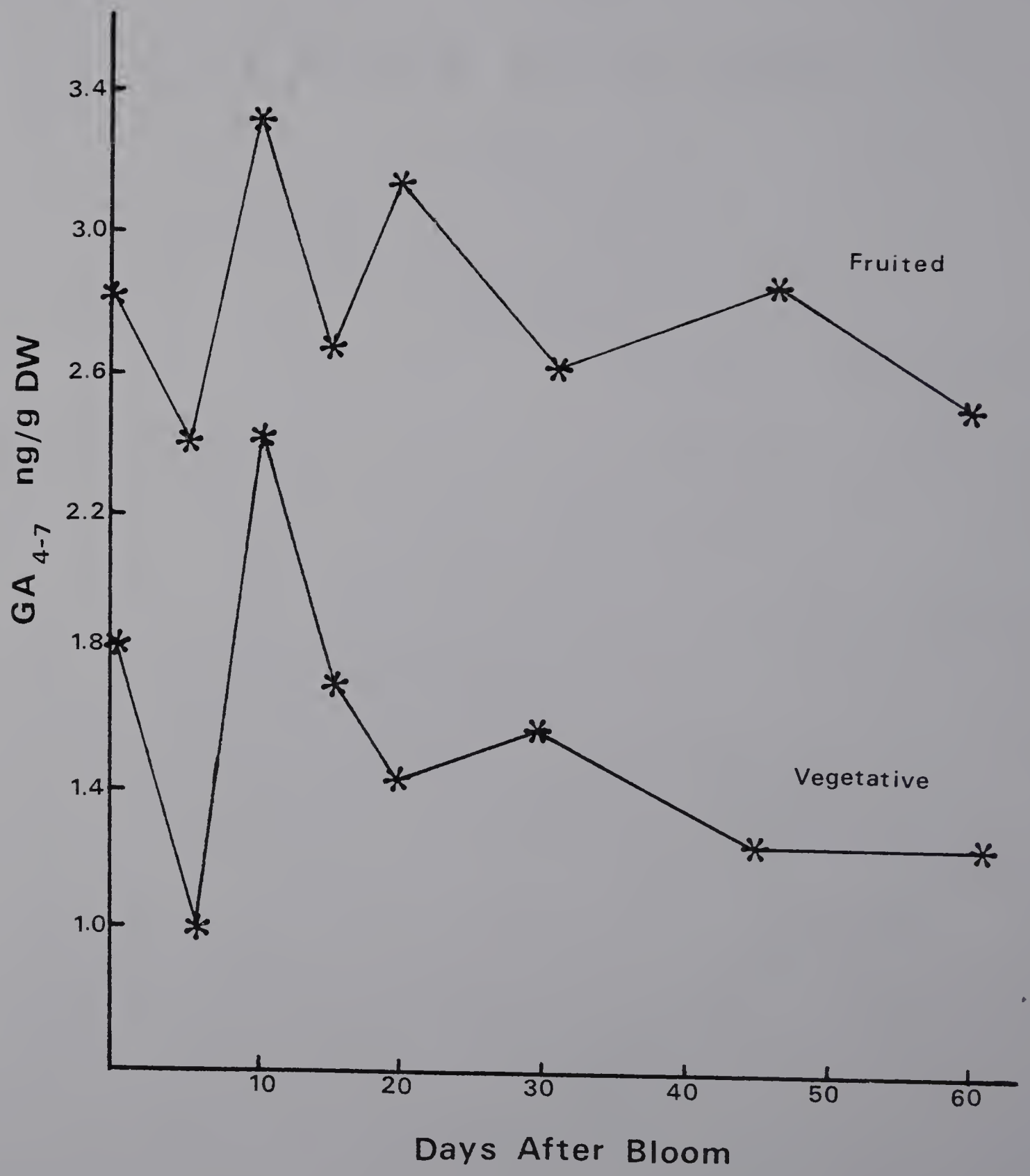
Table 16. Levels of Gibberellin A_{4-7} in Spurs.

Date of Harvest (FB +)	Actual GA_{4-7} Vegetative ng/g DW	Actual GA_{4-7} Fruited ng/g DW
0	1.78 ± 0.16	2.81 ± 0.40
5	0.98 ± 0.19	2.39 ± 0.35
10	2.55 ± 0.63	3.30 ± 0.49
15	1.73 ± 0.24	2.65 ± 0.36
20	1.43 ± 0.24	3.14 ± 0.50
30	1.56 ± 0.38	2.65 ± 0.35
45	1.25 ± 0.50	2.85 ± 0.43
60	1.28 ± 0.51	2.41 ± 0.29

Analysis of Variance

Source	F (5%)
Trees	Not significant
Days	Significant
Spurs	Significant
Trees-Days	Not significant
Trees-Spurs	Not significant
Days-Spurs	Not significant

Figure 8. Levels of Gibberellin A_{4-7} in Fruited and Vegetative Spurs.


3.4
3.0
2.6
2.2
1.8
1.4
1.0
ng/g DW
GA 4-7
Fruited
Vegetative
10
20
30
40
50
60
Days After Bloom

DISCUSSION

Partition Coefficients

The time of partitioning necessary to bring the distribution of the growth regulators to equilibrium was established by choosing conditions which would yield a quantitative partition coefficient. The growth regulator was added to the organic phase (petroleum ether) and partitioned into the aqueous phase. Under these conditions a bioassay of the organic phase yielded a partition coefficient approaching zero, which was easily determined. In the time course extraction each growth regulator came to equilibrium within 15 seconds (table 2). Since the basic structure and physico-chemical properties of a class of growth regulators are the same, it was assumed that equilibrium was achieved within 15 seconds for each solvent and pH (41). In partitioning of tissue extracts the time was increased to one minute to account for other substances.

Several trends developed in the partition coefficients (table 3). The solvents, petroleum ether and hexanes, partitioned no growth regulators at all pH values. Hence, these, the lipid solvents, were used to remove fats, chlorophyll, or inhibitors but to leave the growth regulators in the aqueous phase (72, 93). The growth regulators which contain a carboxylic acid group displayed an acid-base relation to the pH of the buffer. At high pH values when the carboxylic acid group would be ionized, the growth regulators were water soluble. At lower pH values the carboxylic

acid group was in the unionized form and being uncharged and less polar was more lipid soluble. All carboxylic acid group growth regulators except gibberellin A_{4-7} had an actual partition coefficient value of zero at pH 9. For gibberellin A_{4-7} extraction with ethyl acetate had to be increased to pH 10 for an actual value of zero (41). It is, therefore, necessary to partition gibberellin A_{4-7} carefully so that it is not mistaken for a neutral growth regulating compound. The solvent n-butanol (water saturated) did not partition these growth regulators according to acid-base equilibria. This may be due to the large amount of water held by the n-butanol (10%) or the ability of n-butanol to stabilize the charged carboxylic acid group (41). As would be expected, neutral auxins did not partition according to pH. Therefore, at a high pH the neutral and acidic compounds can be completely separated by partitioning (88). Ethyl acetate was the best solvent to remove the neutral auxins and, at low pH values the acidic growth regulators. Indoleacetaldehyde is an extremely unstable compound in the free state (137). It turned an orange-brown within 30 minutes of conversion from the bisulfite addition complex. The compound formed was active in the Avena test and its values were recorded.

The cytokinins contain nitrogen atoms which are weakly basic and would be expected to have the largest partition coefficients at higher pH values; however, the effect was not as extreme as the carboxylic acid containing growth regulators. For each cytokinin, n-butanol was the solvent which gave the largest value. Compared

to the non-sugar containing cytokinins, the sugar containing cytokinins were more soluble in the aqueous layer and the hydroxylated zeatin and zeatin riboside were also more soluble in the aqueous layer than the non-hydroxylated cytokinins. This may be caused by the increased hydrogen bonding. The partition coefficients were recorded within the range of 0.01 to 15.0; outside of this, differences are negligible.

Hemberg and Westlin (72) using kinetin in partitioning as a model cytokinin assumed that other cytokinins would act similarly. This assumption is not valid considering the partition coefficients presented here, those of Letham (93), Purse (136), and Park (129). While the partition coefficients presented here differ somewhat from those of Letham or Purse, the transformation of the value expressed as a percentage extracted is in closer agreement. This is because the partition coefficient is a quotient. This also holds for a comparison of the gibberellin values determined by Durley and Pharis (41). Larsen (88) determined that indoleacetic acid can be completely partitioned from an extract using ether-aqueous volume proportions of 1:1 or 2:3 in the pH range of 2.5 - 4.7 with three subsequent extractions. Atsumi et al. (6) reported ethyl ether and dichloromethane to be effective in partitioning indoleacetic acid. The above two reports are in close agreement with the indoleacetic acid values determined here. When the partition coefficients of Durley and Pharis (41) for gibberellin A_3 and A_{4-7} are inverted, there is a close quantitative agreement with those

determined here. Therefore, major emphasis was placed on partitioning as a means of clean up (chlorophyll, lipids) or separation of the growth regulators (neutral from acidic) in the extraction method.

Method of Separation of the Growth Regulators

Conditions were determined to streamline purification and separation of each class of growth regulators with respect to time, work, and efficiency. A maximum recovery was desired for separation of those growth regulators known or thought to be found in apple. The criteria of the isolation procedure were as follows:

1. Percent Recovery - efficiency
2. Percent Loss - justifiable discards
3. Sensitivity - minimum detection
4. Qualitative Design - maximum effect of separation
5. Quantitative Aspects - assay system's sensitivity, precision, specificity, accuracy.

The techniques used were to take advantage of the physico-chemical properties of the molecules; hence, the properties to be exploited are solubility, charge, and adsorption. A logical sequence of the techniques was determined for maximum effect and from knowledge of the partition coefficients.

A titer solution of pure growth regulators was separated to maximize the conditions of isolation. Differences between the recovery values of the pure compounds and those of an extract would be caused by interferences from within the extract. The sequence of

techniques for the maximum recovery of pure compounds and clean up of an extract follows:

Partitioning→Cation Exchange→Partitioning→Thin Layer Chromatography→Bioassay/Gas Chromatography

In table 4, the percent recoveries of the auxin determinations were analysed by gas chromatography as bioassay results varied greatly. Indoleacetaldehyde was too unstable to be included. The percentage of indoleacetamide is somewhat low due to tailing of its peak on the chromatogram indicating breakdown in the column and an inexact triangulation of peak areas. Indoleacetic acid is an unstable compound toward some isolation procedures as noted by Mann and Jaworski (106), and manipulation of the fraction containing it was carried out at an alkaline pH to convert it to the more stable ionic form.

The acidic and neutral growth regulators were recovered from the column quantitatively in the first 250 ml wash of 10% ethanol, as a second 250 ml wash contained no growth regulators. The cytokinins were shown to be held tightly on the column as a 750 ml wash of the bed released no detectable amounts, allowing this to be a major clean up of cytokinins. A 600 ml volume of eluant (8% NH_4OH, 10% EtOH) removed 80 to 87% of the cytokinins with a second 250 ml volume of eluant releasing no detectable amounts. These data suggest that binding is ionic and not adsorptive as previously stated (172). The polar and non-ionic 10% ethanolic solution did not desorb the cytokinins, whereas the ion (NH_4^+)

containing eluant released them by an exchange. These volume data and the partition coefficients were considered in the flow chart of the isolation procedure (figure 2) to justify discarding all the fractions not shown. To insure that a minute amount of the growth regulators present in an extract would not be irreversibly bound or lost in the isolation procedure, a minimum detection was performed (table 5). The titer solution contained that amount which would be just detectable if assayed directly; therefore, the assay system chosen would be limiting in detection. In triplicate separations each growth regulator was detected each time. Therefore, the techniques or materials employed have no binding of the growth regulators in themselves.

Two differences were included in the general use of the cation exchange columns (90, 117) which became evident with the use of tissue extracts. First, with a cytokinin concentration of 10^{-6} - 10^{-7} molar, competition for the exchange sites would probably favor the mineral cations lessening the efficiency of the column unless a large bed volume would compensate. Therefore, a block of partitioning conditions was included to extract the neutral, acidic, and basic growth regulators into the organic layers before the use of the column. The mineral cations remained in the aqueous phase. Besides the competition factor, large amounts of acid would be generated with exchanging. Second, the extract was not acidified before addition to the column for similar reasons. Acidification to pH 2-3 greatly increases the chance of the hydrogen ion replacing

the exchanged cytokinins in the column. A strong cation exchange resin can protonate and thereby charge the cytokinin itself. There were reports of the hydrolysis of cytokinin ribosides with the use of strong cation exchange resins (33, 42, 82, 116, 162). This was not found with this method of addition and elution of the column (table 7). To demonstrate this, isopentyl adenosine and zeatin riboside were added to the column and recovered 90% and 95% respectively, with no isopentyl adenine or zeatin detectable. Therefore, with this addition and elution, there was no hydrolysis of these cytokinins. In the reports hydrolysis could have been attributed to acidification of the extract, acid generation in the exchange of other cations, or heat generation on elution.

It was stated by Mann and Jaworski (106) that indoleacetic acid was unstable to oxygen and was sublimable. An antioxidant and work under nitrogen were necessary along with gentle conditions in flash reduction. Here, indoleacetic acid has not been found to be unstable toward oxygen during actual isolation (table 4) as no antioxidant or nitrogen was added, and the percent recovery was acceptable (79%). However, with flash reduction, comparing sublimable properties of indoleacetic acid in the non-dissociated and dissociated forms, the recoveries were 0% in the former and 85% in the latter form (table 7). This suggests either sublimation or acidic destruction (106, 88) in the non-dissociated form, whereas, there were stability and no sublimation in the dissociated form. Therefore, in flash reduction, when relatively high temperatures were needed to reduce

the aqueous solutions, the pH was adjusted to alkalinity (pH 8) because of the indoleacetic acid. A check for sublimation of indoleacetonitrile and indoleacetamide was performed with recoveries, 94% and 85% respectively, high enough to exclude loss by sublimation.

Extraction of Apple Seed

The extraction of apple seed, a rich source of growth regulators, was chosen to test the method and determine percent recovery from a natural source (table 6). All the previously separated growth regulators were sought; however, only abscisic acid, gibberellin A_{4-7}, and zeatin were detected. The literature contains references of the presence of gibberellin A_3 (71) in special circumstances and gibberellin A_9 (153) in trace amounts. Zeatin riboside, an apple cytokinin (92), was not detected. Though present in apple (39, 40, 143), no auxins were detected, but the percent recoveries of the extraction exclude the possibility they were lost in isolation.

For the gibberellins and cytokinins the respective areas of the thin layer plates were scraped for the compounds sought (gibberellin A_3 and A_{4-7}, isopentyl adenine, isopentyl adenosine, zeatin and zeatin riboside). Then, the rest of the plate was eluted and bioassayed for any other activity, none being found. However, inhibitors present could mask their detection.

The column of the 'endogenous amount' represents that quantity of the hormone actually found in the tissue for it includes the percent recovery factor of the extract. An assumption being

made is that all of the endogenous hormone is initially being extracted by methanol from the tissue. Methanol is considered by McDougall and Hillman (111) to be an efficient solvent for the initial extraction as it causes breakdown of plastids and has denaturing properties. The arbiter of the tissue extraction was taken to be the removal of a pigment. Although Milborrow and Mallaby (114) reported the formation of methyl (+)-abscisate when methanol was selected as a extractant under neutral or basic conditions, our percent recovery precludes its formation.

The greatest variation of the tissue hormonal levels was for the cytokinin zeatin. The only percent recovery which differed from the corresponding values of pure compounds was that of gibberellin A_{4-7} (table 4, 6). This suggests a loss caused by some interference of the extract.

The Involvement of Gibberellins in Biennial Bearing

The inhibiting effect of flowering by gibberellin spray treatments is well documented for apple (34, 55, 64, 100, 107, 108, 161, 175) and other species (5, 17, 77, 149). The data in table 8 reveal that a concentration of 30 ppm for both gibberellin A_3 and A_{4-7} was not sufficient to reduce flowering of spurs. At a concentration of 300 ppm there was a significant reduction of 20% for gibberellin A_3 and 40% for gibberellin A_{4-7}. The latter, being the naturally occurring gibberellin, had twice the inhibiting effect. An interesting point of these data is no significant difference between the single dose and repeat application. This non-difference

may be a saturation effect at this concentration, or it may be that exogenous gibberellin was no longer effective at the time of the repeat application (Luckwill, 100). There was no visual difference in flower quality (clusters with more than two well-developed flowers; Tromp, 164) caused by treatments, contrary to that observed by him. An indication of the biennialness of 'Early McIntosh' can be seen from the complete return bloom of the control treatment. In the year of treatment it was totally vegetative (table 8).

Data of spur growth (table 9) were analysed for differences caused by the gibberellin treatments. There was no statistical difference in length caused by tree variability, the gibberellin treatments, or their interaction. Since there is no difference in length within the spurs because of the different treatments, a reduction in flowering caused by increased growth is eliminated, and the flowering reduction is attributed to a more direct hormonal action. A significant difference exists in a comparison of the spurs: flowering versus vegetative. The greater growth of flowering spurs (growth not caused by gibberellin treatments) must be an endogenous effect proper to flower initiation or coinciding with it.

All spurs were vegetative the year of treatment. Until the time of flower initiation, there would be no distinction in the spurs and there would not be any growth differences. Once flower initiation has taken place there is a distinction between

the spurs and biochemical differences probably exist. Hence, the greater growth of the flowering spurs begins after flower initiation and this greater growth of flowering spurs opposes the negative correlation of growth and flowering.

The data of tables 10 and 11 demonstrate the effect of gibberellin spray treatments on the flowering and growth of one-year-old wood. There was large variation in the tree replicates and a good test of significance was not obtained for either. Three ways of expressing the flowering are presented. When the flowering was calculated on a bloom/cm shoot length, a decrease in flowering with increasing gibberellin concentration was obtained. At 30 ppm gibberellin A_3 caused a significant reduction while gibberellin A_{4-7} did not. Each 300 ppm concentration had a large effect on flower reduction but did not differ significantly between the gibberellins or between the single and repeat application. When flowering was expressed as the percentage of the buds which broke on one-year-old wood, only gibberellin A_3 (R) at 300 ppm had a significant effect in reducing the flowering. When the flowering was expressed as bloom/cm-limb circumference, the effect was erratic as one treatment had a greater effect (gibberellin A_3 30 ppm) and one treatment a lesser effect (gibberellin A_{4-7} 300 ppm) than the control, making this the least accurate way of expressing flower reduction.

Data of shoot length were taken to determine if the gibberellin spray treatments had an effect on growth of one-year-old wood. There was no effect on the average shoot length (length of laterals/

no. of laterals) and the treatments increased total growth (sum of laterals) only for 300 ppm gibberellin A_{4-7}. Since there is only one treatment in both growth analyses where the gibberellins acted as a promoter of vegetative growth (gibberellin A_{4-7} 300 ppm) the greater number of effective treatments in the reduction of flowering (bloom/cm shoot length) is not the result of the inverse relation between growth and flowering. It is attributed to a more direct hormonal action though more variable than with spurs. Tromp (164) drew essentially the same conclusions from his data. He found gibberellins to be inhibitors of flowering with gibberellin A_{4-7} of greater potency than gibberellin A_3 and, for their greatest effect, they had to be applied soon after bloom. While he made a distinction for flower quality, we found no visual difference in quality caused by the treatments. He also presented data on mean shoot growth. His results were very similar to those reported here (table 11), that while variability was large, there was no statistical effect on growth. From this and with his data on succinic acid dimethylhydrazide, he argued that growth and flowering were independent phenomena.

Time Interval of Flower Initiation

To determine the time interval when the spurs would initiate flowers, a fruit removal experiment was undertaken (table 12, figure 4). This would rely on the natural inhibiting effect on flowering by the seed in the fruit, an effect demonstrated by Chan and Cain (26). Fruit were periodically removed from full bloom

to harvest to determine the time interval when flowering would be inhibited. A plot of the return bloom versus time of removal yielded a graph where the interval of inhibition was later than expected. The time when flower initiation can be influenced in 'Early McIntosh' seems to vary from year to year possibly depending on the amount of bloom. In 1948, Southwick and Weeks (154) obtained no return blossoming with thinning at full bloom. In 1952, with adequate thinning at 10 days after petal fall, the same authors (156) obtained an increase in return bloom. Southwick et al. (157) increased flower bud initiation at 3-4 weeks after bloom in 1963 with scoring and naphthaleneacetic acid on partially defoliated spurs. Lord et al. (98) increased flowering 4.5 fold on 'Early McIntosh' at 44 days after bloom. In the strongly biennial 'Emneth Early' , it was found that fruits only exert a strong inhibiting influence on flower initiation when they reach the age of 6-8 weeks (Luckwill, 100). There was a 100% return bloom until the removal date of 45 days after bloom and then a decrease to 0% at harvest (figure 4). The percent return bloom for removal date FB +60 is not an accurate value with respect to the number of trees or observations per tree. A different tag was used for this date and many of these, being larger than the others, were lost during two winter blizzards. The bienniality of this variety was again evident in the extreme to which the spurs differentiate over the course of the growing season. If fruit were removed early, a complete return bloom was obtained; complete inhibition was

obtained if fruit were left on until harvest. The intention of the time interval of flower bud initiation was to determine if the gibberellins appeared before or during the interval, a condition necessary to be considered the causal agent of inhibition.

Time Course Extraction of Tissues

The percent recovery of the extraction procedure was determined for diffusate, seed, and spurs to arrive at the actual internal levels of the tissues (table 13). The diffusate, being the cleanest extract, had a recovery of 94% with the seed and spurs having a recovery of 81% and 80% respectively. The inverse of the percent recovery was multiplied by the endogenous measurement to obtain the actual hormonal levels of the tissues which were plotted as such.

Seed were harvested from 5 days to 60 days after bloom, extracted, and a time course profile of gibberellins was plotted (table 14, figure 5). Peak levels of gibberellin were found at FB +5, +15, and +45 to +60. Dennis (36) found peak levels at FB +20 and FB +58 in 'Golden Delicious'. Luckwill et al. (99) found gibberellins to peak at 9 weeks after bloom in 'Cox's Orange Pippin', 'Emneth Early', and 'Laxton's Superb'. Hoad (76) found gibberellin activity to peak in 'Laxton's Superb' and 'Cox's Orange Pippin' from the 8th to 11th week after bloom.

The fruit diffusate was collected in a buffer containing citrate (86) and analysed for gibberellin levels. The gibberellin levels were then calculated on a ng/g fresh weight and pg/seed

basis (table 15). The actual amount of gibberellins collected decreased ~6 fold with time. When plotted as ng/g FW (figure 7), a hyperbolic curve was obtained with highest levels immediately following bloom and a rapid decrease to an equilibrium from 30 days after bloom onward. The hyperbolic effect of the levels is due mainly to the ~24 fold increase in fresh weight of the fruit. When the gibberellin levels were plotted on a pg/seed basis (figure 6), there was a steady amount of gibberellin levels until FB +20, a sharp increase to a peak at FB +45, and high levels until FB +60. The harvest date FB +5 was analysed for gibberellin levels but not connected on the graphs. This was a date of harvest three days following a snowstorm, and frost damaged flowers, being indistinguishable, were included in the samples for diffusate collection. Frost damage was appreciable and became distinguishable for the next harvest date, FB +10. In his work with fruit diffusate, Hoad (76) determined two peak gibberellin levels, one at the 4th and one at the 8th week after bloom in the biennial variety 'Laxton's Superb'.

A comparison of the gibberellin levels of fruit bearing and vegetative spurs was desired to determine if higher amounts are present in the fruit bearing spurs, spurs which would not initiate flowers (table 16). The gibberellin levels of the spurs were plotted on a ng/g dry weight basis and had the lowest levels of the three tissues analysed (figure 8). Over the harvest dates the vegetative spurs contained between 1.0 and 2.5 ng GA_{4-7}/g DW,

and fruited spurs contained between 2.4 and 3.3 ng GA_{4-7}/g DW. The higher levels are attributed to the presence of the fruit. During the period of time in which the gibberellins in the diffusate were peaking (FB +45→60; pg/seed basis), there was no corresponding peak in the fruit bearing spurs. This is probably metabolic regulation of the influxing hormone by the spur. Luckwill (100) found low non-varying growth promoter levels in defruited bourses; however, he found fruit bearing bourses to contain strong flushes of growth promoters occurring over the period of 5-9 weeks after bloom. This may not be contrary data, for he used the Avena mesocotyl test which measures all growth promoting activity (auxins, gibberellins, cytokinins).

Hypothesis of Biennial Bearing

Chan and Cain (26) have demonstrated that in apple the inhibiting source of annual flower initiation is the seed. Fulford (49) suggested that spurs will flower unless specifically inhibited. From Fulford's view inhibition must be caused. With the seed as the source of inhibition and the initiating bud on the spur, a hormonal messenger seemed necessary. Gibberellins are an inhibiting factor of flower initiation from data presented and from the literature for both apple (34, 55, 64, 107, 108, 161, 168) and other species (5, 17, 77, 149). Seed was found to be a relatively rich source of gibberellins in apple (35, 99, 102) with a concentration difference of ~3000 fold greater than fruit flesh (Dennis, 36). At comparable times the diffusate of apple fruit had shown

gibberellins to be always present (table 15). Hoad (76) reported two intervals of several weeks duration of gibberellin activity in fruit diffusate of 'Laxton's Superb'. This source of gibberellin must be the seed for Grochowska (61) has demonstrated that only growth regulators of the seed are diffusable.

Chan and Cain (26) found a highly significant negative correlation between the number of seed and the percent return bloom, making the appropriate basis for diffusate analysis pg/seed. With the ability of the biennial bearing varieties to set more fruit per spur and contain more seed per fruit (179), the total amount of gibberellin arriving in the spur would be proportionately increased. Grochowska and Karaszewska (62) offered evidence of a greater content and rate of auxin and gibberellin translocation in biennial cultivars. Hoad (76) presented similar data of larger levels diffusing from a biennial cultivar. Since the flowers were initiated after FB +60 (or FB +45), relatively large levels of gibberellin were found in the diffusate before or during the period of initiation.

The higher levels of gibberellin in the fruit bearing spurs are attributed to the gibberellins of the fruit diffusate. The lack of a large gibberellin peak in the fruit bearing spur, corresponding in magnitude or time to the peak in the diffusate, might be a metabolic regulation of the spur to the influxing hormone. This strict regulation can be further substantiated by the observation of Harley et al. (70), that of Chan and Cain (26),

and by our personal observation that the inhibiting effect of flowering is localized. Harley et al. (70) stated that there was no effect of unsprayed and undefoliated branches on the flowering of naphthaleneacetic acid sprayed and defoliated branches. Chan and Cain (26) observed that seedless fruit spurs 2-5 cm from seeded fruit spurs would flower the following year. We observed that some spurs with two buds were out of phase (one was vegetative, one was fruited). Since there was no peak of gibberellin in the fruit bearing spur, a peak threshold seems unlikely as the flower inhibiting stimulus. Rather, low but non-varying gibberellin levels reside in the fruited spur, from the fruit diffusate, and similar to peas (80, 83, 97) this becomes inhibitive when the spur becomes susceptible by its own timing.

SUMMARY

The partition coefficients and a method of isolation were determined for the growth regulators of apple. The involvement of gibberellin in the biennial bearing of 'Early McIntosh' was investigated.

1. Partition Coefficients

The partition coefficients of the growth regulators of apple were determined with petroleum ether, hexanes, ethyl ether, ethyl acetate, and n-butanol at all pH values between 3 and 9 and for 5% bicarbonate solution. Each partitioned as would be expected by acid-base equilibria. Hexanes and petroleum ether removed no growth regulators at all extraction conditions, hence, they were used to clean up extracts. Ethyl acetate removed the largest amount of the neutral and acidic growth regulators and was used for their extraction. Cytokinin partitioning was most effective with n-butanol.

2. Separation Method

The growth regulators were separated using pure compounds to maximize efficiency and an apple seed extract as a natural source. Zeatin, gibberellin A_{4-7}, and abscisic acid were detected in the apple seed extract. No auxins were determined present.

3. Growth Regulator Stability

Auxins and cytokinin ribosides were checked for stability to the isolation techniques of flash evaporation and cation exchange.

In the ionic form (pH 8) there was no loss of indoleacetic acid by sublimation. There was no loss of the neutral auxins by sublimation. The cytokinin ribosides were recovered quantitatively from the exchange resin. Therefore, these two techniques were employed in the isolation of the growth regulators.

5. Gibberellin Spray Treatment of 'Early McIntosh'

Gibberellin spray treatments were applied to 'Early McIntosh' to inhibit flower initiation and, thereby, show it to be the possible natural inhibitor. Both gibberellin A_3 and A_{4-7} were effective in reducing flowering on spurs without concomitantly influencing their growth. Direct hormonal action was attributed to their effect.

6. Fruit Removal

Fruit were periodically removed and the time of flower initiation was found to be between FB +45 and FB +105. Peak levels of gibberellin in the fruit diffusate were found to precede the time interval of flower initiation. This is a necessary condition for gibberellin to be considered the causal agent.

7. Tissue Extraction

Fruit diffusate, seed, vegetative spurs, and fruited spurs were periodically harvested and extracted for gibberellin content. Seed levels were high immediately following bloom and at FB +60. Diffusate levels increased with time and peaked at FB +45. The fruited spurs contained significantly greater amounts of gibberellin than the vegetative spurs. This is attributed to the gibberellin in the fruit diffusate and is sufficient to cause

flowering to be inhibited. There was no peak of gibberellin in the time course extraction of the fruited spurs. This eliminated a peak threshold as the triggering response.

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