ISOLATION, CHARACTERIZATION AND ROLE OF ENDOGENOUS AUXINS AND CYTOKININS IN SOUR CHERRY (Prunus cerasus L. cv. Montmorency) FRUIT DEVELOPMENT

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ABSTRACT

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By

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Endogenous auxins and cytokinins were isolated from developing sour cherry (Prunus cerasus L. cv. Montmorency) fruit and the changes in level of each promotive substance related to the development of the fruit. Fruit were collected at regular intervals from anthesis, immediately frozen, lyophilized, and divided into seed and pericarp tissues. Methanolic extracts of these tissues were further purified by partition and paper chromatography. Chromatograms were tested for auxinic substances by the Avena first internode and Avena curvature bioassays. Ethanolic extracts of lyophilized whole fruits were also purified by partition and paper chromatography. Chromatograms were tested for cell division factors by the radish cotyledon and soybean callus bioassays. Increased levels of butanol-soluble cytokinin were correlated with increased cell division in

the pericarp although the cessation in pericarp cell division was not correlated with low levels of this cytokinin. Changes in free (ethyl ether-soluble) seed auxin levels were correlated with the development of the nucellus and integuments, endosperm and embryo. No correlation could be established between these auxin levels and the levels of free auxin in the pericarp. Furthermore, free auxin levels in the pericarp could not be correlated with periods of rapid fruit development. Base hydrolysis of the water-soluble fraction resulted in the isolation of a further auxin (bound auxin) and a highly potent inhibitor of cell enlargement. This inhibitor was found only in pericarp tissues and, as such, may be associated with the inhibition of cell enlargement during fruit growth stage II. The level of bound auxin in the seed paralleled the decrease in free seed auxin content. The level of bound auxin in the pericarp was inversely associated with the level in the seed and was considered to be physiologically significant to cell enlargement during fruit growth stage III. Fractionation of the seed or pericarp acidic-ether and neutral-ether phases by repeated paper chromatography resulted in the isolation of one acidic auxin and two neutral auxins (IIA and IIB) that were highly active in the Avena first internode and curvature bioassays. The acidic auxin was identified as indole-3-acetic acid by

gas-liquid chromatography and combined gas-liquid chromatography-mass spectrometry. Neither of the neutral auxins were chromatographically similar to standard auxins nor did they yield chromogenic reactions with reagents specific for indolic compounds. Gas-liquid chromatography of the neutral auxin IIB resulted in further resolution into three compounds. Combined gas-liquid chromatography-mass spectrometry confirmed the non-indolic nature of the three compounds. However, insufficient evidence was available to assign a structure to any of the three compounds. Fractionation of the seed or pericarp basic-ether phase, or aqueous phase, by paper and silicic acid column chromatography did not result in the isolation of any consistent growth promoting activity in the Avena first internode bioassay. Base hydrolysis of the aqueous phase, however, resulted in the release of an ether-soluble auxin that was chromatographically distinct from either of the neutral auxins or the acidic auxin. Fractionation of ethanolic extracts of whole fruits resulted in the isolation of a water-soluble cytokinin and a butanol-soluble cytokinin active in both the radish cotyledon and soybean callus bioassays. These two cytokinins were tentatively identified as zeatin and zeatin ribonucleotide, respectively.

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By

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INTRODUCTION

Manipulation of post fertilization fruit growth by application of exogenous growth regulating substances has become an increasingly important aspect of horticultural production. The use of auxin to set tomato flowers (greenhouse production) or of gibberellin to stimulate Thompson Seedless grape berry growth has resulted in striking increases in crop production (Luckwill, 1957b; Crane, 1964; Wittwer, 1968). Auxin (naphthaleneacetic acid) has found wide usage as a chemical thinning agent when applied during the initial stages of fruit growth, while auxin application during the later stages of fruit growth has been found to prevent pre-harvest fruit drop. Gibberellin application to developing navel orange and lemon fruits has been used to delay the onset of rind disorders (Wittwer, 1968). A further example is the use of ethylene in the pineapple industry as a flower-inducing agent (Cooke and Randall, 1968). Yet the practical applications of such growth-promoting substances has arisen from empirical experimentation rather than from an understanding of the hormonal mechanisms involved in fruit development.

Seeds, at various stages of their development, have been shown to be a rich source of growth-promoting substances

(Crane, 1964) and a number of investigators have sought to correlate the changes in seed hormone content with concomitant changes in fruit development. In some cases, correlations have been established between seed cytokinin levels and post-fertilization fruit growth (Letham, 1963a, 1964) and between seed auxin (and gibberellin) levels and the initial period of rapid cell enlargement in the fruit (Wright, 1956; Nitsch et al., 1960; Coombe, 1961; Jackson and Coombe, 1966; Jackson, 1968). Correlations between any of these seed hormones and the later stages of fruit growth have been seldom demonstrated. Similar correlations between the level of pericarp hormone levels and fruit development or even between the levels of pericarp hormones and those of the seed have seldom been sought.

Evaluation of such results has been impaired for two reasons. Firstly, and with the exception of Blumenfeld (1970), the levels of auxins, gibberellins, cytokinins and inhibitors have not been determined, simultaneously for any one fruit cultivar growing at the same location. Secondly, rigorous identification of isolated growth-promoting substances has not always been attempted, partly because of the problem of low concentrations present and partly because the physical-chemical methodology has not been available.

Furthermore, recent studies have shown that the control of fruit growth is much more complex than was originally envisaged. Fruit development, or the control

of it, is now believed to be the result of the net balance between promotive and inhibitory substances and not merely the result of fluctuations in the level of promotive substances.

The present study was undertaken with three main objectives: (1) to relate the levels of seed and pericarp auxins to the development of respective tissues in the sour cherry fruit; (2) to relate the levels of fruit cytokinins with fruit development; and (3) to characterize and identify the auxins and cytokinins in fruit tissues.

LITERATURE REVIEW

The relationship between seed development and concomitant fruit growth was initially shown as correlations between seed number and fruit size, and between seed distribution and fruit shape (Heinicke, 1917; Brittain and Eidt, 1933; Olmo, 1946; Roberts, 1946). Premature fruit abscission in apple (Malus sylvestris Mill.) was also associated with a lower seed number per fruit than that found for fruit remaining on the tree (Brittain and Eidt, 1933; Roberts, 1946).

Single seeded fruit also exhibit a relationship between seed development and fruit growth. Fruit from different Prunus sp. (single seeded as the result of one ovule aborting) often display marked carpel asymmetry with the larger side being more colored, flavored and earlier ripening. Tukey (1936a) related this asymmetry with seed attachment to the larger side. That the seed is intimately involved in fruit growth was shown by Tukey (1936b) by destroying peach (Prunus persica L.) and cherry (Prunus cerasus L.) embryos during different stages of their development. Destruction during early stage II of fruit growth (Conners, 1919) resulted in an abrupt check to ovary wall development and ultimate abscission. As embryo

destruction was successively delayed, fruit growth approached that of controls. Tukey concluded that the presence of a developing embryo during the initial stages of fruit growth was essential to the development of the fruit.

Although the association between seed development and fruit growth had been firmly established, it was results from studies on fruit set, rather than on fruit growth, that led to the concept of fruit growth being dependent on specific growth factors produced in the seed. In 1909, Fitting reported that aqueous extracts of living or dead, orchid pollen grains would stimulate orchid ovary swelling. Subsequently, Thimann (1934) showed that chloroform extracts of Sequoia pollen were highly active in the Avena curvature bioassay, implicating auxin as the active moiety. Subsequently Yasuda (1935) injected aqueous extracts of Petunia pollen into egg plant (Solanum melongena L.) ovaries and obtained parthenocarpic fruit. In order to test the hypothesis that auxin was involved in parthenocarpic fruit development Gustafson (1936) applied synthetic auxins, in lanolin paste, to cut styles of a number of plants. Auxin application stimulated ovary swelling in many of the plants tested and ultimately resulted in mature, parthenocarpic tomato (Lycopersicon esculentum Mill.), petunia (Petunia hybrida Vilm.), salpiglossus (Salpiglossus variabilis Hort.), and pepper (Capsicum sp.) fruits. Gustafson concluded that

definite chemical substances, which seemed to be closely related to the auxins, would cause the ovary of a flower to develop into a fruit.

Evidence that auxins were present in seeds was published independently by Dolfus (1936) and Meyer (1936). Dolfus (1936) showed that a diffusible substance from pollinated ovaries was active in the Avena curvature test. No similar substance could be detected in diffusates from the ovary wall. Similar results were obtained by Meyer (1936) using an alcohol extraction procedure rather than diffusates. Dolfus concluded that, "pollinated ovaries provide the necessary hormones for the growth of the fruit wall."

In 1939, Gustafson determined the auxin content in seeded and naturally parthenocarpic orange (Citrus sinensis Osbeck), lemon (Citrus limonia burm.) and grape (Vitis sp.) fruits at various times before and after anthesis. The auxin content of ovaries from parthenocarpic varieties was shown to be higher at anthesis than at any time during fruit growth. The opposite was found for the seeded varieties. Gustafson proposed that the auxin content in flowers from parthenocarpic varieties was sufficiently high to induce the ovary to grow even though fertilization had not taken place. Continued partheocarpic fruit growth was thought to be under the influence of auxin synthesized in the fruit itself or translocated to the fruit from the

leaves. In an attempt to test the hypothesis that parthenocarpic fruits can synthesize, or at least contain, auxin Gustafson (1939b) extracted auxin from seeded and parthenocarpic tomato fruits. Parthenocarpic fruit, induced with phenylacetic, indolebutyric, or naphthaleneacetic acids (all of which are only slightly active in the Avena curvature bioassay utilized) were shown to contain appreciable amounts of auxin in the pericarp and central axis. Although the question of synthesis or translocation could not be answered, similar determinations on seeded fruit showed that the seed was always a richer source of auxin than the pericarp.

In earlier publications Gustafson (1937, 1938) had implicated pollen auxin in the initiation of ovary swelling and had shown that in one plant, crookneck squash (Cucurbita moschata Duchesne), exogenous auxin could substitute for the developing ovules to yield a normally maturing fruit. This evidence, in conjunction with studies on auxin distribution in fruits, led Gustafson to propose that fruit growth was initiated by auxin in pollen grains, and tubes, and continued by additional auxin diffusing from the developing seeds. This, then, was the essence of a theory that was to be explored and expounded for the next thirty years.

In 1946, Luckwill showed that aqueous extracts of apple seeds would stimulate parthenocarpic fruit development

in tomato. Stimulatory activity, however, could only be detected in seed extracts between the third and tenth week after petal fall. Luckwill associated the absence of activity after the tenth week with the cessation of rapid seed development and the occurrence of "June drop." Subsequent experimentation (Luckwill, 1948, 1953) established that stimulatory activity could be shown in seed extracts of several apple varieties and that this activity occurred as two peaks during seed development. The first peak was associated with cytokinesis in the endosperm. The second, and more intense peak was associated with the development of the embryo. Seed extracts were also shown to delay abscission of debladed ivy (Hedera helix L.) petioles. In addition, seed extracts from "June drop" fruit were found to contain considerably lower levels of activity than were found in similar extracts from persisting fruit. Luckwill concluded that the extractable stimulus prevented premature fruit abscission and played no part in fruit growth, per se. However, the evidence that growth promoting activity could only be demonstrated at certain stages of seed growth was to prove to be a major point in subsequent experimentation.

In a series of elegant experiments, Nitsch (1950) demonstrated that the growth of the strawberry (Fragaria sp.) receptacle was dependent on the presence of fertilized achenes. Asymmetric achene removal resulted in asymmetric

fruit growth and this effect could be overcome if the excised achenes were replaced by β-naphthoxyacetic acid. On the basis that achenes produced some substance, possibly auxin, that diffused into the receptacle and caused receptacle growth, Nitsch extracted both achenes and receptacle tissue and assayed for activity with the Avena curvature test. Auxin was found only in achene extracts. Moreover, the level of achene auxin was greatest just prior to rapid receptacle enlargement.

Attempts to identify the active moieties in seed extracts of apple (Luckwill and Woodcock, 1951; Luckwill, 1952; Teubner, 1953; Luckwill and Powell, 1956) and strawberry (Nitsch, 1955) met with little success other than the recognizance that extracts contained a number of different growth-promoting and growth-inhibiting substances.

Wright (1956) extracted three growth promoters and one growth inhibitor from black currant (Ribes nigrum L.) fruit. The levels of two of these promoters were correlated with the rate of fruit enlargement while the concentration of the third growth promoter was negatively correlated with premature fruit drop. The inhibitor concentration increased with fruit development and could not be correlated with any stage of fruit growth. Premature fruit drop in black currant was shown to be due to seed abortion, rather than failure

of fertilization, suggesting a nutritional regulation of fruit drop. Wright hypothesised that growth promoting substances (auxins) were produced as a consequence of protein synthesis in fruit meristems and thus fruit growth, and retention, would be dependent on the maintenance of active protein synthesis. Competition between berries on the same raceme for available nutrients was thought to result in some berries receiving insufficient nutrients to maintain the necessary protein synthesis, and hence insufficient auxin would be produced to prevent abscission.

In contrast to Wright's findings on black currant, Crane et al., (1959) could find no evidence that the rate of fig (Ficus carica L.) fruit enlargement was in any way dependent on fruit auxin content. These workers showed that two acidic auxins occurred in both seeded (Calimyrna) and parthenocarpic (Mission) fruits. One of these auxins, which exhibited chromatographic and chromogenic properties similar to those of indoleacetic acid, was predominant during growth stage I. The second auxin was shown to be the major hormonal constituent associated with endosperm development during the period of reduced fruit growth. Crane et al., considered that this latter auxin was involved in the prevention of fruit abscission.

Stahly and Thompson (1959) reported that peach ovules contained three auxins. The presence of two of these auxins was associated with rapid development of the

endosperm and embryo although some growth-promotive activity was detected throughout much of seed growth. In contrast to Luckwill (1946, 1953), Wright (1956) and Crane et al., (1959) who related endosperm and embryo auxin levels with the prevention of fruit abscission, Stahly and Thompson associated these auxin levels with inhibition of mesocarp cell enlargement during growth stage II. However, Crane (1964) has pointed out that since parthenocarpic peaches have a similar growth curve to that of seeded peaches (Crane et al., 1960), auxin originating from developing seeds could not be the factor responsible for growth stage II as no seed development occurs.

In an attempt to understand the physiology of parthenocarpic fruit growth, Nitsch et al., (1960) compared growth regulator changes in Concord grape (Vitis labrusca L.) and its presumed mutant, Concord Seedless. These workers showed that the level of growth substances active in the Avena first internode bioassay was higher in Concord Seedless than in Concord immediately after anthesis. The rate of fruit growth was similarly higher in Concord Seedless. Subsequent growth promoter activity in both cultivars was associated with nucellus and endosperm development although endosperm degeneration in Concord Seedless was associated with a rapid decline in promoter levels. Growth promoters were detected throughout the development of seedless fruits and, surprisingly, their

levels were higher during final fruit swell than those found in seeded fruits. However, Nitsch et al., (1960) concluded that while there could be some association between growth substance levels and fruit growth in growth stage I, no similar association could be found during growth stage II. Aqueous extracts of fruit from both cultivars were shown to contain unknown growth factors which stimulated the growth of Jerusalem artichoke tuber tissue (Nitsch and Nitsch, 1956b). Although the levels of these unknown factors were found to be lower in Concord Seedless than in Concord, no conclusions were made as to their role in fruit growth.

In a similar study, Coombe (1961) extracted growth-promoting substances from fruit of seeded, stenospermocarpic, and parthenocarpic grapes (Vitis vinifera L.). Crude extracts were assayed by the Avena first internode test. Correlations between detected growth-promoting activity and fruit growth could only be established for seeded cultivars during growth stage I even though some activity was detected during the greater part of fruit growth in all cultivars tested. Crude extracts were also assayed for gibberellin-like activity by the d_1 maize bioassay. Although gibberellin-like activity could be detected in stenospermocarpic and parthenocarpic fruits, no activity could be shown in extracts from seeded cultivars.

Measurement of berry sugar content showed that the rate of sugar accumulation increased dramatically at the onset of growth stage III. This observation was true of all cultivars. Coombe hypothesised that the sugar influx, in the presence of auxin, would cause cell enlargement and hence fruit growth. If this were the case, trunk girdling should increase fruit size. When this hypothesis was tested it was found that girdling increased the size and weight of mature stenospermocarpic and parthenocarpic berries. In both fruit types, sugaring did not commence until the onset of growth stage III, yet the rate of fruit growth increased immediately after girdling suggesting that the growth enhancement was due to movement of substances other than sugars into the fruit.

Attempts to understand the relationship between seed hormones (auxins and gibberellins) and fruit growth using parthenocarpic varieties as reference points (Crane et al., 1959; Nitsch et al., 1960; Coombe, 1961) proved to be as unsuccessful as earlier attempts with seeded fruits (Luckwill, 1953, 1957). Moreover, it became increasingly clear that auxins were not the only seed hormones to be considered; gibberellins (Phinney et al., 1957), unknown cell division stimulants possessing activity similar to that of kinetin (Miller et al., 1956), and inhibitors (Luckwill, 1952) were also implicated.

The report that developing seeds were relatively rich sources of gibberellin-like substances (Phinney et al., 1957), focused attention on the possible relationship between seed gibberellins and fruit growth. In general, the levels of seed gibberellin have been correlated only with development of the seed (Skene and Carr, 1961; Murakami, 1961; Corcoran and Phinney, 1962; Ogawa, 1963a, 1963b) although Ogawa (1963a) showed that the level of gibberellin-like substances in lupin (Lupinus luteus L.) pods was correlated with pod weight and length. The finding that levels of seed gibberellins were not correlated with fruit growth was surprising since exogenous gibberellin had been shown to be more effective than auxin in stimulating parthenocarpic fruit development (Prosser and Jackson, 1959; Luckwill, 1959; Davison, 1960; Bukovac, 1963).

Coconut (Cocos nucifera L.) endosperm had long been recognized as a rich source of cell division factors (van Overbeek et al., 1941) but it was not until 1961 that similar cell division factors [cytokinins (Letham, 1967)] were conclusively demonstrated in fruitlet extracts (Letham and Bollard, 1961). Ethanolic extracts of apple, peach, plum (Prunus domestica L.), and pear (Pyrus communis L.) fruitlets were all highly active in the carrot secondary phloem explant test (Letham and Bollard, 1961) or the

tobacco stem pith test (Bottomley et al., 1963). Similar extracts of quince (Cydonia oblonga L.) and pumpkin (Cucurbita pepo L.) were shown to contain lower amounts of these cell division factors while none could be demonstrated in avocado (Persea americana Mill.) or tomato extracts (Letham and Bollard). Subsequent experimentation showed that cytokinins were present at high concentrations during the period of intense cell division in apple (Letham, 1963a; Bottomley, et al., 1963) and plum (Letham, 1963a) fruit. Cytokinin levels began to decline just prior to cessation of cell division. No activity could be detected in extracts of mature fruit (Letham and Bollard, 1961; Letham, 1963a) although some evidence was presented for both apple and peach that cytokinins were present during endosperm and embryo development (Bottomley, et al., 1963; Powell and Pratt, 1964).

Cytokinins were also demonstrated in tissues other than the developing fruit. Apple flower cluster bases, pedicels, petals, and leaves were all shown to possess cytokinin activity (Letham and Bollard, 1961). Moreover, such activity could be demonstrated in apple flower parts as early as 21 days before anthesis (Bottomley, et al., 1963). Although this evidence detracts from the hypothesis that developing seeds are the primary source of factors inducing cell division in fruit tissues, Letham and Bollard

(1961) showed that seed removal from apple fruitlet explants led to growth cessation unless exogenous cytokinins were supplied.

In a comprehensive review, Crane (1964) evaluated the role of growth substances in fruit setting and development. Although Crane conceded that developing seeds were possible synthesis sites for auxins, gibberellins, and cytokinins the major conclusion from this review was that "no relationship has been proven between the levels of growth substances in developing seeds and fruit growth." This conclusion is misleading in so much as it is based on results derived from inadequate technique. Firstly, growth promoter levels have seldom been determined in both the seed and pericarp fractions of the same fruit (Nitsch, 1950, 1955; Luckwill, 1957; Letham and Bollard, 1961; Ogawa, 1963; Letham, 1963; Bottomley, et al., 1963). More commonly, growth promoter levels were only determined in the seed fraction (Luckwill, 1946, 1953; Stahly and Thompson, 1959) or in the entire fruit (Wright, 1956; Crane, et al., 1959; Nitsch, et al., 1960; Coombe, 1961). In this regard no evidence has been obtained that changes in growth promoter levels in the seed are reflected by comparable changes in the fruit, nor has evidence been obtained on possible growth promoter metabolism in the fruit.

Secondly, and more importantly, the evidence presented by Crane was obtained from reports where crude extracts had been bioassayed directly (Luckwill, 1946; Nitsch, 1950), or at best subjected to various dilutions before bioassay (Luckwill, 1953; Nitsch, 1960; Coombe, 1961). Promotive activity detected by either technique is merely the expression of the net balance between inhibitors and promoters. Extract purification by one dimensional paper chromatography does not necessarily result in complete separation between inhibitors and promoters (Wright, 1956; Luckwill, 1957; Crane, 1959; Stahly and Thompson, 1959).

Crane, however, overlooked these experimental discrepancies in order to propose that the sole function of the seed was to initiate and maintain a metabolic gradient between the ovules and ovary, and the vegetative organs. Growth substances in the seed were implicated as the causal agents. Crane cites the case of Black Corinth grape to explain parthenocarpy. Pollination without fertilization was proposed to establish the metabolic gradient and Coombe (1961) showed that auxin levels were high at this time. Yet sugaring did not commence until 40 days after anthesis, a point in time when seed auxin and gibberellin levels were shown to be extremely low. Furthermore, seed destruction or removal at the beginning of final fruit

swell of cherry, peach (Tukey, 1936b), and apple (Abbott, 1958; Southwick, et al., 1962) had no deleterious effect on the rate of subsequent fruit growth, suggesting that the mobilizing capability of the seed is of little consequence during the later stages of fruit growth. That fruit are strong mobilizing centers through much of their development is not the point in question; rather, it is whether or not this mobilization is the cause, or consequence, of growth that must be answered.

More recent studies have utilized the techniques of thin-layer chromatography in conjunction with gas-liquid chromatography, and mass spectrometry, to the extent that some of the gibberellins, cytokinins, and inhibitors occurring in developing seeds have been identified. Little progress, however, has been made with physical chemical identification of the seed auxins. Reappraisal of the relationship between seed hormones and fruit growth using these new techniques has not received commensurate attention.

Comparison of growth promoter levels detected in seed of early, mid, and late maturing peach varieties failed to demonstrate any relationship between seed hormones and either fruit abscission or fruit growth (Powell and Pratt, 1966). The validity of this conclusion should be questioned since unfractionated extracts were

bioassayed directly. Column chromatography of selected extracts, however, yielded two biologically active substances which accounted for much of the total detectable activity. Comparison of results obtained from different years showed that fluctuations in levels of either growth substance occurred at comparable dates. Subsequently, one of these growth promoters was identified by gas-liquid chromatography and spectrophotofluorometry as indoleacetic acid.

When crude methanolic extracts of apricot fruits were bioassayed by the barley endosperm test, gibberellin-like substances were detected in both seed and pericarp components (Jackson and Coombe, 1966). Levels of gibberellin-like substances increased in all tissues to a maximum at 20 days after anthesis and subsequently declined. However, levels in the mesocarp declined at a slower rate than comparable levels in the seed and endocarp. These workers were able to correlate the levels of gibberellin-like substances in all three tissues with their growth rate until 60 days after anthesis. Insufficient data were obtained, however, to show a similar correlation between gibberellin-like activity and mesocarp growth during the final stages of fruit growth.

In a similar study, Jackson (1968) compared the levels of gibberellin-like substances detected in different

peach tissues. Thin layer electrophoresis was used to separate gibberellin-like substances from other compounds active in the barley endosperm bioassay. The seed was shown to be a rich source of gibberellin-like substances during the period of active nucellus, endosperm, and embryo growth. No association could be found between levels in the seed and cell division in the integuments or embryo although these same levels of gibberellin-like substances were correlated with cell expansion in the nucellus, endosperm, and embryo. Similar relationships were shown for the endocarp and mesocarp. Jackson suggested that endocarp and mesocarp development was not directly dependent on gibberellin-like substances synthesized by the seed. Rather, the presence of gibberellin-like substances during final fruit growth was thought to be due to synthesis in the mesocarp.

Gibberellins A_4 and A_7 have been identified in apple seed extracts by thin layer co-chromatography (Dennis and Nitsch, 1966) and gas-liquid chromatography (Luckwill, et al., 1969). When seasonal fluctuations of these two gibberellins were determined by bioassay, appreciable gibberellin activity could not be detected in seeds until six weeks after anthesis (Luckwill, et al., 1969). Subsequent to this time, gibberellin activity increased to a maximum at completion of embryo growth and then declined.

Gibberellin levels in the fruit were not determined. However, a further two unidentified substances, which were highly active in the Avena first internode bioassay, were located in seed extracts. The maximum concentration of one of these substances preceded the appearance of gibberellin while the second substance reached its maximum concentration after the peak of gibberellin activity. Luckwill, et al., (1969) could advance no reasons as to the functional significance of these hormones other than to suggest that they may be involved in the mobilization of essential metabolites to the fruit.

Cytokinin activity in a number of fruits has been shown to be maximum during the period of intense cell division following fertilization (Letham, 1963a). More recently, Letham and Williams (1969) extracted apple ovaries and receptacle tissue and showed that seed extracts were considerably more active in the carrot phloem bioassay than were extracts of receptacle tissue. Further purification of seed and receptacle extracts yielded three cytokinins common to both tissues which were tentatively identified as zeatin, its riboside and ribotide. These authors considered the developing seed to be the site of cytokinin synthesis although extracts of fertilized and unfertilized flowers showed similar levels of activity at petal fall. Cytokinin activity in unfertilized flowers was thought to originate from either cluster bases or receptacle vascular tissue

since zylem sap was shown to contain detectable cytokinin activity 7 to 14 days after petal fall.

Two cytokinins were detected in extracts of watermelon (Citrullus lanatus [Thunb] Mansf.) seeds (Prakash and Maheshwari, 1970). Contrary to Letham's (1963a) finding, the levels of these two cytokinins reached a maximum of activity after the seed had completed its most intense growth phase. Cytokinin levels in the fruit were low in comparison to those in seeds.

The most complete assessment, to date, of auxin, gibberellin, cytokinin and inhibitor levels occurring in one fruit has been presented for avocado (Blumenfeld, 1970). Growth substance levels were determined in mesocarp, seed coat, endosperm and embryo tissues. No correlation could be established between auxins detected in the seed and seed coats and mesocarp growth. High gibberellin-like levels were detected in the endosperm and seed coats of young fruits. Gibberellin-like activity decreased in these tissues as the rate of mesocarp growth diminished. Although cytokinin levels were high in the endosperm, seed coats, and embryo, no activity could be found in the mesocarp later than three months after fruit set even though growth continued by cell division. However, hydrolysis of mesocarp extracts yielded appreciable amounts of cytokinin which were positively correlated with mesocarp

growth. Two major inhibitors were also detected in mesocarp extracts. One of these, inhibitor B, possessed properties similar to abscisic acid. The levels of the second inhibitor, inhibitor C, increased with increasing fruit maturity. A negative correlation was established between levels of this inhibitor and the rate of mesocarp cell division. When growth substance levels detected in the mesocarp of seeded and seedless fruits were compared, a marked difference was observed only in the level of cytokinins, suggesting that mesocarp growth is primarily dependent on cytokinins. However, Blumenfeld concluded that no specific growth substance is solely responsible for any specific stage of fruit development, but that a number of substances acting in concert were causal to fruit growth.

The precise significance of the relationship between seed development and fruit growth remains today as one of the major unsolved problems of fruit physiology. Admittedly, a considerable research effort has been expended investigating the "cause versus correlation," yet identification of the hormonal factors involved is still incomplete, especially for the auxins. Even when identification has been achieved by physical-chemical methods, technical problems have negated the use of such methods to routinely determine the levels of auxins, gibberellins, and cytokinins. A further problem, and with the exception of

Blumenfeld (1970), has been the lack of an in-depth study of the levels, and identity of growth promoters and inhibitors in different tissues of fruit grown under the same environmental conditions.

The evidence that has been compiled from bioassay data of crude or semi-purified extracts has shown that correlations can be drawn between hormone levels and successive stages of seed development (Crane, 1964). Correlations between these seed hormone levels and fruit growth have been demonstrated in few fruits (Wright, 1956; Nitsch et al., 1960; Coombe, 1961; Letham, 1963; Jackson and Coombe, 1966; Jackson, 1968; Blumenfeld, 1970); even so, these correlations are only tenable during the initial stages of fruit growth. The inability to demonstrate similar correlations during the later stages of fruit growth is perhaps to be expected in the light of Tukey's and Abbott's findings that fruit growth is independent of seed presence during final fruit growth. This evidence suggests that the stimulus for fruit growth originates in the seed during the initial stages of fruit growth but is resident in the pericarp by the onset of final fruit growth. Such a hypothesis is fully tenable with parthenocarpic fruit development since pollination without fertilization or subsequent seed abortion would provide the necessary initial hormonal stimulus.

Drupaceous fruit develop from a single carpel containing two ovules, one of which usually aborts before anthesis (Tukey, 1936a). Subsequent to fertilization the endocarp and mesocarp undergo a rapid increase in size by firstly cell division and secondly cell enlargement (Tukey and Young, 1939). This stage I of fruit growth is followed by a period of reduced mesocarp enlargement (stage II), progressive thickening and hardening of the endocarp, and rapid growth and differentiation of the embryo. During stage III of fruit growth the mesocarp undergoes rapid cell enlargement. Changes between consecutive growth stages are relatively abrupt enabling different developmental stages of the seed to be related to fruit growth. Moreover, the initial growth of the fruit has been shown to be dependent on the presence of a seed.

For reasons such as these, the sour cherry (Prunus cerasus L., cv. Montmorency) was selected as an eminently suitable fruit to reinvestigate the relationship between seed development and fruit growth. This study will report on the auxins and cytokinins occurring in sour cherry fruit and will relate both hormones to fruit growth.

THE AUXINS

Introduction

The relationship between seed auxin content and concomitant fruit growth in various Prunus sp. has seldom been investigated, even though Tukey had shown as early as 1936 that the growth of the fruit was highly dependent on the developing embryo. Moreover, the few investigations that have been made have either dealt exclusively with seed extracts (Stahley and Thompson, 1959; Schulte and Holm, 1964; Pillay, 1965) or have given the pericarp fraction only cursory examination (Powell and Pratt, 1966). As a result of these studies, no conclusive relationship could be established between the level of seed auxins and development of the fruit.

The source of the detected auxins in peach and cherry seeds has been ascribed to the developing nucellus, integuments, endosperm and embryo (Stahley and Thompson, 1959; Schulte and Holm, 1964; Powell and Pratt, 1966). Purification of peach seed extracts, obtained during periods of rapid nucellus, endosperm, or embryo development, revealed two highly active growth-promoting substances (Stahley and Thompson, 1959; Powell and Pratt, 1966). One of these promoters was tentatively identified by gas-liquid

chromatography and spectrophotofluorometry as indole-3-acetic acid. The second promoter, as characterized by paper chromatographic mobility and column chromatographic elution patterns, exhibited properties similar to ethyl-3-indoleacetate. Although similar investigations have been reported for sour cherry, no evidence has ever been presented (Schulte and Holm, 1964).

Materials and Methods

Source of Material

In 1969, fruit were harvested from mature, fifteen-year-old, trees growing at the Horticulture Research Center, East Lansing. Similar harvests in 1971 were made from vigorous six-year-old trees. In both years, fruit were collected at weekly intervals from anthesis; care being taken to harvest a random sample, with pedicels attached, from a number of trees. Harvested fruit were bulked, quickly frozen in the field with dry ice, and stored at -25°C until lyophilized.

The General Method

Extraction

Lyophilized fruit were divided into seed and pericarp tissues and extracted by one of two methods.

Procedure A.--Seed or pericarp tissues were homogenized in a Wareing Blender with cold (-25°C) 90%

MeOH (redistilled) for 2 minutes. The resulting homogenate was extracted for 20 hours, in the dark, at -25°C. The residue, obtained from filtration through glass wool, was repeatedly washed with cold 90% MeOH. Filtrates were combined, and stored in the dark at -25°C.

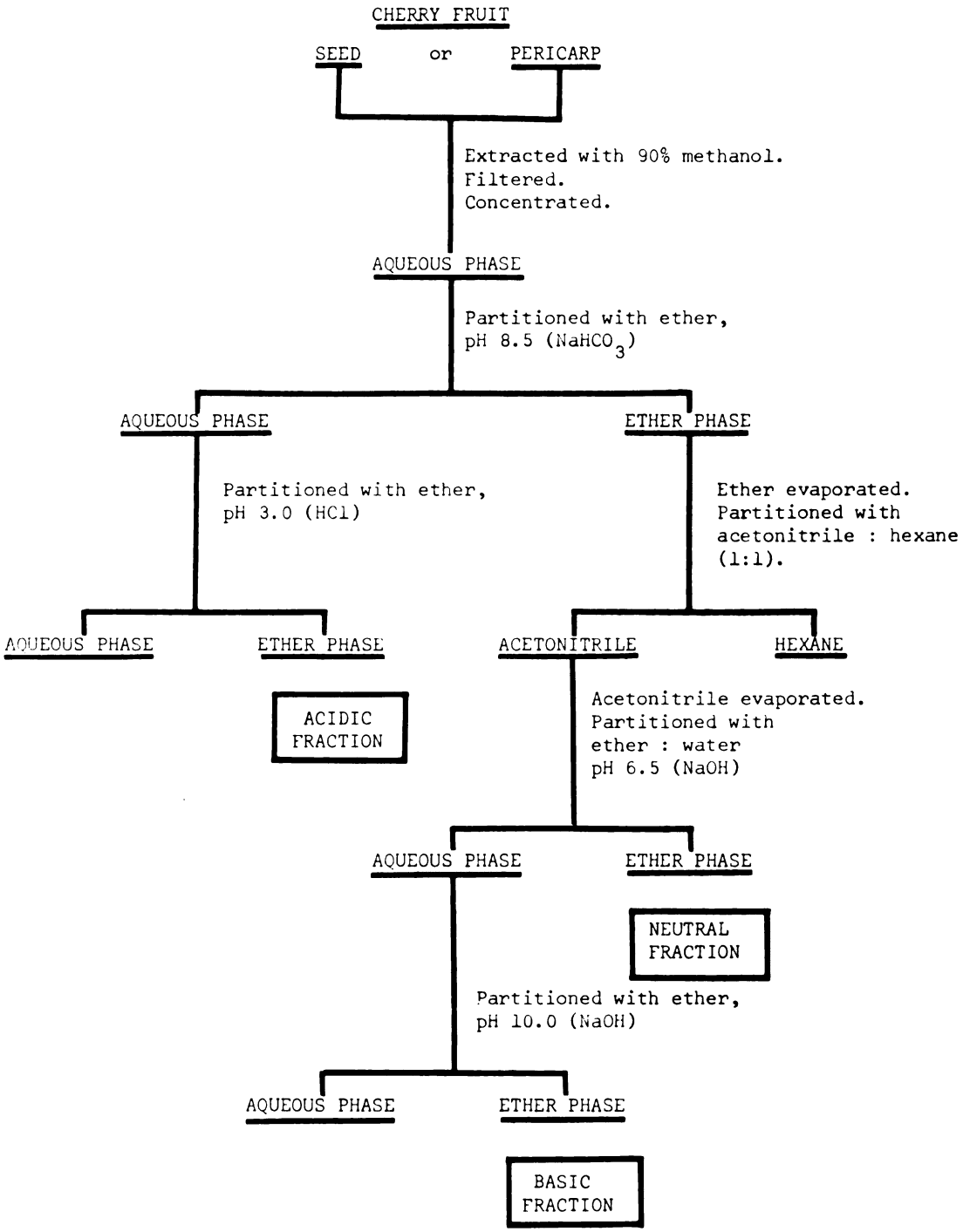
Procedure B.--Lyophilized seed or pericarp tissues were ground in a Wiley Mill to pass a 20 mesh screen. Ground material was extracted with 90% MeOH (redistilled) for 8 hours, in the dark, at -25°C. The residue, after filtration through glass wool, was washed several times with 90% MeOH and re-extracted for two further periods of 8 hours. All washings and filtrates were combined and stored in the dark at -25°C.

Fractionation

Partition chromatography.--The combined filtrates from either extraction procedure were evaporated to the aqueous phase under reduced pressure (flash evaporator, water bath temperature 30°C). The aqueous phase were adjusted to pH 8.5 and partitioned against ethyl ether (Figure 1) at least 3 times or until all chlorophyll residues were removed (Kefford, 1955). In all instances, ethyl ether had been previously shaken with acidic $FeSO_4$, to remove peroxides, and then redistilled.

The aqueous phase, containing acidic substances, was adjusted to pH 3.0 and partitioned 3 times against ethyl

Figure 1.--Flow diagram showing the procedure for extraction, and fractionation, of acidic, neutral, and basic substances.


CHERRY FRUIT
SEED
or
PERICARP
Extracted with 90% methanol.
Filtered.
Concentrated.
AQUEOUS PHASE
Partitioned with ether,
pH 8.5 (NaHCO3)
AQUEOUS PHASE
ETHER PHASE
Partitioned with ether,
pH 3.0 (HCl)
Ether evaporated.
Partitioned with
acetonitrile : hexane
(1:1).
AQUEOUS PHASE
ETHER PHASE
ACETONITRILE
HEXANE
ACIDIC
FRACTION
Acetonitrile evaporated.
Partitioned with
ether : water
pH 6.5 (NaOH)
AQUEOUS PHASE
ETHER PHASE
NEUTRAL
FRACTION
Partitioned with ether,
pH 10.0 (NaOH)
AQUEOUS PHASE
ETHER PHASE
BASIC
FRACTION

ether. The ethyl ether phases were combined, cooled at -25°C to remove residual aqueous phase, and evaporated to near dryness under reduced pressure. Acidic, ethyl ether-soluble substances were taken up in MeOH (redistilled) and stored in the dark at -25°C. Unless the extracted substances were to be further purified immediately, all fractions were flushed with nitrogen prior to storage (Kefford, 1955).

The ethyl ether phase obtained from the initial partitioning was taken to dryness under reduced pressure, resuspended in acetonitrile (50 ml, redistilled), and partitioned against hexane (50 ml, redistilled) (Nitsch, 1955) until all chlorophyll residues had been removed from the acetonitrile. The acetonitrile phase, containing neutral and basic substances, was taken to dryness under reduced pressure, resuspended in distilled water (pH 6.5, NaOH) and partitioned 3 times against ethyl ether. The ethyl ether phases, containing neutral ethyl ether-soluble substances, were combined and treated as for the acidic substances. The remaining aqueous phase was adjusted to pH 10.0 and again partitioned 3 times against ethyl ether (Kefford, 1959). The ethyl ether phases, containing basic ethyl ether-soluble substances, were combined and treated as above.

Paper chromatography.--Extracted acidic, neutral, or basic substances were further separated by ascending

paper chromatography. Extracted substances were quantitatively streaked on Whatman No. 3, or No. 20 paper and equilibrated over the developing solvent. In all cases where applied substances were to be quantitated by bioassay, chromatograms were pre-washed in the developing solvent to remove potential growth inhibitory substances present in the paper (Burnett, et al., 1965). Similarly, all solvents were redistilled prior to use.

After development to a distance of 15, 20, or 30 cm, chromatograms were dried in a cool air stream and stored at -25°C in a nitrogen atmosphere (Kefford, 1955).

Detection

The Avena first internode bioassay.--Avena sativa L., cv. Brighton, seed, which had been screened for a transverse diameter of 1.62 to 1.93 mm, were imbibed in running tap water for 2 hours and then sown on moist vermiculite. Seeds were germinated in the dark at 25°C. Seedlings, 3-4 cm in length, were selected and a 4 mm section was cut 2 mm below the first node on a modified guillotine (Crowe, 1963). Sections were floated in distilled deionized water for 1 hour prior to their introduction to the assay vial (Nitsch and Nitsch, 1956a). All manipulations were carried out under green safe-lights (Vegetable Green fluorescent tubes covered with 2 layers of No. 24 green Cinemoid and 1 layer of No. 4 amber Cinemoid; maximum transmittance at 552 nm.).

Chromatograms to be tested were divided into 10 or 15 equal sections, each section being shredded with a paper cutter. Control sections, equal in size to treatment sections, were taken from the area below the start line. Shredded sections were placed in 13 x 100 mm vials and 1-2 ml of citrate-phosphate buffer (pH 5.0), containing 2% sucrose and 0.01% Tween 80 polyoxyethylene sorbitan monooleate), added. Initially, 10 sections were added to each vial but in later experiments the number was reduced to 5 sections. In order to standardize the growth response, known concentrations of indole-3-acetic acid (IAA) were bioassayed in the presence of "blank" shredded chromatogram strips that had been cut from chromatograms.

Assay vials were incubated on a clinostat (1 r.p.m.) for 18-24 hours, in the dark, at 23°C. Subsequent to this period, sections were removed from the vials and placed on moistened glass slides. The glass slides were inserted into a photographic enlarger and the length of the enlarged section image measured. In some instances, the enlarged image was recorded as a developed shadowgraph (Kodabromide F5 photographic paper) and measurements were taken at a later date. In either case, initial section lengths were recorded so that growth increments could be measured directly from the enlargement.

Computation of response.--The mean length of the sections in each vial was calculated and the growth

increment (dL) determined by subtracting the initial section length (L). The increment in growth, expressed as a percentage of the initial length ($\frac{dL}{L}$ x 100), was plotted against chromatogram Rf in the manner of a histogram. When necessary, 5% fiducial limits of the control line were calculated (Wright, 1956) outside of which a treatment must fall in order to be significant.

The Avena curvature bioassay.--Avena sativa L., cv. Brighton, seeds were selected for size as described above, imbibed for 1 hour in running tap water and planted in moist vermiculite. Seeds were germinated in the dark at 26°C. The seedlings were exposed to a fluorescent red light source (General Electric-Red) for two periods of 1.5 hours during the time of coleoptile emergence. All subsequent manipulations followed the method of Kaldewey, et al.,(1969). To mention the method briefly, 20-30 mm long coleoptiles were slid into specially grooved Plexiglass holders. After removal of the apical 4 mm and the projecting basal parts, the remaining 10 mm long section was clamped by its acropetal end to the holder. Since the test substance, in agar, must be applied to the lower half of the cut acropetal end it was found expedient (Kaldewey, et al., 1969) to apply a single strip of agar to 6 adjacent coleoptiles; each coleoptile section then represented a replicate of the applied treatment. The preparation of

the test substance and agar strip was accomplished by the following method.

Chromatograms to be tested were divided into 10 equal sections, shredded and eluted successively with 2 ml portions of MeOH (redistilled), 90% MeOH, 40% MeOH, and water. The MeOH was removed under reduced pressure and 2 agar strips (14 x 2 x 2 mm; 1.5%) were equilibrated in the aqueous phase overnight at 6°C. Agar strips were similarly treated with known concentrations of IAA.

One of the agar strips was applied to a set of 6 coleoptiles held in the horizontal plane by the Plexiglass holder. All manipulations were carried out under a green safe light. Each Plexiglass holder was kept in a moist Petri dish in the dark, at 25°C, for 4 hours. After this period, the coleoptiles were lifted from the holder, turned through a 90° angle, and placed on a moistened glass slide. The degree of curvature was determined from shadowgraphs (Kodabromide F5 photographic paper) prepared with a photographic enlarger.

Computation of response.--The mean degree of curvature was calculated for each treatment and was plotted against chromatogram Rf in the manner of a histogram.

Changes in Auxin Levels During Fruit Development, 1969

Seed and pericarp tissues were individually extracted by procedure A and partitioned into acidic-ether and

acetonitrile phases. The acidic-ether and acetonitrile phases were combined and chromatographed in the following solvents (Nitsch and Nitsch, 1955):

Isopropanol : water (4:1)

Hexane : water (9:1)

After development, the chromatograms were divided into 10 equal sections and bioassayed by the Avena first internode test.

Changes in Auxin Levels During Fruit Development, 1971

Selected seed and pericarp tissues (harvests 21, 28, 42, and 56 days after anthesis) were extracted by procedure B, partitioned into acidic-ether and neutral-ether phases and chromatographed in the following solvents:

Isopropanol : water (9:1) (Acidic-ether phase)

Hexane : water (upper phase) (Neutral-ether phase)

After development, zones corresponding with chromatographed standards (IAA, indole-3-acetonitrile [IAN]) were cut from the chromatograms and bioassayed by the Avena first internode test.

Characterization of Seed Auxins

Anthesis + 21 days.--The seed extract (procedure B) from fruits collected in 1969 was partitioned into acidic, neutral, and basic-ether phases. Each phase was chromatographed with known indole compounds (IAA, IAN,

indole-3-propionic acid (IPA), indole-3-butyric acid (IBA), tryptamine; 1 µg) in isopropanol : water (9:1). After development, chromatograms were cut into 3 longitudinal strips. One strip, which consisted of chromatographed standards and a 1 cm wide section of the streaked unknown, was sprayed with either Salkowski (Stahl, 1969) or Ehrlich (Stahl, 1969) reagents. The remaining 2 strips, containing equal amounts of the streaked unknown, were bioassayed by the Avena first internode and Avena curvature tests.

Zones of growth-promoting activity, detected as above, were cut from duplicate chromatograms. Promotive substances were eluted with CH_3CN (2 times) and 95% MeOH (3 times) and the eluates re-chromatographed in the following solvents (Kefford, 1959; Thompson et al., 1959):

Isopropanol : ammonia (30%) : water (10:1:1) (Acidics)

Water (Neutrals)

Water (Basics)

After development, chromatograms were again cut into longitudinal strips which were either sprayed with chromogenic reagents or bioassayed.

Further separation of growth promoting substances was achieved by eluting detected growth promoting zones from duplicate chromatograms (developed in the secondary solvent systems) and chromatographing the eluates in the following solvent systems (Nitsch and Nitsch, 1955):

Acetonitrile : H_2O (4:1) (Acidics)

Hexane : water (upper phase) (Neutrals)

After development, chromatograms were cut into longitudinal strips and either sprayed with chromogenic reagents or bioassayed.

Anthesis + 42 days.--The seed fraction was extracted, partitioned, and chromatographed as previously described (anthesis + 21 days). Chromatograms were cut into 3 longitudinal strips; one strip was sprayed with chromogenic reagents, one was bioassayed by the Avena first internode test and the remaining strip stored for later elution. Detected growth promotive zones were eluted from stored chromatograms and re-chromatographed in the following solvents:

Isopropanol : water (4:1) (Acidic)

Acetonitrile : water (4:1) (Acidic)

Hexane : water (upper phase) (Neutral)

After development, chromatograms were cut into longitudinal strips and either sprayed with chromogenic reagents or bioassayed by the Avena first internode test.

Characterization of Pericarp Auxins

Anthesis + 42 days.--Experimental details were identical to those previously described for seed auxins (anthesis + 42 days).

Further Characterization of the Neutral Auxins

Chromatography in two solvents.--Equal gram equivalents of the seed neutral auxin (anthesis + 42, 1969 fruit harvest, extraction procedure B) were chromatographed in isopropanol : water (9:1) and hexane : water (upper phase). Developed chromatograms were bioassayed by the Avena first internode test.

Dilution curves.--Seed neutral auxins (anthesis + 42 days, extraction procedure B) were diluted to 1.0, 0.1, 0.01, 0.001 gram equivalents and bioassayed directly. All treatments were replicated 3 times.

Factorial combination.--Seed neutral auxins (anthesis + 42 days, extraction procedure B) were chromatographed in hexane : water (upper phase). After development, the zones between Rf 0.0 - 0.2 and between Rf 0.4 - 0.7 were eluted with the CH_3CN-95% MeOH schedule and the resulting eluates diluted to 0.5, 0.25, 0.1 and 0.05 gram equivalents. Fractions, so obtained, were combined as a 5 x 5 factorial with 3 replicates and bioassayed by the Avena first internode test.

Characterization of the "Bound" Auxins

Fractionation of the aqueous phase.--Seed and pericarp tissues (anthesis + 28 and + 42 days, 1969 fruit harvest) were extracted by procedure B. The acidified aqueous phase remaining after partition chromatography was adjusted to

pH 7.0 and lyophilized. Silicic acid (100 mesh, 8 gm) was hydrated with 5 ml HCOOH (0.5M), slurried in acetonitrile (distilled) and packed into 10 x 400 mm glass columns (Powell, 1960). Approximately 100 mg of lyophilized material was mixed with 0.5 gm silicic acid (hydrated with 0.3 ml HCOOH [0.5M]) and introduced to the column. Columns were serially eluted (2-3 ml/min) with 100 ml portions of 0, 10, 25, and 75% water in acetonitrile (saturated with 0.5 M HCOOH). Samples (2 ml) were taken from each 10 ml fraction collected. Each sample was evaporated under reduced pressure and bioassayed by the Avena first internode test.

Changes in "bound" auxin levels during fruit development.--The acidified aqueous phase remaining after partition chromatography of seed and pericarp extracts (anthesis + 21, + 28, + 42, + 56 days, 1971 fruit harvest) was adjusted to pH 7.0 and lyophilized. Each lyophilized phase was made 1N with respect to NaOH and hydrolyzed in the presence of excess ethyl ether for 15 minutes at 25°C (Ueda and Bandurski, 1969). At the completion of hydrolysis the hydrolysate was adjusted to pH 3.5 and partitioned against ethyl ether (3 times). Ether phases were combined, frozen to remove the residual aqueous phase, and chromatographed in water. Developed chromatograms were bioassayed by the Avena first internode test.

Gas-Liquid Chromatography of the Acidic I and Neutral IIB Auxins

Fractionation.--Seed from a number of harvests (1971; 21, 28, 35, 42 and 56 days after anthesis) were extracted by procedure B, partitioned into acidic and neutral phases, and each phase evaporated to near dryness. Each phase, in ethyl ether, was added to 0.5 gram silicic acid (hydrated with 0.3 ml HCOOH [0.5M]) and the carrier solvent evaporated under reduced pressure. The resulting, dry, silicic acid was added to the top of 10 x 400 mm columns that had been packed with 8.0 gram silicic acid (100 mesh, hydrated with 5 ml HCOOH [0.5M]) in hexane. Each column was serially eluted with 30 ml portions of 0, 1.0, 1.5, 2.0, 2.5, 10.0, and 20.0% ethylacetate in hexane (saturated with 0.5M HCOOH) at a flow rate of 2-3 ml/min. (Powell, 1960).

Previous column chromatography had established that the neutral auxin was eluted between fractions 8-14 (1.5 to 2.5% ethyl acetate) (Figure 6, Appendix A) while Salkowski-positive reactions could only be obtained with aliquots from fractions 20-22 (20% ethyl acetate). Column chromatography of standard IAA also yielded Salkowski-positive reactions for fractions 20-22. Accordingly, fractions 8-14 from the fractionation of the neutral phase and fractions 19-23 from the acidic phase were collected, bulked, taken to near-dryness and chromatographed in water.

Narrow strips from the edge of the chromatographed streak were sprayed with 4-dimethylaminocinnamaldehyde reagent (Stahl, 1969) and zones of color development compared with chromatographed standards. The chromatogram zone corresponding with standard IAA was eluted and re-chromatographed in isopropanol : water (9:1). Since no chromogenic reaction was observed on neutral auxin chromatograms with either the 4-dimethylaminocinnamaldehyde or Ehrlich reagents, the zone of presumed growth promotive activity (Rf 0.4 - 0.7, Figure 13a) was eluted, evaporated to near dryness, and re-chromatographed in hexane : water (upper phase). Developed chromatograms (acidic auxin) were sprayed with both Salkowski and 4-dimethylaminocinnamaldehyde reagents and the zone corresponding to the most intense color development was eluted. Eluted substances, after solvent evaporation and re-solubilization in MeOH, were dried over anhydrous Na_2SO_4 and stored at -25°C under a nitrogen atmosphere. Although the neutral auxins did not give a chromogenic response, growth-promotive activity presumed to be located at Rf 0.5 - 0.7 (Figure 13b) was eluted and stored as above.

Derivatization.--Acidic and neutral auxins were methylated by the micro-method of Powell (1964). To briefly describe the method, the carrier solvent (MeOH) was evaporated under a stream of nitrogen, 1 ml of ethyl ether : MeOH (9:1) added, and a stream of diazomethane bubbled through the

solvent for 1.5 minutes. The diazomethane was generated by the action of 60% KOH and carbitol (2-[2-ethoxy ethoxy] ethanol) on diazald (N-methyl-N-nitroso-p-toluene sulfonamide). At the completion of methylation, the ether : MeOH was evaporated under a stream of nitrogen and the trifluoroacetic anhydride (TFA) derivatives were synthesized (Seeley and Powell, 1970). These derivatives were synthesized under "dry-box" conditions, by the addition of 100 µl of trifluoroacetic anhydride and 0.5 mg Na_2SO_4 to the methylated auxins. After allowing the reaction to proceed for 1 hour, the unreacted TFA was removed under reduced pressure and the fluorinated derivatives diluted to 0.5 ml with ethyl acetate (anhydrous).

Gas-liquid chromatography.--The TFA derivatives of the acidic and neutral IIB auxins were subjected to gas-liquid chromatography (Packard 7300) on 2 x 152 mm glass columns packed with either 3% SE - 30 (methyl silicone gum) coated Gaschrome Q (60-80 mesh) support or 3% DC - 200 coated Gaschrome Q (60-80 mesh). Column packings were prepared by adding the stationary phase (Gaschrome Q) to the liquid phase (SE - 30, DC - 200) in excess $CHCl_3$. After thorough mixing, the $CHCl_3$ was evaporated under reduced pressure. The resulting liquid-coated stationary phase was packed into silanized glass, "U" shaped, columns to within 50 mm of the column inlet and outlet. Column inlet and outlets were filled with silanized glass

wool plugs and stoppered with silicone rubber septums. Columns were conditioned at 210°C for 24 hours before use.

Gas chromatograph inlet block and electron capture detector ovens were maintained at 250°C. Column gas flow rates (nitrogen) were 23 ml/minute (SE - 30) or 42 ml/minute (DC - 200) while scavenger gas flow rates (nitrogen) were maintained at 67 ml/minute or 78 m./minute, respectively.

Combined gas-liquid chromatography--mass specrometry.-- The TFA derivatives of the acidic and neutral IIB auxins were subjected to combined gas-liquid chromatography--mass spectrometry on a LBK 9000 spectrograph. Samples of endogenous (acidic and neutral IIB) or synthetic (IAA, Calbiochem) auxins were introduced to the spectrograph after fractionation on a 2 x 153 mm coiled glass column packed with 3% SE - 30 (stationary phase : Supelcoport [100 - 120 mesh]) and maintained at 130°C. The major peak fractionated from the endogenous acidic auxin (retention time equal to synthetic sample) was introduced to the spectrograph and the fragmentation pattern recorded as a percentage of the most intense ion. Fractionation of the neutral IIB auxin revealed three major peaks (total ion scan). Each peak was introduced to the spectrometer and the fragmentation pattern recorded as a percentage of the most intense ion. The background mass spectrum was subtracted from each spectrum obtained.

Results

Changes in Auxin Levels During Fruit Development, 1969

Growth-promoting substances detected in seed and pericarp tissues (Figure 2, 3) exhibited both quantitative, and qualitative, changes with development of the fruit. Histograms of seed extracts chromatographed in isopropanol : water (4:1) (Figure 2) exhibited two zones of growth promotion and two zones of growth inhibition. The first of these promoters (Rf 0.5 $\pm$ 0.1) increased from a low level (anthesis + 7, + 14; whole fruit) to a peak of activity 21 days after anthesis. Subsequently, this promoter decreased (anthesis + 28) and then increased (anthesis + 35, + 42, + 49). The shift in promoter Rf (Rf 0.7 $\pm$ 0.1) can be attributed to a decrease in ambient temperature. The second promoter (Rf 0.9 $\pm$ 0.1) increased dramatically from a low level (anthesis + 7, + 14) to a maximum 21 days after anthesis. Subsequently, this zone of growth promotion was replaced by one of inhibition (anthesis + 28, + 35, + 42, + 49) but reappeared during the later stages of fruit growth. A further zone of growth inhibition was detected at Rf 0.4 $\pm$ 0.1 (anthesis + 49, + 70).

In contrast to the seed fraction, the pericarp exhibited only one growth promoter (Rf 0.7 $\pm$ 0.1) which did not markedly fluctuate with development of the fruit.

Figure 2.--Histograms depicting the Avena first internode bioassay response to growth promoting substances in seed and pericarp tissues (1969). Solvent system:- isopropanol : water (4:1). Control value (——). Upper and lower 5% fiducial limits (---).

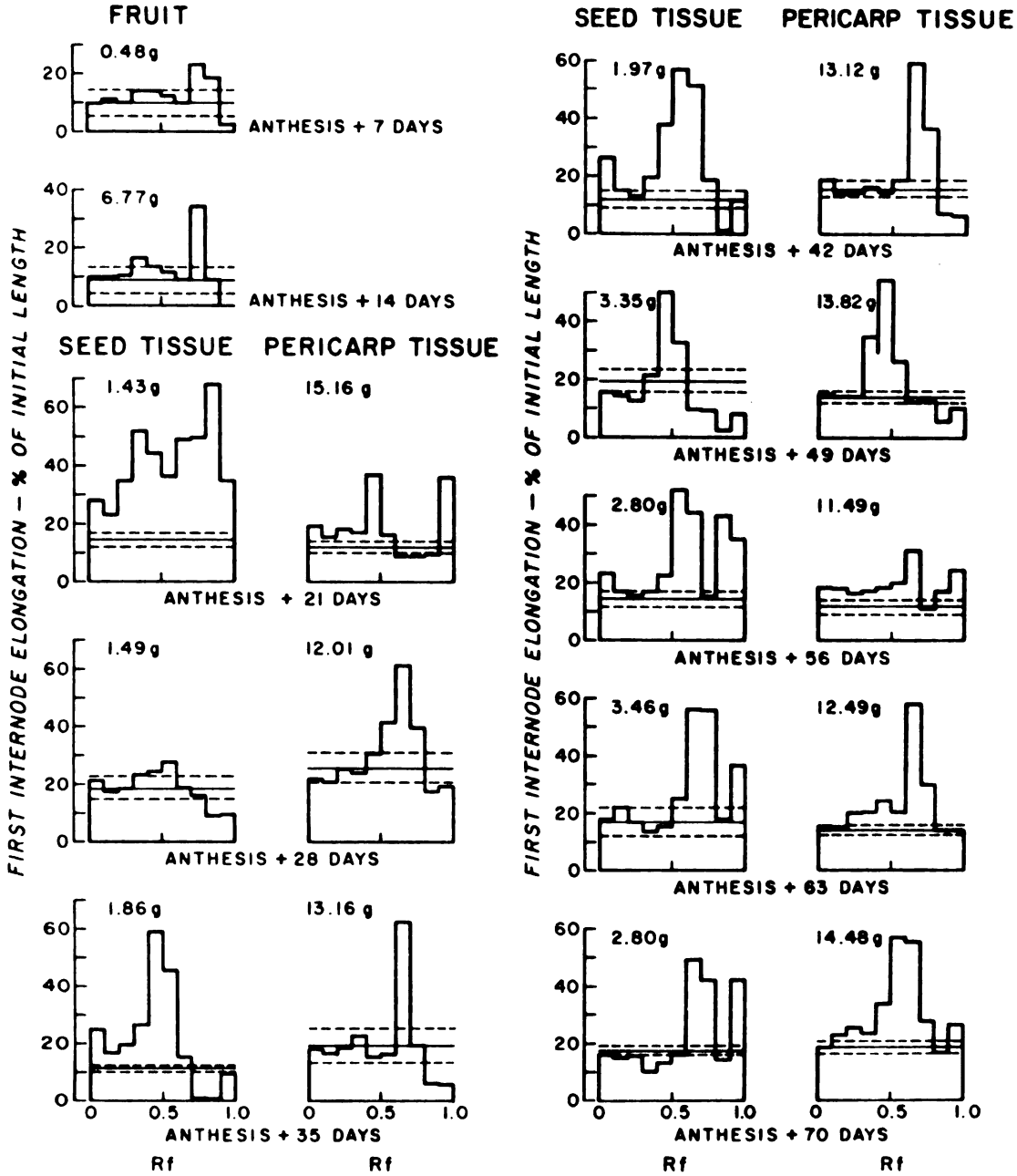
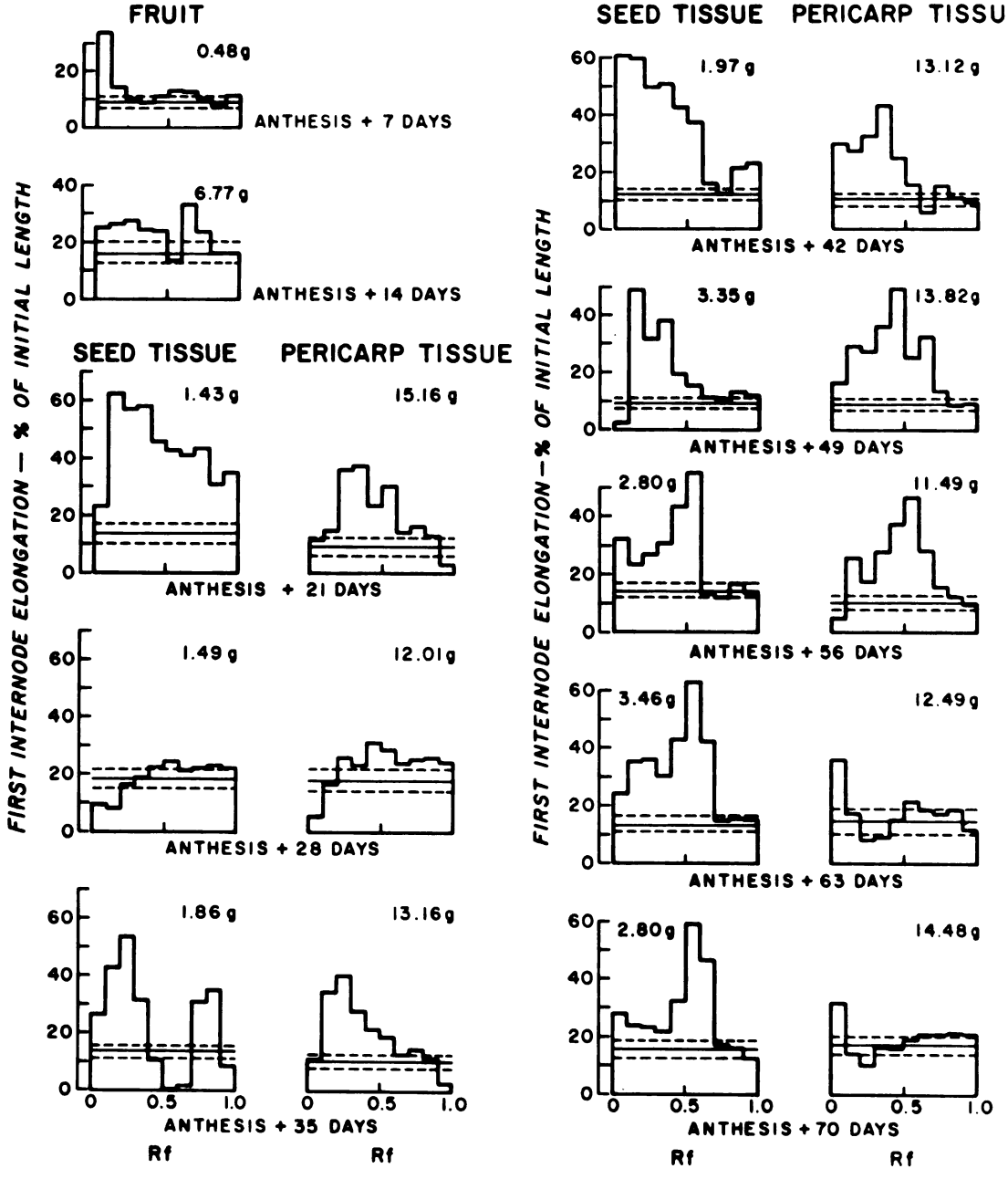

FRUIT
SEED TISSUE
PERICARP TISSUE
0.48g
ANTHESIS + 7 DAYS
6.77g
ANTHESIS + 14 DAYS
1.43g
15.16 g
ANTHESIS + 21 DAYS
1.49g
12.01 g
ANTHESIS + 28 DAYS
1.86g
13.16 g
ANTHESIS + 35 DAYS
1.97g
13.12 g
ANTHESIS + 42 DAYS
3.35g
13.82g
ANTHESIS + 49 DAYS
2.80g
11.49g
ANTHESIS + 56 DAYS
3.46g
12.49g
ANTHESIS + 63 DAYS
2.80g
14.48g
ANTHESIS + 70 DAYS
FIRST INTERNODE ELONGATION — % OF INITIAL LENGTH
Rf

Figure 3. Histograms depicting the Avena first internode bioassay response to growth promoting substances in seed and pericarp tissues (1969). Solvent system:- hexane : water (9:1). Control value (——). Upper and lower 5% fiducial limits (---).


FRUIT
0.48g
ANTHESIS + 7 DAYS
6.77g
ANTHESIS + 14 DAYS
SEED TISSUE
PERICARP TISSUE
1.43g
15.16g
ANTHESIS + 21 DAYS
1.49g
12.01g
ANTHESIS + 28 DAYS
1.86g
13.16g
ANTHESIS + 35 DAYS
Rf
SEED TISSUE
PERICARP TISSUE
1.97g
13.12g
ANTHESIS + 42 DAYS
3.35g
13.82g
ANTHESIS + 49 DAYS
2.80g
11.49g
ANTHESIS + 56 DAYS
3.46g
12.49g
ANTHESIS + 63 DAYS
2.80g
14.48g
ANTHESIS + 70 DAYS
Rf
FIRST INTERNODE ELONGATION — % OF INITIAL LENGTH

Again, a zone of inhibition (Rf 0.9 ± 0.1) occurred in all fractions from 28 to 49 days after anthesis.

Chromatography of seed and pericarp fractions in hexane : water (9:1) resulted in promoter and inhibitor zones (Figure 3) that were not as well defined as shown previously (Figure 2). A general zone of promotion (Rf 0.2 ± 0.1) found in seed fractions, presumed to be acidic in nature, increased to a maximum 21 days after anthesis, declined (anthesis + 28) and then increased to a second maximum 42 days after anthesis. This behavior is similar to that found previously (Rf 0.5 ± 0.1, Figure 2). The levels of this promoter declined with successively later harvests (anthesis + 49, + 56), (Figure 3). A second general zone of promotion (Rf 0.6 ± 0.1), presumed to be neutral in nature, appeared to be the major histogram component in later harvests (anthesis + 56, + 63, + 70). A third, poorly defined, zone of promotion occurred between Rf 0.3 - 0.5 21, and 42, days after anthesis. This latter zone may represent overlapping of the presumed acidic and neutral promoters.

In comparison to the seed fraction, the pericarp exhibited only one zone of growth promotion (Rf 0.2 ± 0.1) (Figure 3) during the initial stages of fruit growth (anthesis + 21, + 28, + 35). A second, but different promotive zone (Rf 0.6 ± 0.1), shown by histograms at 49 and 56 days after anthesis, was not detected during the later stages of fruit growth (anthesis + 63, + 70).

In order to relate detected growth-promotive activity to fruit growth, histogram peaks delineated by the upper 5% fiducial line (Figure 2, 3) were converted to IAA equivalents by means of a standard curve (Figure 1, Appendix A). Since organic solvents used in the extraction and fractionation procedures were without activity in the Avena first internode bioassay (Figure 2, Appendix A), the computed growth response is due entirely to extracted substances. Results obtained from the standard curve were converted to IAA equivalents/gram dry weight or IAA equivalents/fruit and expressed as a function of time after anthesis.

Comparison of IAA equivalents/gram dry weight between harvest dates (Figure 4A) showed that seed growth substance levels were initially low, increased to a maximum at day 21 and then declined. Up to this point, there was good agreement between the two solvent systems. The low level of growth substances detected at the 35th (hexane : water) and 42nd day after anthesis (isopropanol : water) can be attributed to zones of inhibition at Rf 0.5 - 0.7 (Figure 3) and Rf 0.8 (Figure 2), respectively. As will be shown later, this effect was due to superimposition of two neutral substances, the result of which was strong inhibition. By day 49, levels of growth substances had again fallen to a low level (Figure 4A).

(In contrast to the levels found in the seed, the levels in the pericarp were approximately 2 orders of

magnitude lower (Figure 4B). Moreover, the peak in seed extracts at day 21 is absent. Instead, levels increased slowly to the onset of rapid cell enlargement (day 42), decreased during the next 14 days and then increased just prior to maturity. The absence of a similar increase in hexane : water chromatograms could be due to supra-optimal levels of overlapping promoters (Figure 3).

Expression of detected growth-promoting substances as IAA equivalents/fruit (Figure 5) shifts the emphasis of promoter levels from the initial to the later stages of fruit growth. For example, the peak of seed activity at day 21 (Figure 4A) is markedly reduced while the peaks of activity at days 56 and 63 are dramatically increased. Similarly, the level of promoters in the pericarp during stage III of fruit growth were increased.

Changes in Acidic and Neutral Auxins During Fruit Development, 1971

Acidic and neutral fractions from seed and pericarp extracts were individually chromatographed and bioassayed. Detected auxin activity was converted to IAA equivalents by means of a standard curve and the results (Tables 1, 2) compared with those obtained in 1969 (Figures 4, 5). Direct comparison in the quantitative sense between years is not feasible since fruit growth curves were not identical and, in 1971, acidic and neutral phases were treated separately.

Figure 4.--Changes in levels of endogenous growth substances in seed and pericarp tissues, expressed as IAA equivalents/gram dry weight, in relation to fruit development. IAA equivalents (-0-, -●-). Fruit weight (-▽-).

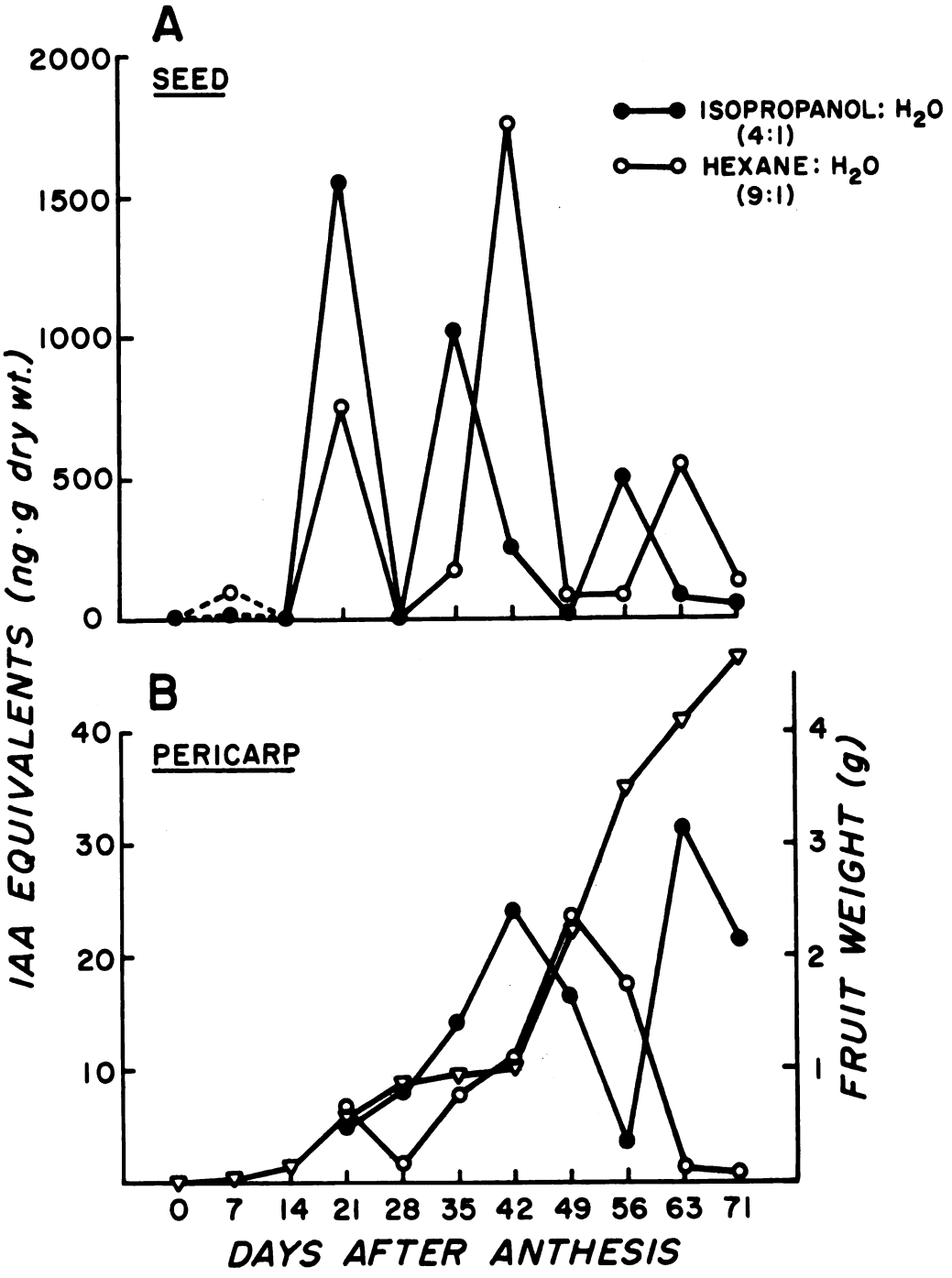

A
SEED
2000
1500
1000
500
0
ISOPROPANOL: H2O
(4:1)
HEXANE: H2O
(9:1)
B
PERICARP
40
30
20
10
4
3
2
1
IAA EQUIVALENTS (ng·g dry wt.)
FRUIT WEIGHT (g)
0 7 14 21 28 35 42 49 56 63 71
DAYS AFTER ANTHESIS

Figure 5. Changes in levels of endogenous growth substances in seed and pericarp tissues, expressed as IAA equivalents/fruit, in relation to fruit development. IAA equivalents (-0-, -●-). Fruit weight (-▽-).

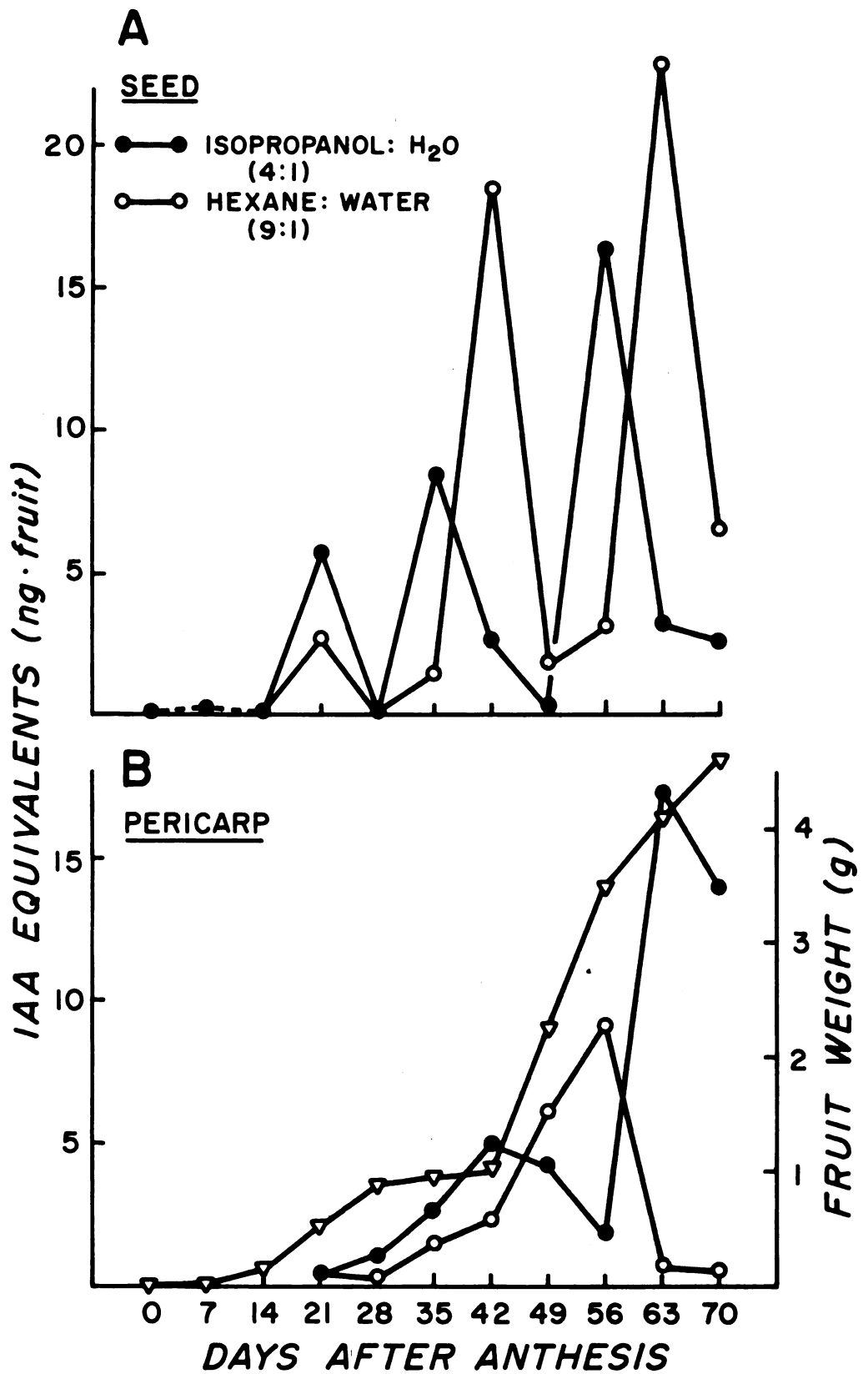

A
SEED
ISOPROPANOL: H2O
(4:1)
HEXANE: WATER
(9:1)
20
15
10
5
B
PERICARP
15
10
5
IAA EQUIVALENTS (ng·fruit)
FRUIT WEIGHT (g)
4
3
2
1
0 7 14 21 28 35 42 49 56 63 70
DAYS AFTER ANTHESIS

TABLE 1.--Comparison of auxin concentrations detected in sour cherry seed and pericarp tissues at different stages of fruit development in 1969 and 1971.

Days after Anthesis	IAA Equivalents (ng/gram dry wt.) Acidic Auxin	Neutral Auxin IIA	Neutral Auxin IIB	Total Auxin (1971)	Total Auxin (1969) IW[1]	Total Auxin (1969) HW[2]
			Seed			
21	1488	1140	2918	5546	1561	742
28	94	880	1182	2156	4.7	5.7
42	28	24	402	454	246	1760
56	53	0	523	576	508	95
			Pericarp			
21	164	0	5.7	169.7	4.8	6.8
28	0	0	0	0	8.0	1.7
42	12	0	117	129	24.1	11.0
56	6	0	3.7	9.7	3.4	17.6

[1]Isopropanol : water (4:1) (Figure 3A, 3B).

[2]Hexane : water (9:1) (Figure 3A, 3B).

TABLE 2.--Comparison of auxin levels detected in sour cherry seed and pericarp tissues at different stages of fruit development in 1969 and 1971.

Days after Anthesis	IAA Equivalents (ng/fruit) Acidic Auxin	Neutral Auxin IIA	Neutral Auxin IIB	Total Auxin (1971)	Total Auxin (1969) IW[1]	HW[2]
			Seed			
21	11.2	8.6	22.1	41.9	5.6	2.6
28	0.8	7.7	10.4	18.9	0.02	0.02
42	0.7	0.6	10.3	11.6	2.6	18.5
56	0.7	0	6.6	7.3	16.4	3.0
			Pericarp			
21	8.7	0	0.4	9.1	0.2	0.4
28	0	0	0	0	1.0	0.2
42	2.2	0	20.8	23.0	5.0	2.3
56	4.1	0	2.5	6.6	1.8	9.1

[1]Isopropanol : water (4:1) (Figure 4A, 4B).

[2]Hexane : water (9:1) (Figure 4A, 4B).

Furthermore, bioassay of known amounts of IAA that had been processed through the extraction and fractionation procedure used to determine changes in auxin levels in 1969 showed that substantial losses occurred. Expression of the amount recovered as a ratio of the amount added gave values of 0.22, 0.18, and 0.23 for three replicates. Part of these losses can be attributed to the solubility of one solvent in another during partition chromatography (Figure 3A, B, Appendix A). Further losses have been reported to occur during chromatogram equilibration and development (Kefford, 1955). Accordingly, all chromatograms involving quantitative determinations (Tables 1, 2) were equilibrated and developed in a nitrogen atmosphere although recovery values were not obtained. Thus the differences between harvest dates are the only valid comparisons.

High concentrations of acidic and neutral auxins (Figure 6B, 17) were located in seed extracts 21 days after anthesis (Table 1). Subsequent to the third week from anthesis all auxin levels, except for seed neutral IIA, decreased (28 and 42 days after anthesis) and then increased. This was also true in 1969 (Figure 4A). In all seed tissues analyzed (1971) the neutral IIB auxin was found to be the major auxinic component. Total auxins in the pericarp (Table 1) decreased from a maximum at day 21 to a non-detectable level at day 28 and then increased (42 days

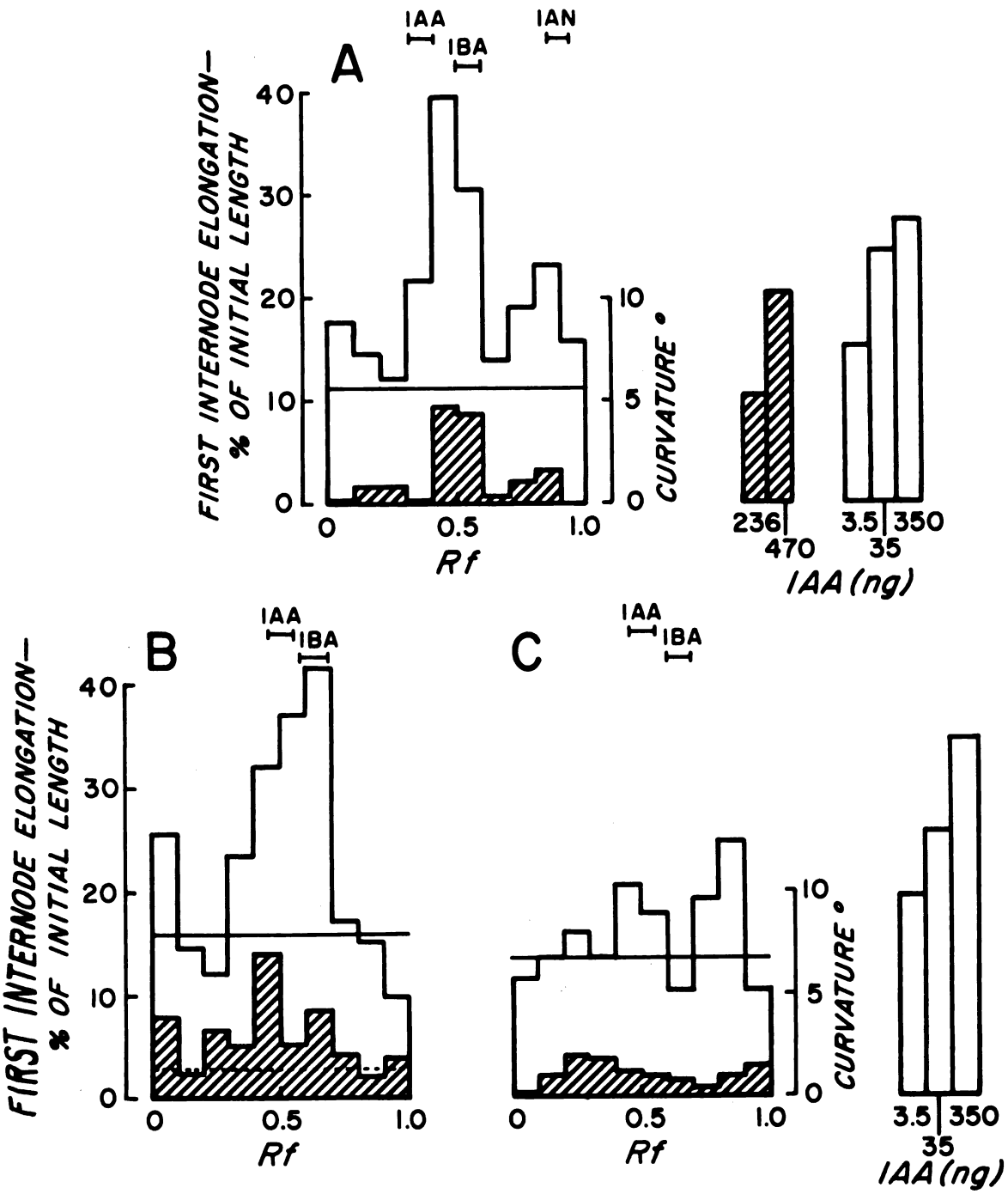
Figure 6.--Histograms depicting the *Avena* first internode (open bars) and *Avena* curvature (shaded bars) bioassay response to acidic seed auxins (21 days after anthesis).

A. 0.3 gram equivalents chromatographed in isopropanol : water (9:1).

B. Re-chromatography of 0.4 gram equivalents Acidic I (Figure 6A, Rf 0.3 - 0.6). Solvent system:- isopropanol : ammonia (30%) : water (10:1:1).

C. Re-chromatrography of 0.4 gram equivalents Acidic II (Figure 6A, Rf 0.7 - 0.9). Solvent system:- isopropanol : ammonia (30%) : water (10:1:1).

Avena first internode bioassay control value (——). *Avena* curvature bioassay control value (---) (Zero value, Figure 6A, 6C).


A
IAA
IBA
IAN
FIRST INTERNODE ELONGATION—
% OF INITIAL LENGTH
40
30
20
10
0
0
0.5
1.0
Rf
10
5
0
CURVATURE °
236
470
3.5
350
35
IAA (ng)
B
IAA
IBA
FIRST INTERNODE ELONGATION—
% OF INITIAL LENGTH
40
30
20
10
0
0
0.5
1.0
Rf
C
IAA
IBA
10
5
0
CURVATURE °
0
0.5
1.0
Rf
3.5
350
35
IAA (ng)

from anthesis). No neutral auxin IIA was detected in pericarp tissues.

When the above data are expressed in terms of IAA equivalents/fruit differences between years are evident (Table 2). The highest level of total seed auxin was found at 21 days after anthesis in 1971 rather than at 42 or 56 days (1969). Seed acidic auxin content rapidly fell to a low, and nearly constant level (anthesis + 28, + 42, + 56), while the level of neutral IIA and IIB auxin declined at a much slower rate. The pericarp fraction, however, had an initially low level of neutral IIB auxin (anthesis + 21, + 28) which increased by day 42 to a maximum and then declined. During the same period, the acidic auxin decreased from an initially high level and then increased with the later stages of fruit growth.

Characterization of Seed Acidic Auxins

Anthesis + 21 days.--Chromatographic separation of acidic substances and subsequent detection by bioassay revealed two peaks of activity (Figure 6A). The first of these (Rf 0.3 - 0.6, designated Acidic I) was active in both the *Avena* first internode and curvature bioassays and chromatographed with applied standards (IAA, IBA). The second peak of activity (Rf 0.8 - 1.0, designated Acidic II) was active only in the straight growth test.

Preparation of a new sample (as described above), elution of Acidics I and II, and re-chromatography of the eluates in a basic solvent resulted in no further resolution of Acidic I (Figure 6B) but separated Acidic II into two substances which were inactive in the Avena curvature test (Figure 6C). The peak of activity located from the Acidic I chromatogram (Rf 0.3 - 0.7, Figure 6B) chromatographed with applied standards (IAA, IBA). Since both standards ran together, the promotive activity at Rf 0.3 - 0.7 (Figure 6B) was again re-chromatographed in an attempt to further separate the active moieties.

Replicate samples of Acidic I were prepared according to the method outlined above. One of these samples was re-chromatographed, in toto, in acetonitrile : water (4:1) and bioassayed (Figure 7A). The other sample was divided and each half separately chromatographed in acetonitrile : water (4:1). One of these latter chromatograms was bioassayed directly (Figure 7B) to provide a replicate of that shown in Figure 7A.

Re-chromatography in acetonitrile : water (Figure 7A) did not resolve Acidic I into further components. This result withstood replication (Figure 7B). However, the peak of activity (Figure 7A, 7B) chromatographed with applied IAA and IBA standards. In an attempt to determine if the active moiety was IAA-like or IBA-like, the remaining chromatogram (see above) was cut at the Rf of IAA (Rf

Figure 7.--Histograms depicting the Avena first internode bioassay response to acidic seed auxins (21 days after anthesis).

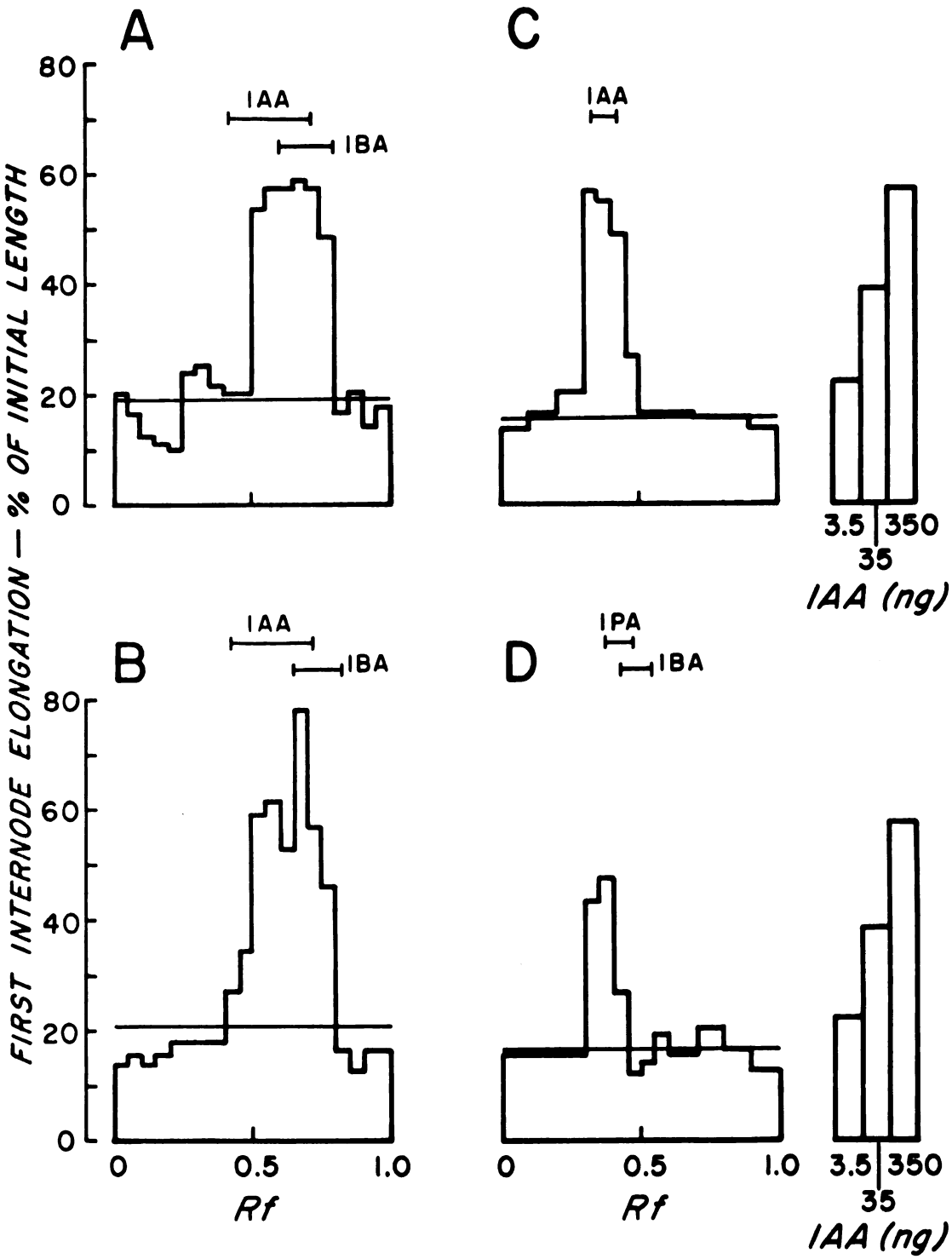
A. Re-chromatography of 0.2 gram equivalents Acidic I (Rf 0.3 - 0.7, Figure 6B). Solvent system:- acetonitrile : water (4:1).

B. Re-chromatography of 0.1 gram equivalents Acidic I (Rf 0.3 - 0.7, Figure 6B). Solvent system:- acetonitrile : water (4:1).

C. Re-chromatography of elute from Rf 0.35 - 0.47 (Figure 7A). Solvent system:- acetonitrile : water (4:1).

D. Re-chromatography of eluate from Rf 0.47 - 0.70 (Figure 7A). Solvent system:- acetonitrile : water (4:1).

Control value (——).


A
B
C
D
IAA
IBA
IPA
80
60
40
20
0
FIRST INTERNODE ELONGATION — % OF INITIAL LENGTH
0
0.5
1.0
Rf
3.5
350
35
IAA (ng)

0.35 - 0.47) and at the Rf of IBA (Rf 0.47 - 0.70) and both strips were individually eluted. Re-chromatography of these eluates in acetonitrile : water (4:1), and subsequent bioassay, showed that each component (Rf 0.35 - 0.47, Rf 0.47 - 0.70) yielded identical peaks (Figure 7C, 7D, respectively). This evidence strongly suggests that the peak of activity shown in Figure 7A, 7B is homogeneous and similar to IAA.

Chromatogram strips, taken from the edge of the developed streaks, were sprayed with Salkowski reagent. With the exceptions of Acidic II, all chromatograms gave the intense red-purple reaction typical of IAA at those zones that would have been adjacent to detected growth promotive activity.

Anthesis + 42 days.--A single, broad, peak of acidic growth promotive activity was located between Rf 0.0 - 0.4 when a relatively concentrated sample (4.5 gram equivalents) was chromatographed in isopropanol : water (9:1) (Figure 8A). Re-chromatography of an eluate of this zone in two further solvents resulted in single peaks of activity (Figure 8B, 8C) at the respective Rf's of IAA. The Rf of the promoter in Figure 8B corresponded with that found earlier (Figure 2) for the seed fraction 28, 35, 42, and 49 days after anthesis. Similarly, the activity shown in Figure 8C occurred at the Rf of the active fraction found in the seed 21 days after anthesis (Figure 7A, 7B).

Figure 8.--Histograms depicting the Avena first internode bioassay response to seed acidic auxins (42 days after anthesis).

A. 4.5 gram equivalents chromatographed in isopropanol : water (9:1).

B. Re-chromatography of 1.5 gram equivalents Acidic I (Figure 8A, Rf 0.0 - 0.4). Solvent system:- isopropanol : water (4:1).

C. Re-chromatography of 1.5 gram equivalents Acidic I (Figure 8A, Rf 0.0 - 0.4). Solvent system:- acetonitrile : water (4:1).

Control value (——).

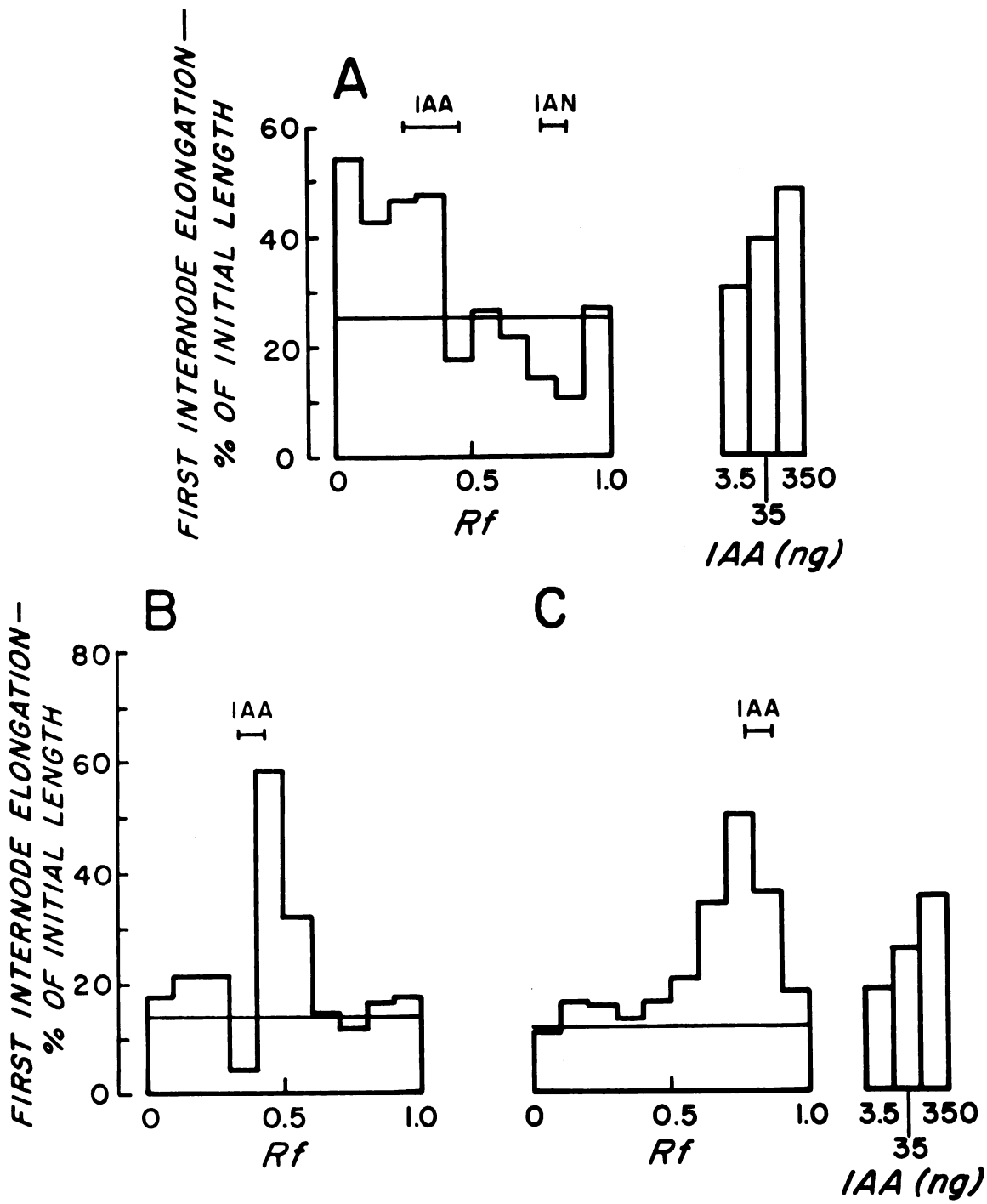

A
IAA
IAN
60
40
20
0
0
0.5
1.0
Rf
FIRST INTERNODE ELONGATION—
% OF INITIAL LENGTH
3.5
350
35
IAA (ng)
B
C
IAA
IAA
80
60
40
20
0
0
0.5
1.0
Rf
0
0.5
1.0
Rf
FIRST INTERNODE ELONGATION—
% OF INITIAL LENGTH
3.5
350
35
IAA (ng)

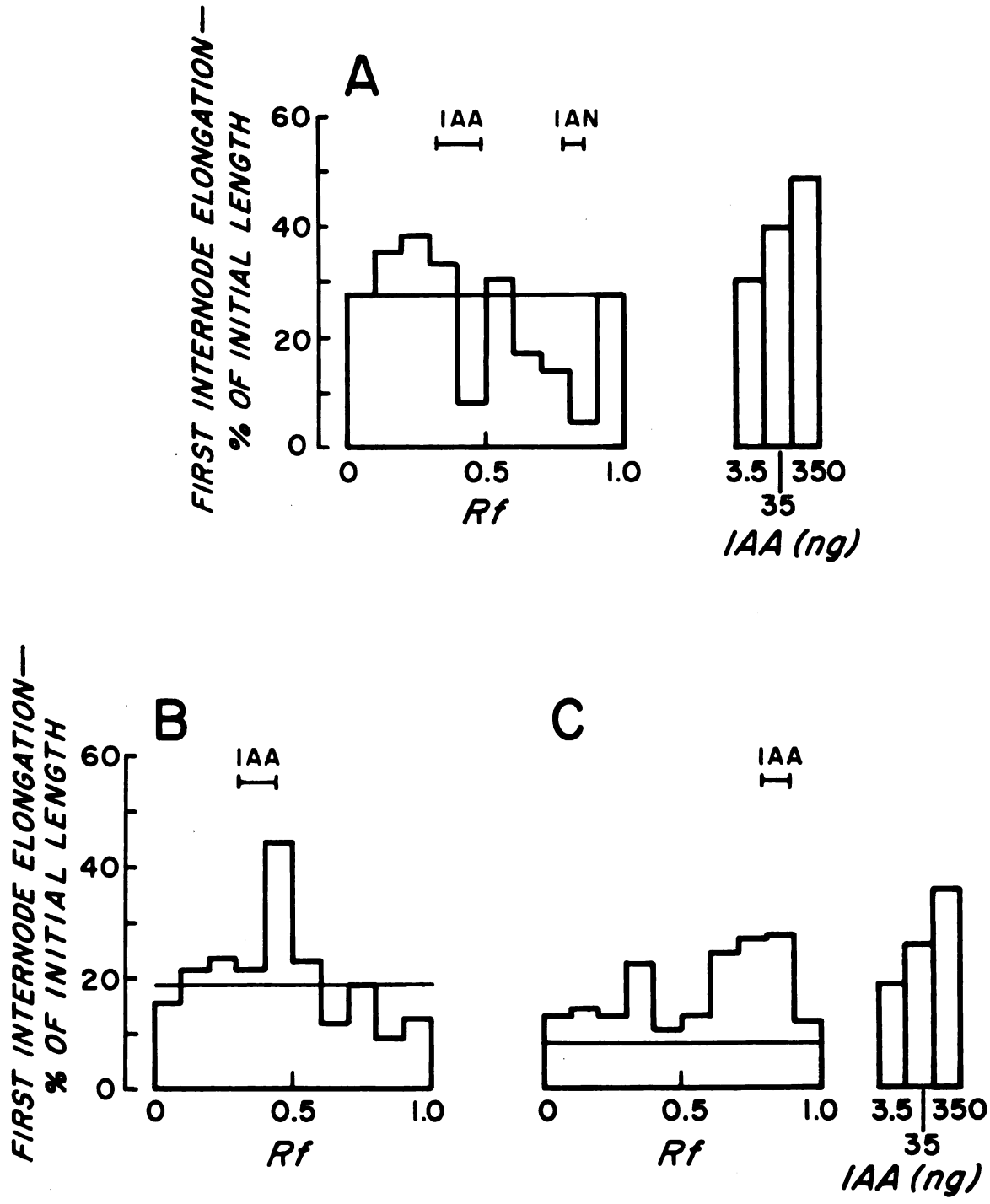
Figure 9.--Histograms depicting the Avena first internode bioassay response to pericarp acidic auxins (42 days after anthesis).

A. 12.0 gram equivalents chromatographed in isopropanol : water (9:1).

B. Re-chromatography of 4.0 gram equivalents Acidic I (Figure 9A, Rf 0.1 - 0.3). Solvent system:- isopropanol : water (4:1).

C. Re-chromatography of 4.0 gram equivalents Acidic I (Figure 9A, Rf 0.1 - 0.3). Solvent system acetonitrile : water (4:1).

Control value (——).


A
IAA
IAN
60
40
20
0
0
0.5
1.0
Rf
FIRST INTERNODE ELONGATION—
% OF INITIAL LENGTH
3.5
350
35
IAA (ng)
B
IAA
C
IAA

Characterization of Pericarp Acidic Auxins

Anthesis + 42 days.--Acidic substances separated by paper chromatography (Figure 9A) show a similar distribution pattern to those previously found for seed extracts (Figure 8A) although the promoter peak (Rf 0.1 - 0.3, Acidic I) is not as pronounced. Re-chromatography of Acidic I in isopropanol : water (4:1) (Figure 9B) and acetonitrile : water (Figure 9C) again showed a promoter distribution similar to that found previously (Figure 8B, 8C).

Gas-liquid Chromatography of the Acidic I Auxin

Gas-liquid chromatographic analysis of the TFA derivatives synthesized from both synthetic and endogenous acidic auxins showed that acceptable resolution could be achieved on 3% SE - 30 liquid phase at a column temperature of 140°C. Injection of 1000 pg of derivatized IAA (synthesized from synthetic IAA purchased from Sigma Chemical Company) yielded a single, large peak with a retention time of 3.75 minutes (Figure 10A). Injection of a further derivatized sample (Calbiochem) again yielded a single, large, peak with a retention time of 3.73 minutes (Figure 10B). Since both samples gave a single, major, peak at equal retention times the assumption was made that the observed peak was due to IAA. When the endogenous derivatized acidic auxin I (Figure 6A) was similarly chromatographed (Figure 10C) the major peak occurred at the same

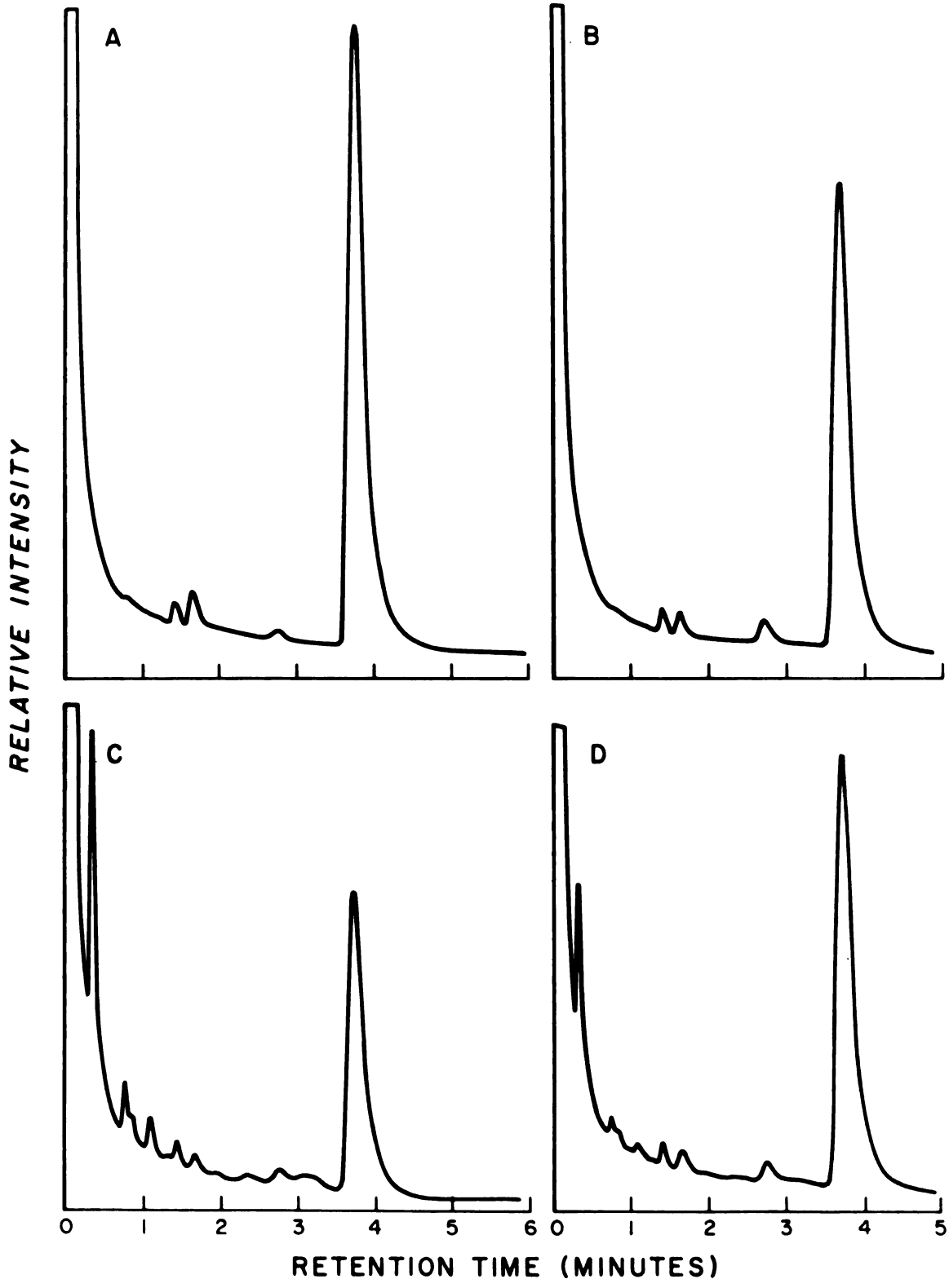
Figure 10.--Gas-liquid chromatographic resolution of synthetic IAA and endogenous acidic auxin I. The trifluoroacetic ether was synthesized from the methyl ester of each auxin prior to injection. Column packing - Gaschrome Q (60/80) coated with 3% SE-30; column temperature - 140°C; column flow rate (N_2) - 23 ml/minute.

A. Standard IAA (Sigma Chemical Company).

B. Standard IAA (Calbiochem).

C. Endogenous acidic auxin I.

D. Co-chromatography of endogenous acidic auxin and standard IAA (Calbiochem).


A
B
C
D
RELATIVE INTENSITY
0
1
2
3
4
5
6
RETENTION TIME (MINUTES)

retention time as synthetic IAA. Co-chromatography of known and unknown auxin did not reveal any dissimilarities in retention time (Figure 10D). Chromatographic analysis of other standards under the above conditions gave retention times of 3.17, 6.00, 9.80 minutes for IAN, IPA, and IBA, respectively.

Chromatography of the endogenous acidic auxin and standards, on a second column (3% DC - 200), isothermal at 140°, gave retention times of 4.12, 4.12, 4.15 and 4.15 minutes for standard IAA (Sigma), standard IAA (Calbiochem), endogenous acidic auxin, and endogenous acidic auxin co-chromatographed with standard IAA (Calbiochem), respectively. Chromatography of other standards gave retention times of 3.37, 6.70, and 10.58 minutes for IAN, IPA, and IBA, respectively. However, both IPA and IBA gave broad peaks with poor resolution.

Combined Gas-liquid Chromatography--Mass Spectrometry of the Acidic I Auxin

Gas-liquid chromatographic resolution of a 3 µl sample of derivatized, synthetic, IAA (Calbiochem) yielded a single peak on 3% SE - 30 liquid phase (stationary phase; Supelcoport, 100 - 120 mesh) with a retention time of 9.4 minutes. The center of this peak was introduced into the mass spectrometer. The spectrum obtained is shown in Figure 11A. Similar manipulations were carried out on a 5.0 µl sample of derivatized, endogenous acidic auxin I

(retention time 9.3 minutes) and the spectrum obtained is shown in Figure 11B.

The molecular ion (M) observed in both spectra occurred at m/e 285. Other features common to both spectra include fragment ions at M - 59 (associated with the loss of the methoxycarbonyl group) and at M - 156 (associated with the loss of the TFA ether group). The ion fragments found at M - 156, M - 183, M - 209, M - 210, M - 211, and M - 234 have been shown to occur as spectral characteristics of IAA (Jamieson and Hutzinger, 1970).

The exact correspondence between the endogenous acidic I spectrum and that of synthetic IAA, the fragmentation pattern obtained, and the correspondence with a published spectrum is conclusive evidence that the extracted endogenous acidic auxin I is indole-3-acetic acid.

Characterization of Seed Neutral Auxins

Anthesis + 21 days.--Chromatographic separation of neutral substances and subsequent detection by the *Avena* first internode bioassay revealed a broken peak of activity at Rf 0.5 - 0.7 (designated Neutral I, Figure 12A). The *Avena* curvature bioassay, however, showed a second, and seemingly unrelated, peak of activity at Rf 0.8 (designated Neutral II, Figure 12A). When re-chromatographed in water, the Neutral I fraction showed no significant promotion in either bioassay (Figure 12B). Re-chromatography of Neutral II, however, yielded a zone of growth promotive activity

Figure 11.--The mass spectrum from combined gas-liquid chromatography-mass spectrometry of the trifluoroacetic ether of methylated endogenous acidic auxin I and IAA. The relative intensity (per cent of base ion) of each fragment was plotted as a function of its mass/charge ratio.

A. 3 µl of presumed TFA-IAA-Me.

B. 5 µl of presumed TFA-acidic auxin I-Me.

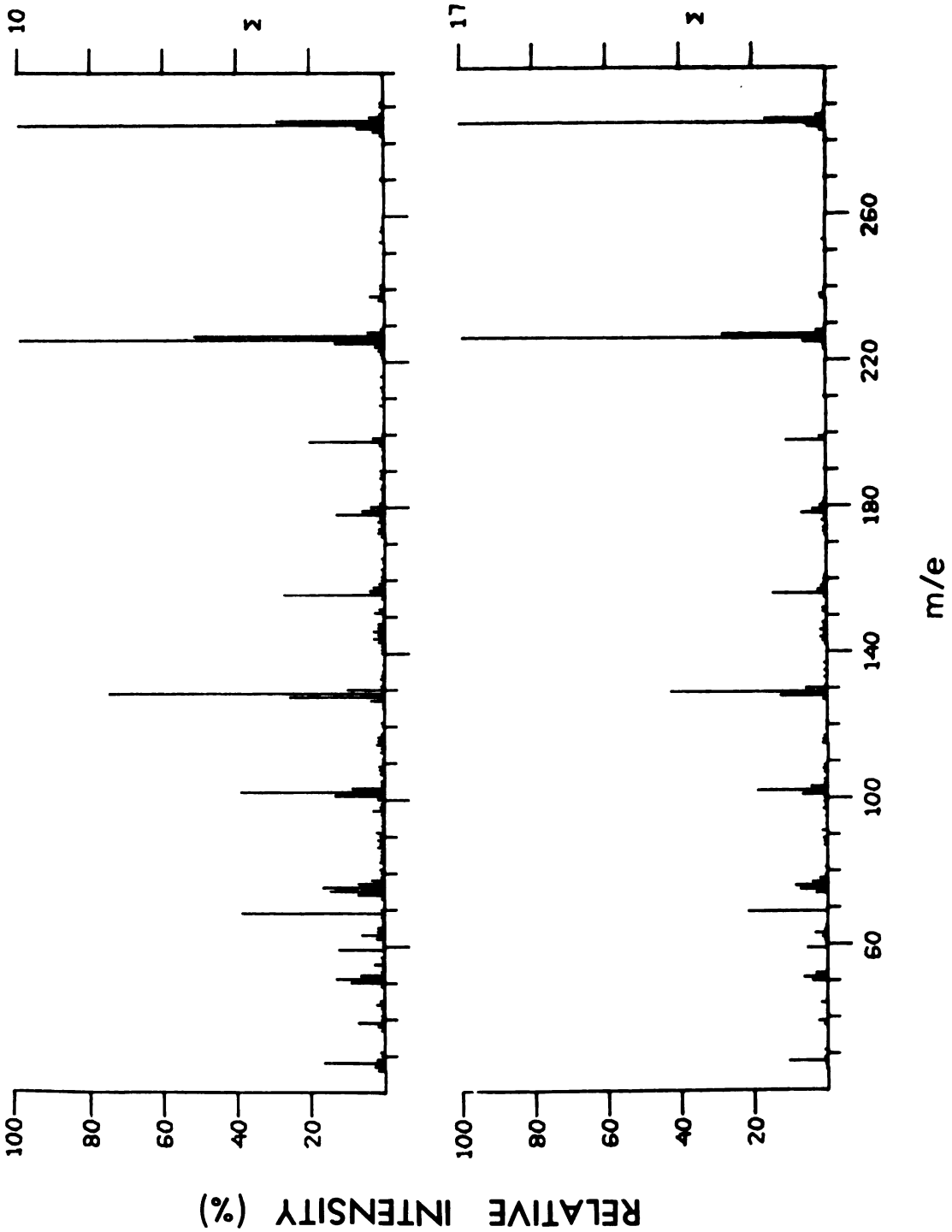
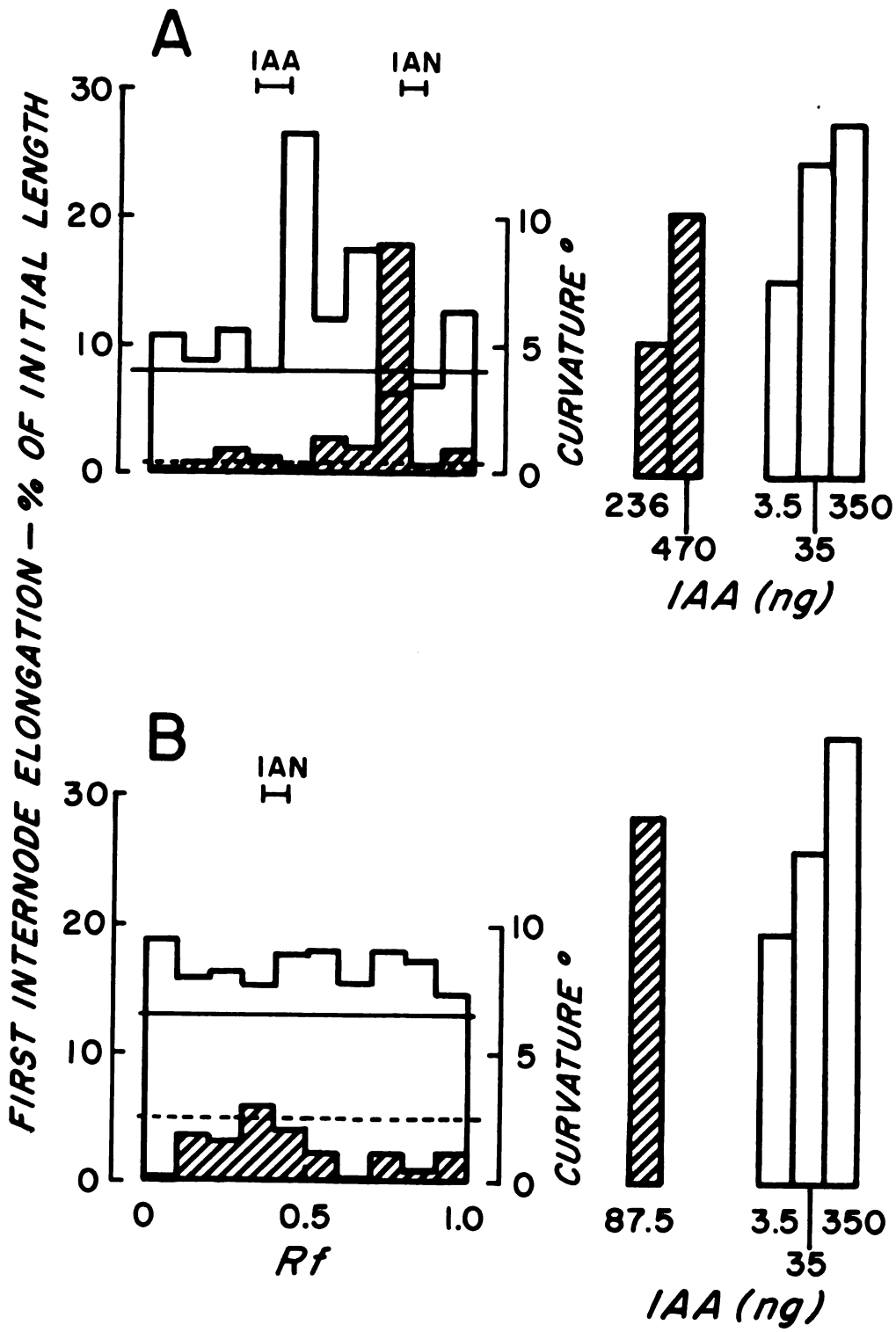

10
17
RELATIVE INTENSITY (%)
100
80
60
40
20
60
100
140
180
220
260
m/e

Figure 12.--Histograms depicting the Avena first internode (open bars) and Avena curvature (shaded bars) bioassay response to seed neutral auxins (21 days after anthesis).

A. 0.3 gram equivalents chromatographed in isopropanol : water (9:1).

B. Re-chromatography of 0.4 gram equivalents Neutral I (Figure 12A, Rf 0.5 - 0.7). Solvent system:- water.

Avena first internode bioassay control value (——). Avena curvature bioassay control value (---).


A
IAA
IAN
30
20
10
0
10
5
0
CURVATURE °
236
470
3.5
350
35
IAA (ng)
B
IAN
30
20
10
0
10
5
0
CURVATURE °
0
0.5
1.0
Rf
87.5
3.5
350
35
IAA (ng)
FIRST INTERNODE ELONGATION — % OF INITIAL LENGTH

(Rf 0.3 - 0.6, Figure 13A) that chromatographed with IAN. When this latter promotive activity (Rf 0.3 - 0.6) was again re-chromatographed in hexane : water (upper phase), resolution into two fractions was achieved (Figure 13B). One fraction remained at Rf 0.0 - 0.1 while the second moved an appreciable distance to Rf 0.6 - 0.7. Neither fraction chromatographed with IAN nor reacted with chromogenic reagents (Salkowski, Ehrlich).

Anthesis + 42 days.--Chromatography of the neutral seed auxins and subsequent bioassay resulted in a zone of weak promotion (Rf 0.5 - 0.6, Neutral I) (Figure 14A) and a zone of strong inhibition (Rf 0.8 - 0.9, Neutral II) similar to that shown previously (Figure 2, anthesis + 28, + 35, + 42, + 49 days, Figure 12A). When both fractions were re-chromatographed separately, only the Neutral II fraction exhibited a promoter peak at Rf 0.6 - 0.7 (Figure 14C). This promoter had been previously shown in the seed fraction 21 days after anthesis (Figure 13B).

Characterization of Pericarp Neutral Auxins

Anthesis + 42 days.--Chromatography of the neutral pericarp fraction (Figure 15A) yielded weak promotive zones at Rf 0.1 - 0.3 and Rf 0.7 and a zone of strong inhibition (Rf 0.8 - 1.0, Neutral II). Since the zone at Rf 0.5 - 0.7 had been previously shown to be weakly promotive (Figure 12A, 14A), the zone at Rf 0.5 - 0.7 (Neutral I, Figure 15A) was

Figure 13.--Histograms depicting the Avena first internode (open bars) and Avena curvature (shaded bars) bioassay response to seed neutral auxins (21 days after anthesis).

A. Re-chromatography of 0.4 gram equivalents Neutral II (Figure 12A, Rf 0.8). Solvent system:- water.

B. 0.3 gram equivalents Neutral II (Figure 12A, Rf 0.8) re-chromatographed in hexane : water (upper phase) after equilibration over water.

Avena first internode bioassay control value (——). Avena curvature bioassay control value (---).

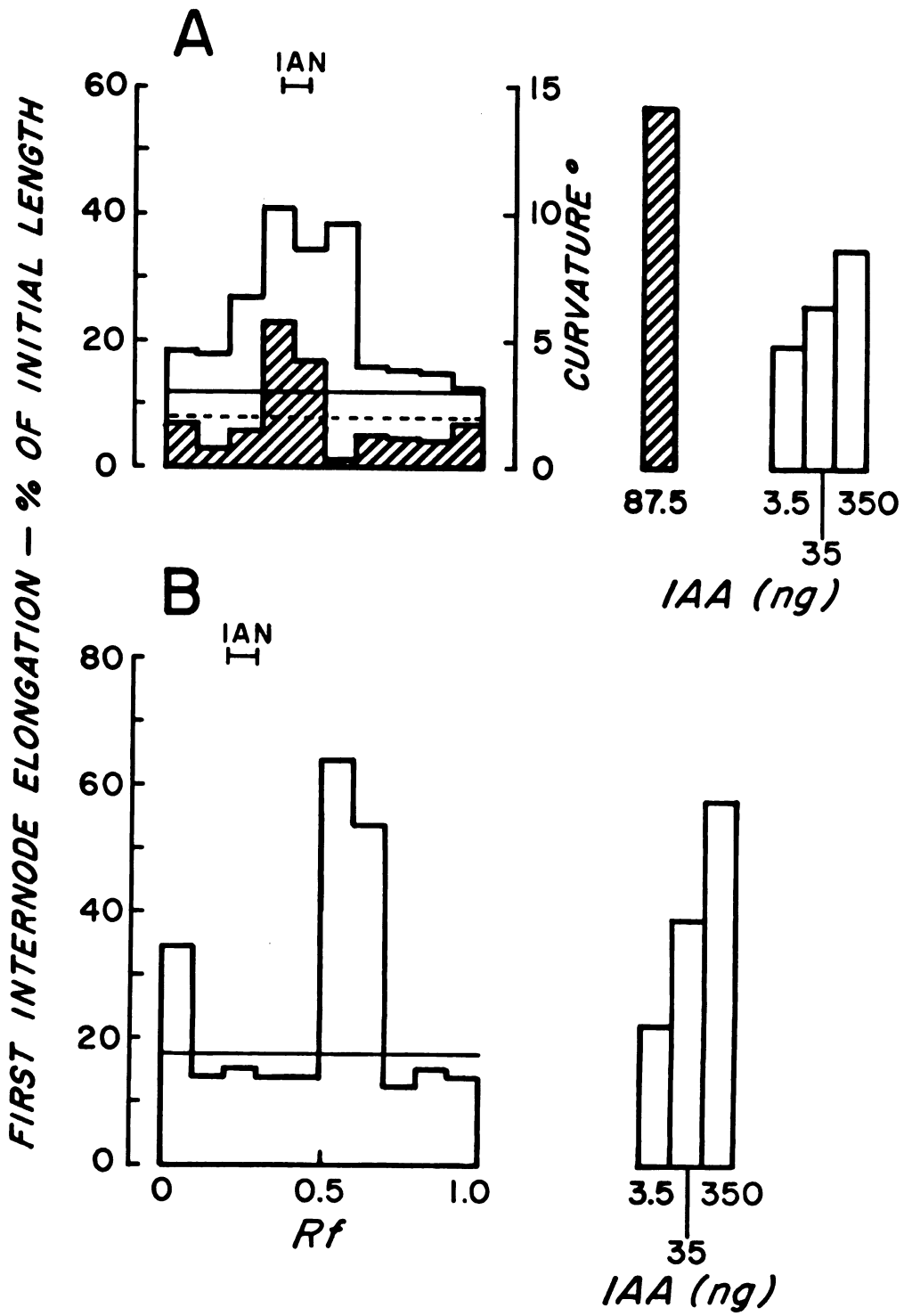

A
IAN
60
40
20
0
15
10
5
0
CURVATURE°
87.5
3.5
350
35
IAA (ng)
B
IAN
80
60
40
20
0
0
0.5
1.0
Rf
3.5
350
35
IAA (ng)
FIRST INTERNODE ELONGATION — % OF INITIAL LENGTH

Figure 14.--Histograms depicting the Avena first internode bioassay response to seed neutral auxins (42 days after anthesis).

A. 4.5 gram equivalents chromatographed in isopropanol : water (9:1).

B. Re-chromatography of 1.5 gram equivalents Neutral I (Figure 14A, Rf 0.5 - 0.6). Solvent system:- hexane : water (upper phase).

C. Re-chromatography of 1.5 gram equivalents Neutral II (Figure 14A, Rf 0.8 - 1.0). Solvent system:- hexane : water (upper phase).

Control value (——).

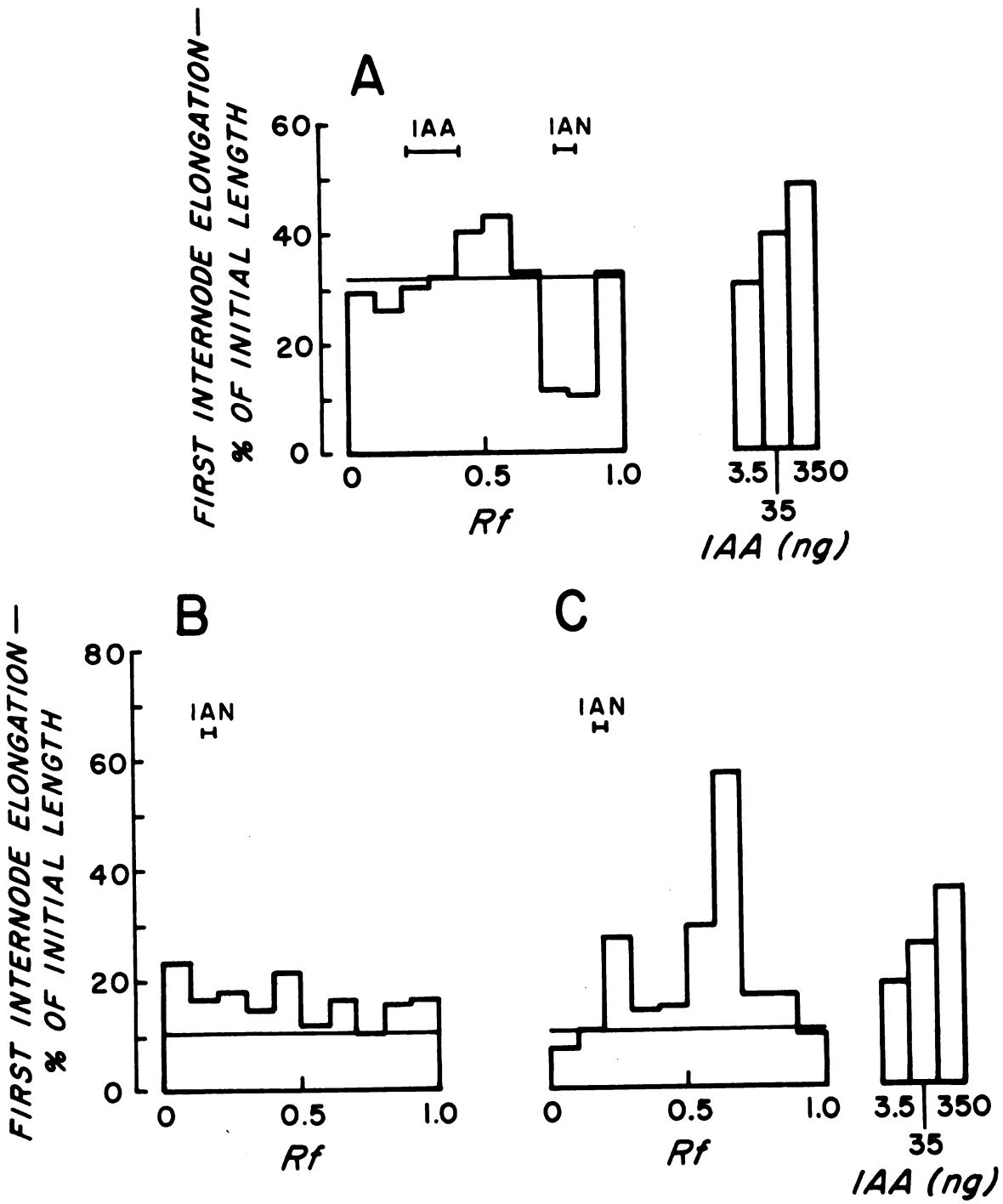

A
B
C
FIRST INTERNODE ELONGATION—
% OF INITIAL LENGTH
IAA
IAN
60
40
20
0
80
0
0.5
1.0
Rf
3.5
350
35
IAA (ng)

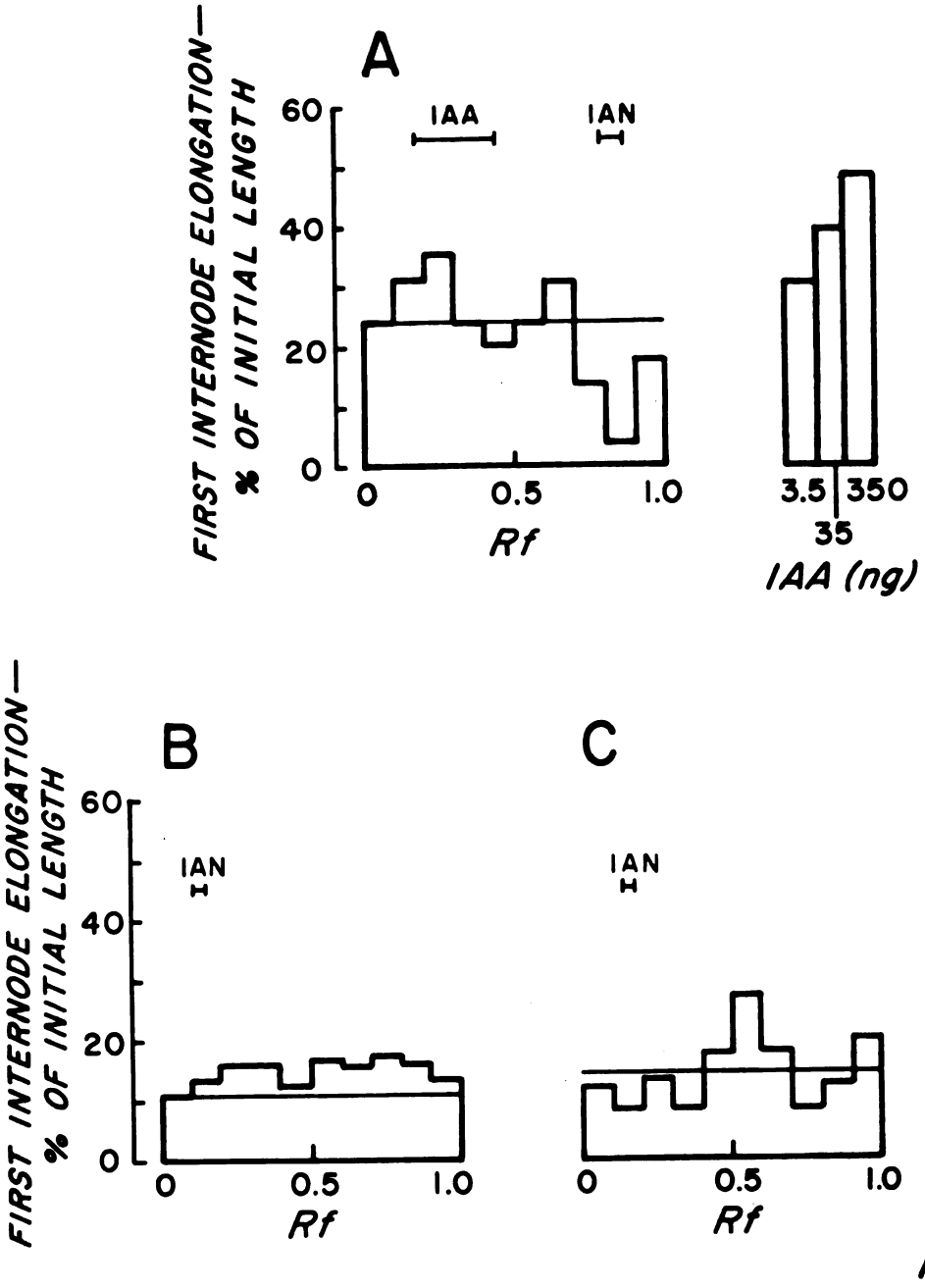
Figure 15.--Histograms depicting the Avena first internode bioassay response to pericarp neutral auxins (42 days after anthesis).

A. 12.0 gram equivalents chromatographed in isopropanol : water (9:1).

B. Re-chromatography of 4.0 gram equivalents Neutral I (Figure 15A, Rf 0.5 - 0.7). Solvent system:- hexane : water (upper phase).

C. Re-chromatography of 4.0 gram equivalents Neutral II (Figure 15A, Rf 0.8 - 1.0). Solvent system:- hexane : water (upper phase).

Control value (——).


A
B
C
FIRST INTERNODE ELONGATION—
% OF INITIAL LENGTH
60
40
20
0
IAA
IAN
0
0.5
1.0
Rf
3.5
350
35
IAA (ng)

eluted from duplicate chromatograms and re-chromatographed (Figure 15B). No promotive peaks were found. Re-chromatography of Neutral II in this latter solvent system (Figure 15C) again demonstrated growth promotive activity at Rf 0.5 - 0.7 similar to that found previously in seed fractions 21 and 42 days after anthesis (Figure 13B, 14C).

Further Characterization of the Neutral Auxins

Chromatography in two solvents.--When equal gram equivalents of the seed neutral fraction (42 days after anthesis) were chromatographed in two dissimilar solvents, separation into two growth promoting substances was achieved with the one (hexane : water) but not with the other (isopropanol : water) (Figure 16). Each of the two growth promoters separated by hexane : water were highly active in the first internode bioassay. The promoter at Rf 0.0 - 0.2 and at Rf 0.5 - 0.7 (Figure 16B) will hereafter be designated as neutral auxin IIA and neutral auxin IIB, respectively.

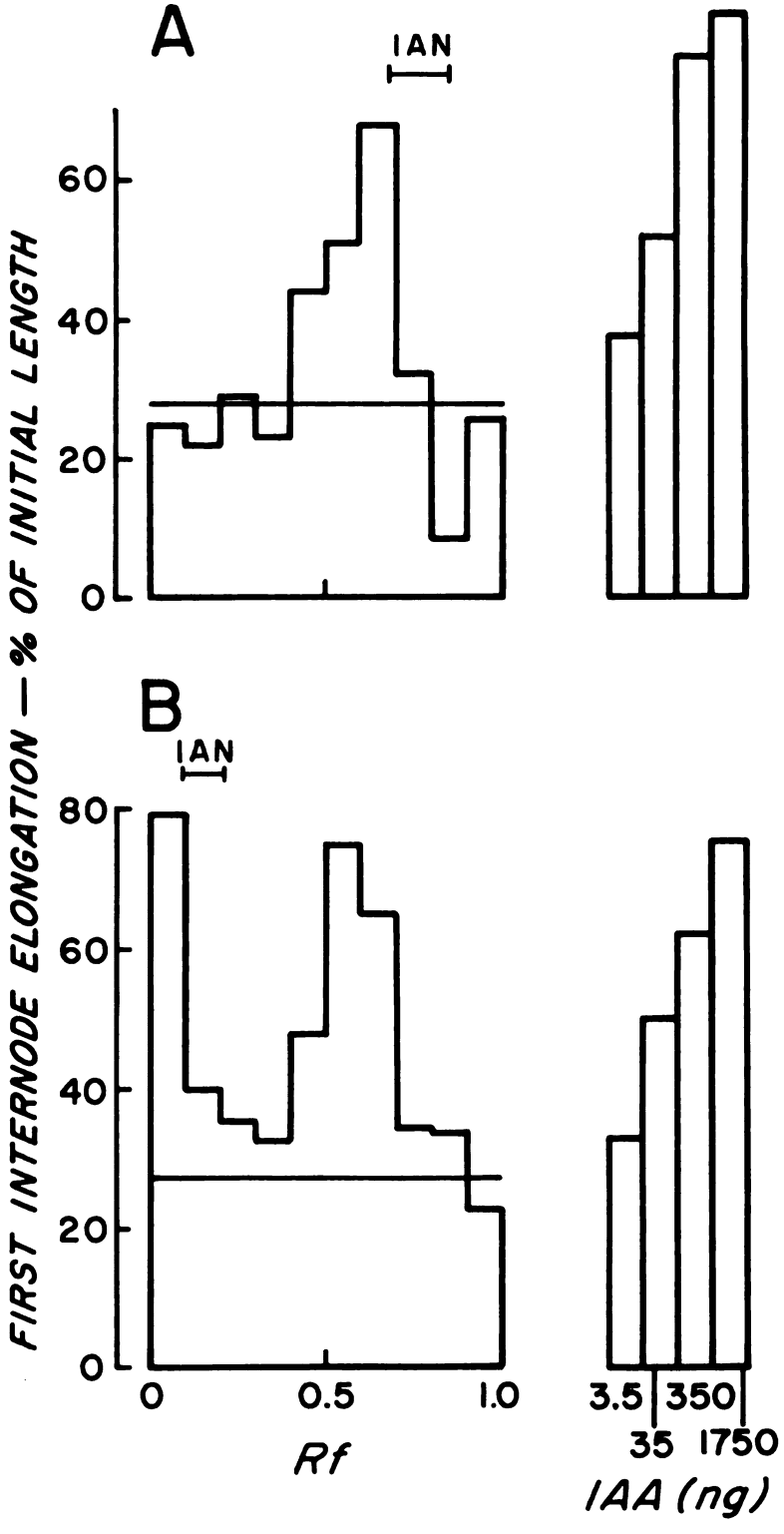
To test the hypothesis that the zone of inhibition (Rf 0.9 - 1.0) from isopropanol : water (9:1) chromatograms was due to overlapping of the two promoters, the neutral phase was chromatographed in hexane : water and the zones Rf 0.0 - 0.2 (neutral IIA) and Rf 0.5 - 0.7 (neutral IIB) eluted. Each eluate (0.1 gram equivalents) was chromatographed individually, or in combination, in isopropanol : water. Bioassay of the developed chromatograms revealed

Figure 16.--Histograms depicting the Avena first internode bioassay response to seed neutral auxins (42 days after anthesis).

A. Chromatography of 1.0 gram equivalents in isopropanol : water (9:1).

B. Chromatography of 1.0 gram equivalents in hexane : water (upper phase) after equilibration over water.

Control value (——).


A
IAN
60
40
20
0
B
IAN
80
60
40
20
0
0
0.5
1.0
Rf
3.5
350
35
1750
IAA (ng)
FIRST INTERNODE ELONGATION — % OF INITIAL LENGTH

weak growth promotive zones at Rf 0.9 ± 0.1 for neutral IIA (Figure 17B) and Rf 0.7 ± 0.1 for neutral IIB (Figure 17C) although the Rf's obtained are the reverse of what would be expected in terms of presumed polarities. Co-chromatography of neutral IIA and neutral IIB in isopropanol : water (Figure 17A) resulted in a single promoter peak at Rf 0.8 ± 0.1 that overlapped the distribution of both auxins (Figure 17B, 17C).

Dilution curves.--The bioassay response to 0.1 gram equivalents of the crude neutral ether fraction (seed tissue, anthesis + 42 days) approximated that obtained with 100 ng IAA (Figure 18). At concentrations greater than 0.35 gram equivalents, growth inhibition occurred which would explain the zone of inhibition (Rf 0.8 - 0.9) on chromatograms developed in isopropanol : water (9:1; 4:1) (Figure 2, 3, 8A, 12A, 15A, 17A) and the higher total auxin content shown in Tables 1 and 2. Although the bioassay response to 0.1 gram equivalents was approximately equal to the maximum IAA response, the two dilution curves deviate markedly at higher, or lower, concentrations suggesting that the biological response of the active endogenous moieties are quite different from IAA.

In order to relate the dose-response curve obtained with the crude neutral ether fraction to neutral auxins IIA and IIB, the neutral ether fraction (42 days after anthesis) was fractionated by paper chromatography (hexane : water)

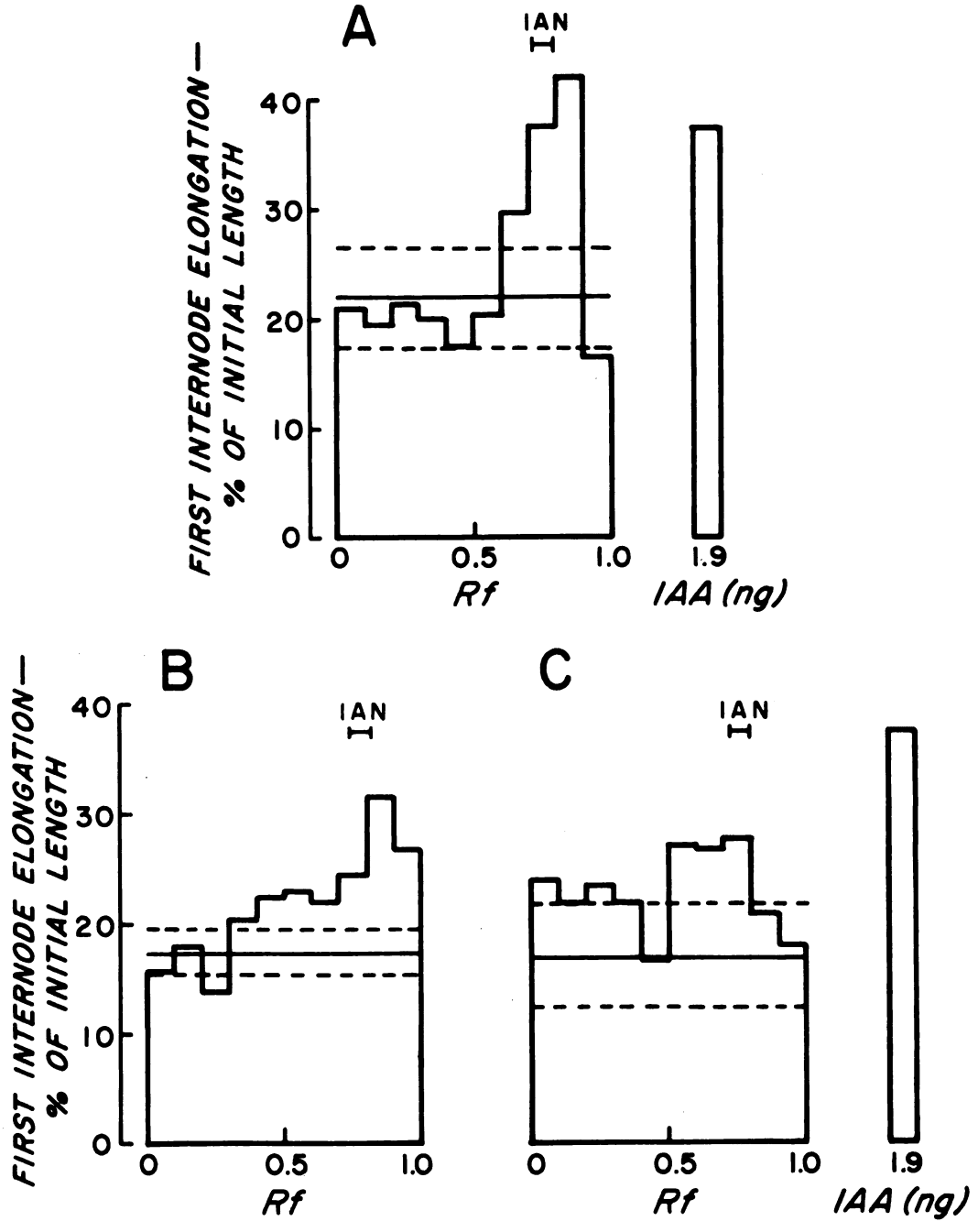
Figure 17.--Histograms depicting the Avena first internode bioassay response to seed neutral auxins (42 days after anthesis).

A. 0.1 gram equivalents of neutral auxin IIA (Figure 16B, Rf 0.0 - 0.2) and neutral Auxin IIB (Figure 16B, Rf 0.5 - 0.7) co-chromatographed in isopropanol : water (9:1).

B. 0.1 gram equivalents of neutral auxin IIA (Figure 16B, Rf 0.0 - 0.2) chromatographed in isopropanol : water (9:1).

C. 0.1 gram equivalents of neutral auxin IIB (Figure 16B, Rf 0.0 - 0.2) chromatographed in isopropanol : water (9:1).

Control value (——).
Upper and lower 5% fiducial limits (---).


A
IAN
FIRST INTERNODE ELONGATION—
% OF INITIAL LENGTH
40
30
20
10
0
0
0.5
1.0
Rf
1.9
IAA (ng)
B
C
IAN
IAN

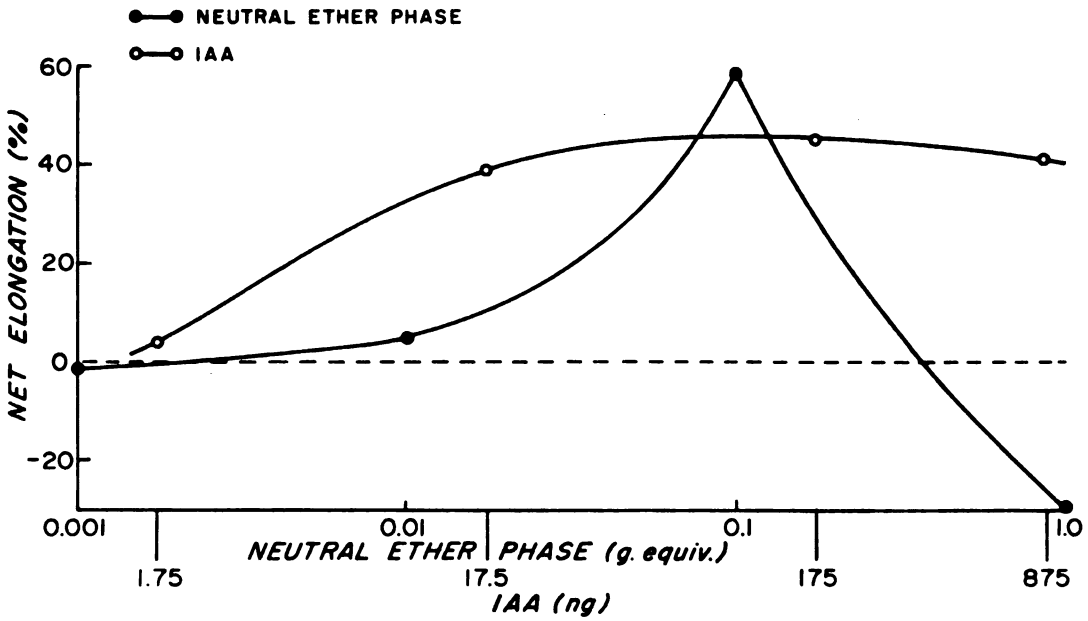
and the zones corresponding to neutral auxin IIA and IIB (Figure 16B) eluted. Eluates were combined to give a total of 0.1 and 0.5 gram equivalents of each auxin and then bioassayed. The mean responses (3 replicates) were 58.3% and -9.5%, respectively. These results fit the dilution curve for the neutral ether fraction (Figure 18) extremely well, suggesting that the response obtained is solely attributable to the presence of neutral auxins IIA and IIB.

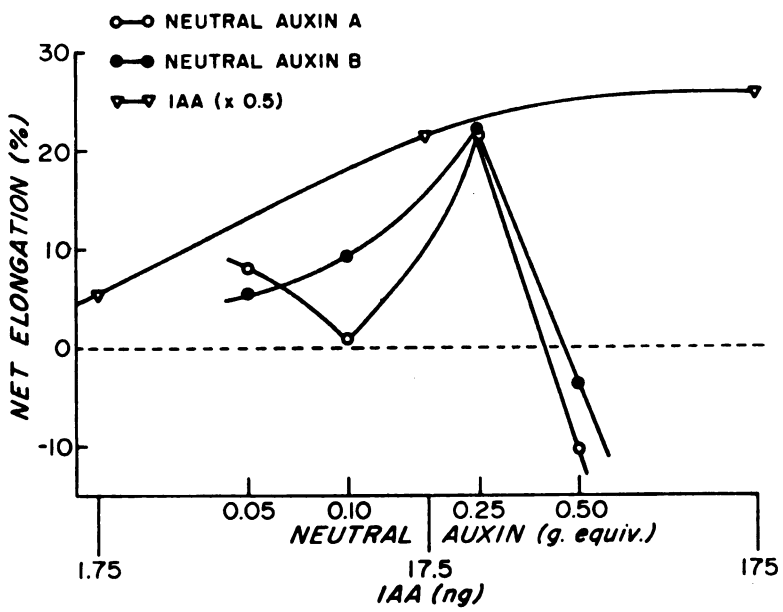
Factorial combination.--Two factors (neutral auxin IIA and neutral auxin IIB, eluted from chromatograms developed in hexane : water) were combined at 5 levels (0, 0.05, 0.10, 0.25, 0.50 gram equivalents) in a randomized complete-block design with 3 replications. Each replicate was blocked to account for possible variation between successive trays of seedlings used for the Avena straight growth bioassay.

The dose-response curves for neutral auxin IIA (in the absence of neutral auxin IIB) and neutral auxin IIB (in the absence of neutral auxin IIA) were found to be nearly identical (Figure 19) although the maximum response was less than half that obtained when both auxins were assayed together (Figure 18). The similarity between curves could possibly be attributed to incomplete chromatographic development of a single substance. However,

Figure 18.--Dose-response curves obtained by Avena first internode bioassay of the crude neutral ether fraction (42 days after anthesis) and known concentrations of IAA. Each value is the mean of 3 replicates. Control value (---).

Figure 19.--Dose-response curves obtained by Avena first internode bioassay of neutral auxin IIA, neutral auxin IIB (seed tissue, 42 days after anthesis), and known concentrations of IAA. Each value is the mean of 3 replicates. Control value (---).


NEUTRAL ETHER PHASE
IAA
NET ELONGATION (%)
60
40
20
0
-20
0.001
0.01
0.1
1.0
NEUTRAL ETHER PHASE (g. equiv.)
1.75
17.5
175
875
IAA (ng)


NEUTRAL AUXIN A
NEUTRAL AUXIN B
IAA (x 0.5)
NET ELONGATION (%)
30
20
10
0
-10
0.05
0.10
0.25
0.50
NEUTRAL AUXIN (g. equiv.)
1.75
17.5
175
IAA (ng)

re-chromatography of neutral auxin IIA, or neutral auxin IIB, in hexane : water did not result in further resolution (Figure 4, Appendix A) strongly suggesting that neutral auxin IIA and neutral auxin IIB are distinctly different entities.

Addition of successive increments of one neutral auxin to fixed levels of the other was expected to result in an increased growth response until the optimal concentration (0.1 gram equivalents) had been reached, followed by a decreasing response as the concentration became supra-optimal. This expectation was realized only in part (Figure 5, Appendix A). Accordingly, the mean increase in section length (final length-initial length) for each treatment was calculated and the data so obtained were subjected to analysis of variance (Table 1, Appendix A). Since the preliminary analysis had indicated significant differences within treatments and interactions, the treatment (and interaction) degrees of freedom and sums of squares were partitioned into single components and retested for significance. The finding that all levels of neutral auxin IIB, in the absence of neutral auxin IIA, and all levels of neutral auxin IIA (except 0.5 gram equivalents), in the absence of neutral auxin IIB, were significantly different can be seen graphically in Figure 19.

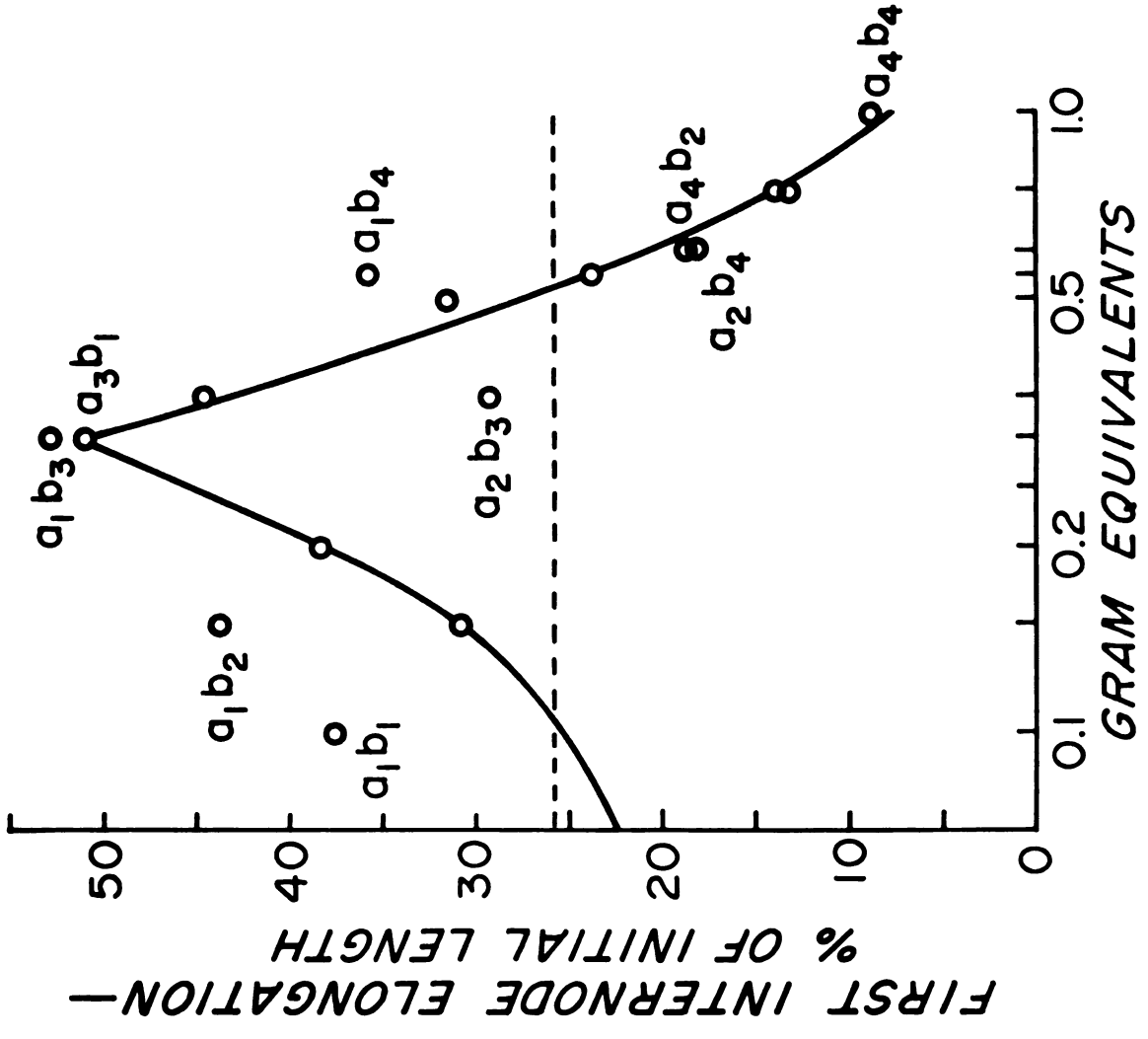
The significant interaction between the first level of neutral auxin IIA and all levels of neutral auxin IIB

(Table 1, Appendix A) is readily apparent when the bioassay response is plotted as a function of total gram equivalents applied (Figure 20). In each case the response is greater than would have been expected, suggesting a synergistic effect between the first level of neutral auxin IIA and any level of neutral auxin IIB. The interaction between 0.10 gram equivalents of neutral auxin IIA and 0.25 gram equivalents of neutral auxin IIB (a_2b_3, Figure 20) resulted in the lowest level of significance found (Table 1, Appendix A) and, as such, could represent a difference between replicates rather than a significant effect on the bioassay, per se. No reason can be advanced to explain the significant interaction between 0.50 gram equivalents of neutral auxin IIA and 0.10 gram equivalents of neutral auxin IIB (a_4b_2, Figure 20). The significant interaction between the highest levels of both auxins (a_4b_4, Figure 20) which yields the lowest growth response and the similarly significant interaction between a_3b_1 (0.25 gram equivalents neutral auxin IIA and 0.05 gram equivalents neutral auxin IIB) which gives the highest response may be attributed to differences between minimum, and maximum, responses.

Gas-liquid Chromatography of the Neutral IIB Auxin

The major growth promoting substance in the neutral ether fractions (seed and pericarp tissues) was shown to be

Figure 20.--Dose-response curve obtained by Avena first internode bioassay of factorial combination of 5 levels of the neutral auxin IIA (0, 0.05, 0.10, 0.25, 0.50 gram equivalents) with 5 levels of the neutral auxin IIB (0, 0.05, 0.10, 0.25, 0.50 gram equivalents). All values are the mean of 3 replicates. Control value (---). Unlabeled points not significantly different interaction values (Table 1, Appendix A).


FIRST INTERNODE ELONGATION—
% OF INITIAL LENGTH
50
40
30
20
10
0
0.1
0.2
0.5
1.0
GRAM EQUIVALENTS
a1b3
a3b1
a1b4
a2b3
a4b2
a2b4
a4b4
a1b2
a1b1

the neutral IIB auxin (Table 1, 2). Consequently, this auxin was prepared for gas-liquid chromatography in the same manner as the acidic auxin even though the methylation step was assumed to be superfluous. Chromatography of the presumed TFA derivative on 3% SE - 30 at 140°C did not yield satisfactory resolution. Improved resolution was achieved at lower column temperatures. Injection at a column temperature of 120°C yielded major peaks at 3.10, 4.50, and 9.95 minutes and minor peaks at 6.10, and 8.50 minutes (Figure 21A). The retention time of standard IAA, at this temperature, was 9.70 minutes.

Chromatography of the neutral IIB auxin on DC - 200 (column isothermal at 120°C) gave major peaks at 3.35, 4.85, and 10.87 minutes and minor peaks at 6.65 and 8.80 minutes (Figure 21B). The shoulder observed at a retention time of 9.45 minutes (SE - 30, Figure 21A) separated as a small peak (retention time, 9.15 minutes) on the DC - 200 column (Figure 21B). The retention time of standard IAA, at this temperature, was 10.70 minutes.

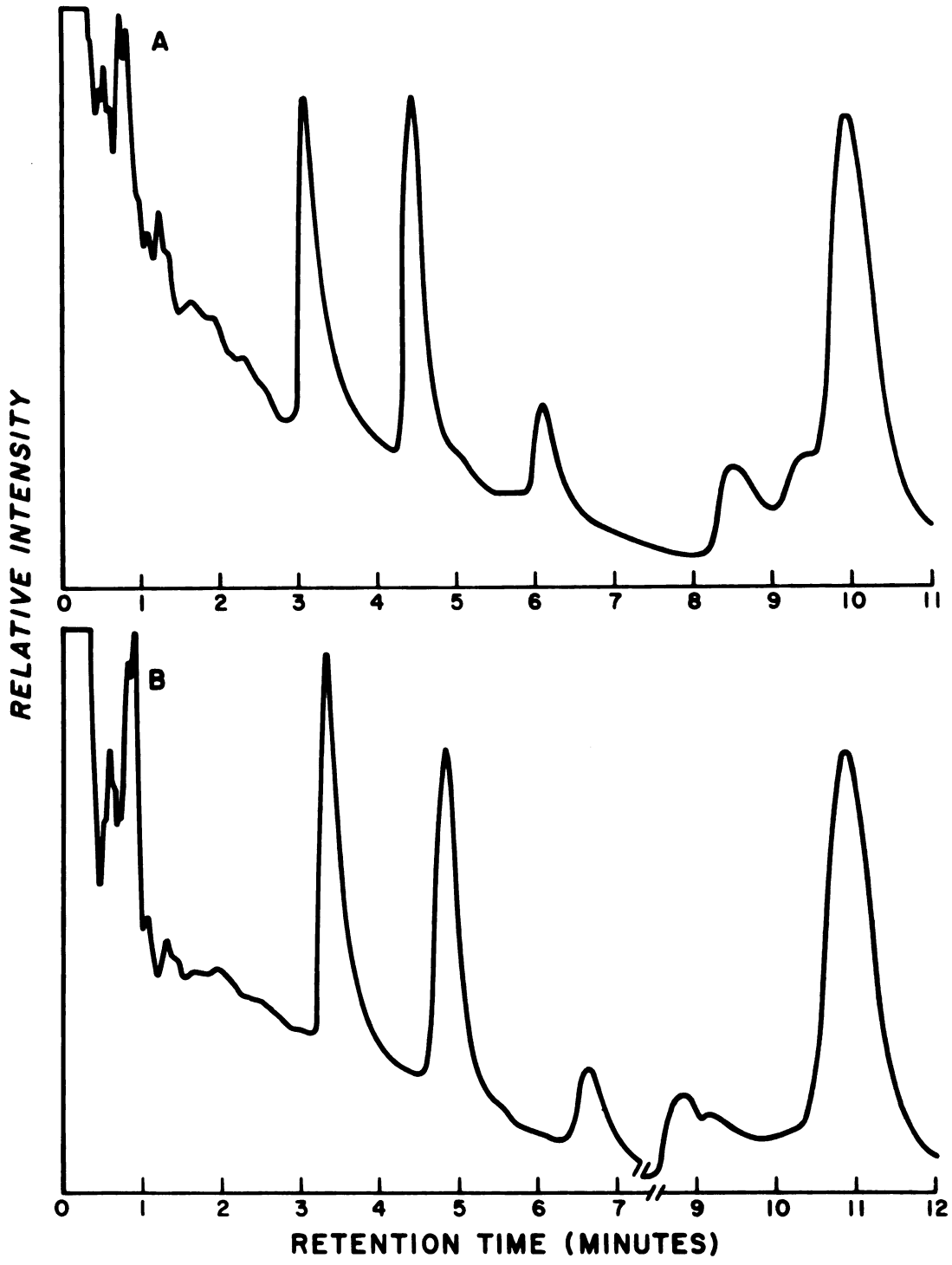
Combined Gas-liquid Chromatography--Mass Spectrometry of the Neutral IIB Auxin

Chromatography of 8.0 µl of presumed TFA - neutral auxin IIB on 3% SE - 30 (column temperature 120°C) revealed three major peaks with retention times of 5.9, 17.7, and 20.4 minutes and three minor peaks at 3.4, 7.2, and 8.6

Figure 21.--Gas-liquid chromatographic resolution of the presumed trifluoroacetic ether of the neutral IIB auxin.

A. Column packing - Gaschrome Q (60-80) coated with 3% SE-30; column temperature -120°C; column flow rate (N_2) - 23 ml/minute. Retention time of TFA-Me-IAA, 9.70 minutes.

B. Column packing - gaschrome Q (60-80) coated with 3% DC-200; column temperature -120°C; column flow rate (N_2) - 42 ml/minute. Retention time of TFA-Me-IAA, 10.70 minutes.


A
B
RELATIVE INTENSITY
0
1
2
3
4
5
6
7
8
9
10
11
12
RETENTION TIME (MINUTES)

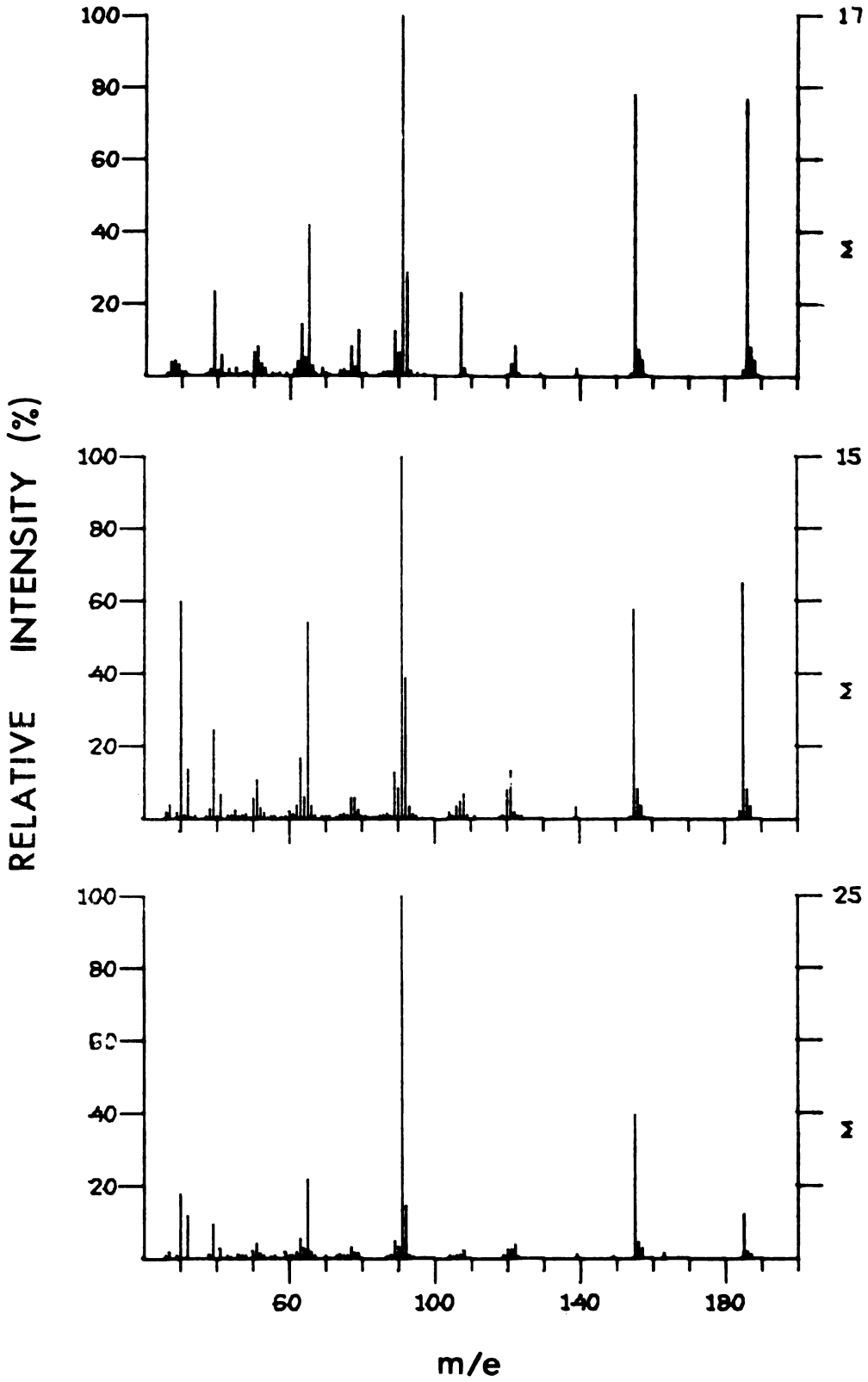
minutes (Table 2, Appendix A). The center of each major peak was introduced into the mass spectrometer. A further sample of TFA - neutral auxin IIB was chromatographed on 3% SE - 30 (column temperature 130°). Major peaks were located at 3.9, 10.5, and 11.9 minutes while minor peaks occurred at 2.3, 4.3 and 5.2 (doublet) minutes (Table 2, Appendix A). The center of the first two major peaks was introduced into the spectrometer.

Spectra obtained from peaks at 5.9 (120°C) and 3.9 (130°C) minutes were identical. Expression of these retention times relative to that of IAA showed that the peaks analyzed corresponded to that with a retention time of 4.50 minutes (Table 2, Appendix A). Spectra obtained from peaks at 17.7 (120°C) and 10.5 (130°C) minutes were also identical. These peaks were found to correspond to the peak at a retention time of 9.95 minutes (Table 2, Appendix A). The mass spectrum of the compound with a retention time of 5.9 minutes (peak 2; Table 2, Appendix A) is shown in Figure 22. The molecular ion (M) is located at m/e 186 and the base peak at m/e 91. The absence of a fragment ion at m/e 69 (Figure 11A, 11B) strongly suggests that the TFA derivative was not synthesized. Similarly, the absence of an intense fragment ion at m/e 130 strongly suggests that the compound is non-indolic (Jamieson and Hutzinger, 1970). The base peak (m/e 91) is indicative of the tropylium ion;

Figure 22.--Mass spectrum of component 2 of the neutral auxin IIB complex. This component was resolved on 3% SE-30 (column temperature 120°C). The relative intensity (per cent of base peak) of each fragment was plotted as a function of its mass-charge ratio.

Figure 23.--Mass spectrum of component 6 of the neutral auxin IIB complex. This component was resolved on 3% SE-30 (column temperature 120°C). The relative intensity (per cent of base peak) of each fragment was plotted as a function of its mass-charge ratio.

Figure 24.--Mass spectrum of component 7 of the neutral auxin IIB complex. This component was resolved on 3% SE-30 (column temperature 120°C). The relative intensity (per cent of base peak) of each fragment was plotted as a function of its mass-charge ratio.


RELATIVE INTENSITY (%)
100
80
60
40
20
17
Σ
100
80
60
40
20
15
Σ
100
80
60
40
20
25
Σ
60
100
140
180
m/e

the compound thus contains a benzene ring with at least one methylene group as a side chain. Other features of the spectrum include fragment ions at M - 31 (associated with the loss of a methoxy group), M - 64 and M - 79. The fragment ion at M - 64 shows the loss of m/e 33 from M - 155 suggesting the loss of a thiol group. Such a suggestion is reasonable in light of the large M + 2 contribution (5.65% of M). The fragment ion at M - 79 could result from the loss of methylmercaptan. Fragment ions at M - 121 and M - 147 are indicative of the loss of acetylene from the ring.

The mass spectrum of the compound with a retention time of 17.7 minutes (peak 6; Table 2, Appendix A) is shown in Figure 23. This spectrum bears a strong resemblance to that found above (Figure 22). The molecular ion (M) is located at m/e 185 and the base peak at m/e 91. Again, the absence of fragments at m/e 69, m/e 130 strongly suggest that the TFA derivative was not synthesized and that the compound is non-indolic. The compound has a benzene ring (tropylium ion, m/e 91) with at least one methylene group as a side chain. Other features of the spectrum include fragment ions at M - 30 (associated with the loss of either the $CH_2 = NH_2^+$ ion, the N = 0 ion or the fragment CH_2O), at M - 64 (associated with the loss of a thiol group; M + 2 contribution, 5.15% of M), and at M - 120 and M - 146

(associated with the loss of acetylene from the ring). The fragment ion at m/e 30 (M - 155) is indicative of the N = 0 ion.

The third mass spectrum obtained (peak 7; Table 2, Appendix A) exhibits a molecular ion (M) at m/e 185 (Figure 24). The base peak occurs at m/e 91 (associated with a tropylium ion). No fragment ions were found at m/e 130 or 69 strongly suggesting that the compound is non-indolic and did not form the TFA derivative. A fragment ion at M - 30 and the presence of a peak at m/e 30 is highly suggestive of the loss of the $CH_2 = NH_2^+$ ion or the N = 0 ion) similar to that found above. However, few fragment ions occur between m/e 91 and m/e 155. In addition, the M + 2 contribution (10.81% of M) is very large suggesting a dithio group. Fragment ions at M - 120 and M - 146 are due to the loss of acetylene from the ring.

Characterization of Seed Basic Substances

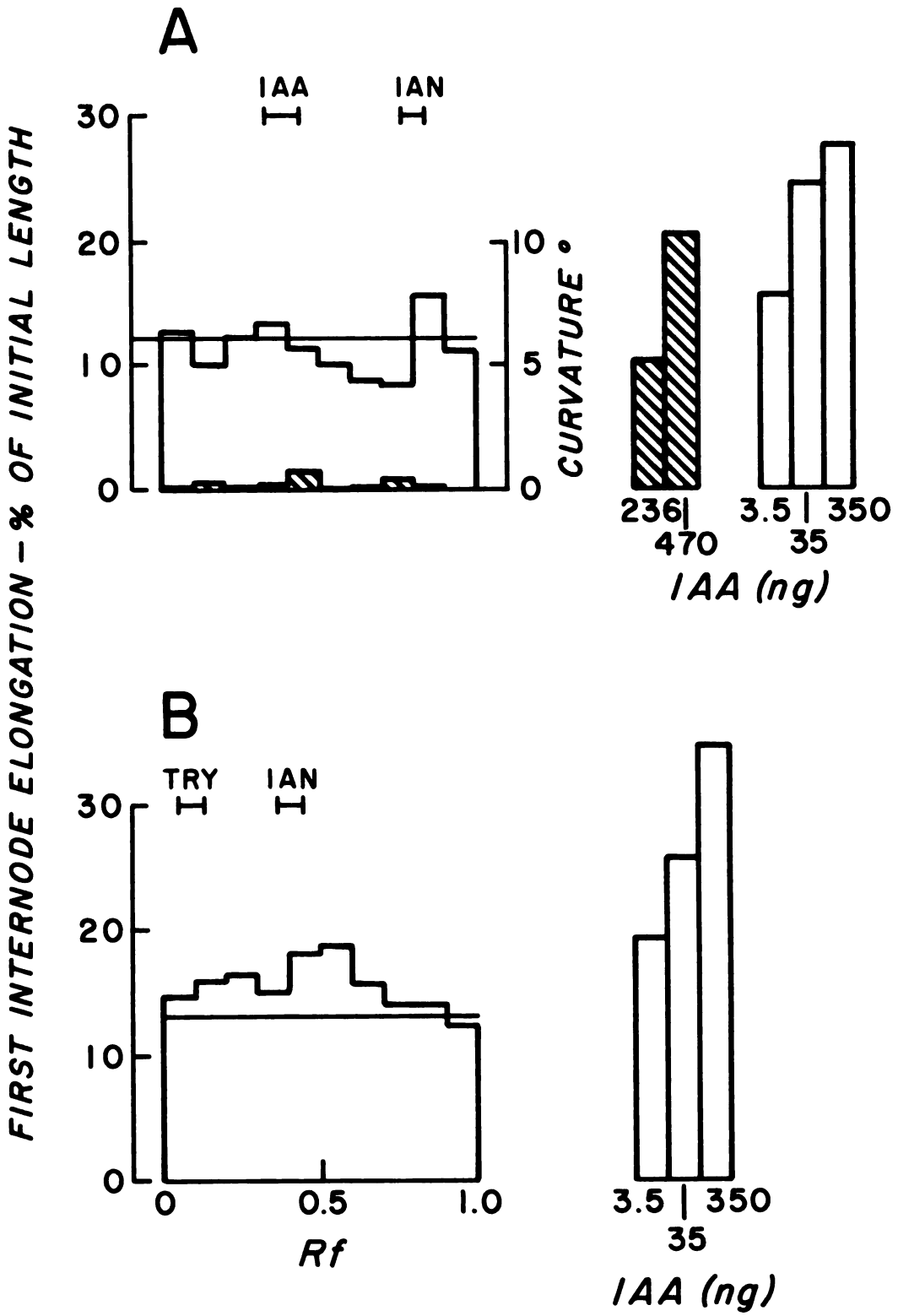
Anthesis + 21 days.--Chromatographic separation of seed basic substances did not yield any distinct promotive activity in either bioassay (Figure 25A). Mindful of the possibility of supraoptimal concentrations, the zone between Rf 0.3 - 0.7 (Basic I) was eluted from duplicate chromatograms and the eluate re-chromatographed in water (Figure 25B). The weakly promotive zone at Rf 0.5 - 0.6 just exceeded the upper 5% fiducial limit (16.3%). Application

Figure 25.--Histograms depicting the Avena first internode (open bars) and Avena curvature (shaded bars) bioassay response to seed basic substances (21 days after anthesis).

A. 0.3 gram equivalents chromatographed in isopropanol : water (9:1).

B. 0.4 gram equivalents Basic I (Figure 25A, Rf 0.3 - 0.7). Solvent system:- water.

Avena first internode bioassay control value (——). Avena curvature bioassay control value (zero).


A
IAA
IAN
30
20
10
0
10
5
0
CURVATURE °
FIRST INTERNODE ELONGATION – % OF INITIAL LENGTH
236
470
3.5
350
35
IAA (ng)
B
TRY
IAN
30
20
10
0
0
0.5
1.0
Rf
3.5
350
35
IAA (ng)

of Salkowski or Ehrlich reagents did not reveal any chromogenic zones on either chromatogram.

Anthesis + 42 days.--Chromatographic separation of the seed basic substances in isopropanol : water revealed a peak of activity at Rf 0.2 (upper 5% fiducial limit 31.2%) (Figure 26). Since this peak of activity was not revealed previously (Figure 25A) no further resolution was attempted.

Characterization of Pericarp Basic Substances

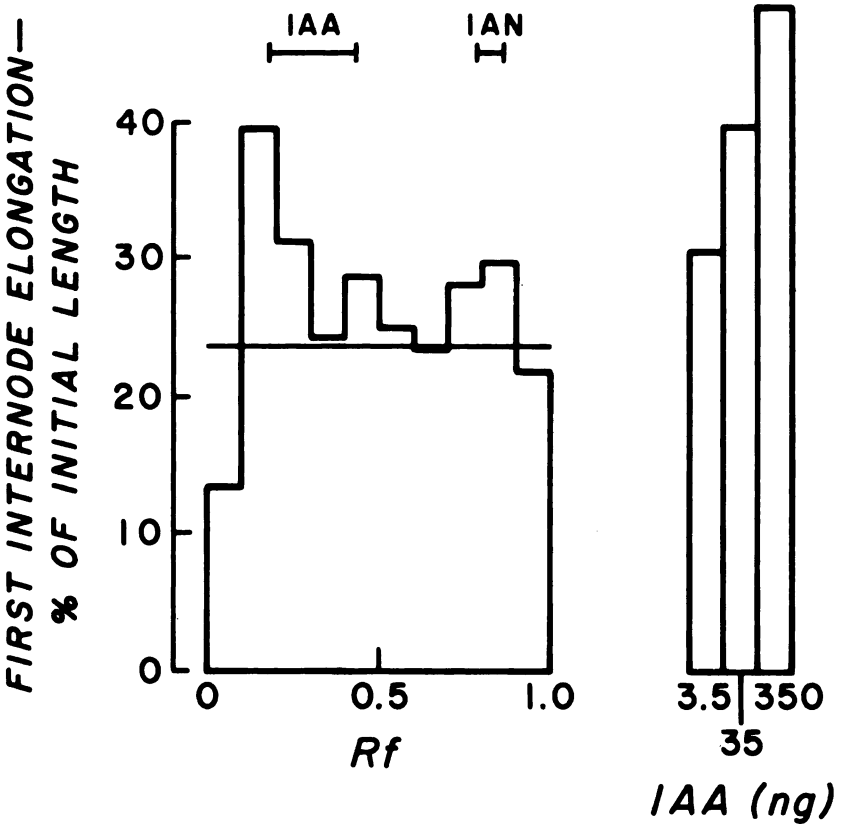
Anthesis + 42 days.--The pericarp basic ether phase showed negligible promotive activity when chromatographed in isopropanol : water (Figure 27). The zone at Rf 0.4 barely exceeded the upper 5% fiducial limits (31.0%). Since this activity did not correspond to that found previously (Figure 25A, 26) no further resolution was attempted.

Characterization of the "Bound" Auxins

Fractionation of the aqueous phase.--Column chromatography of the acidic aqueous fraction (Figure 1), prepared from seed and pericarp extracts (28 and 42 days after anthesis), and subsequent bioassay of individual fractions (Figure 28, 29) revealed two, weakly active, growth promotive substances. One of these substances (Fraction numbers 2 and 3, Figure 28) can be attributed to residual

Figure 26.--Histogram depicting the Avena first internode bioassay response to seed basic substances (42 days after anthesis). 0.45 gram equivalents chromatographed in isopropanol : water (9:1). Control value (——).

Figure 27.--Histogram depicting the Avena first internode bioassay response to pericarp basic substances (42 days after anthesis). 12.0 gram equivalents chromatographed in isopropanol : water (9:1). Control value (——).


IAA
IAN
FIRST INTERNODE ELONGATION—
% OF INITIAL LENGTH
40
30
20
10
0
0
0.5
1.0
Rf
3.5
350
35
IAA (ng)

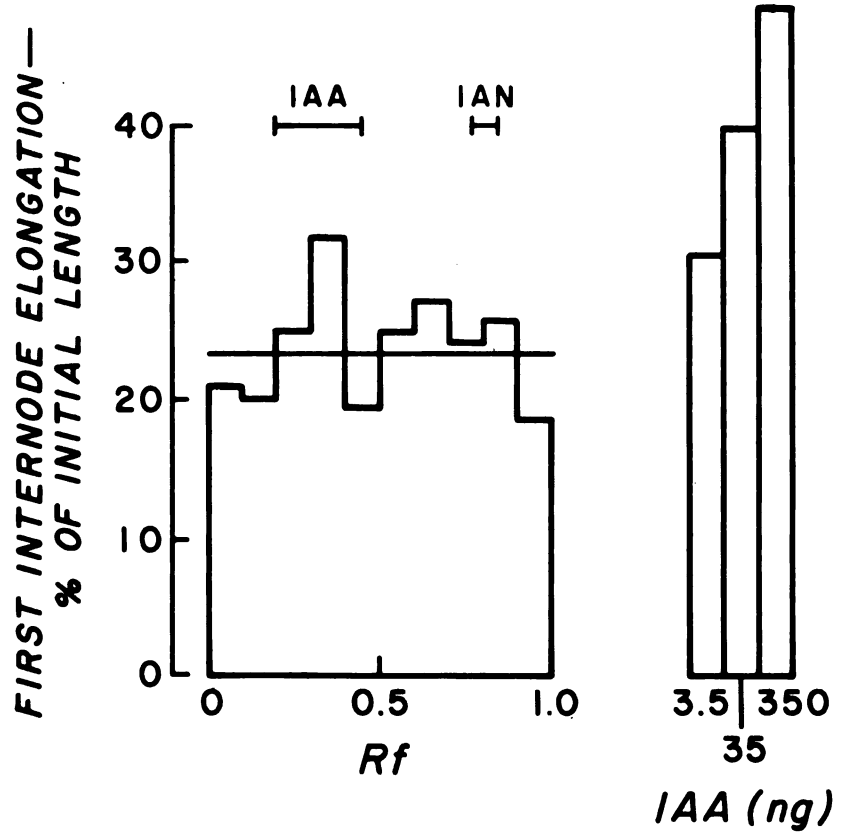
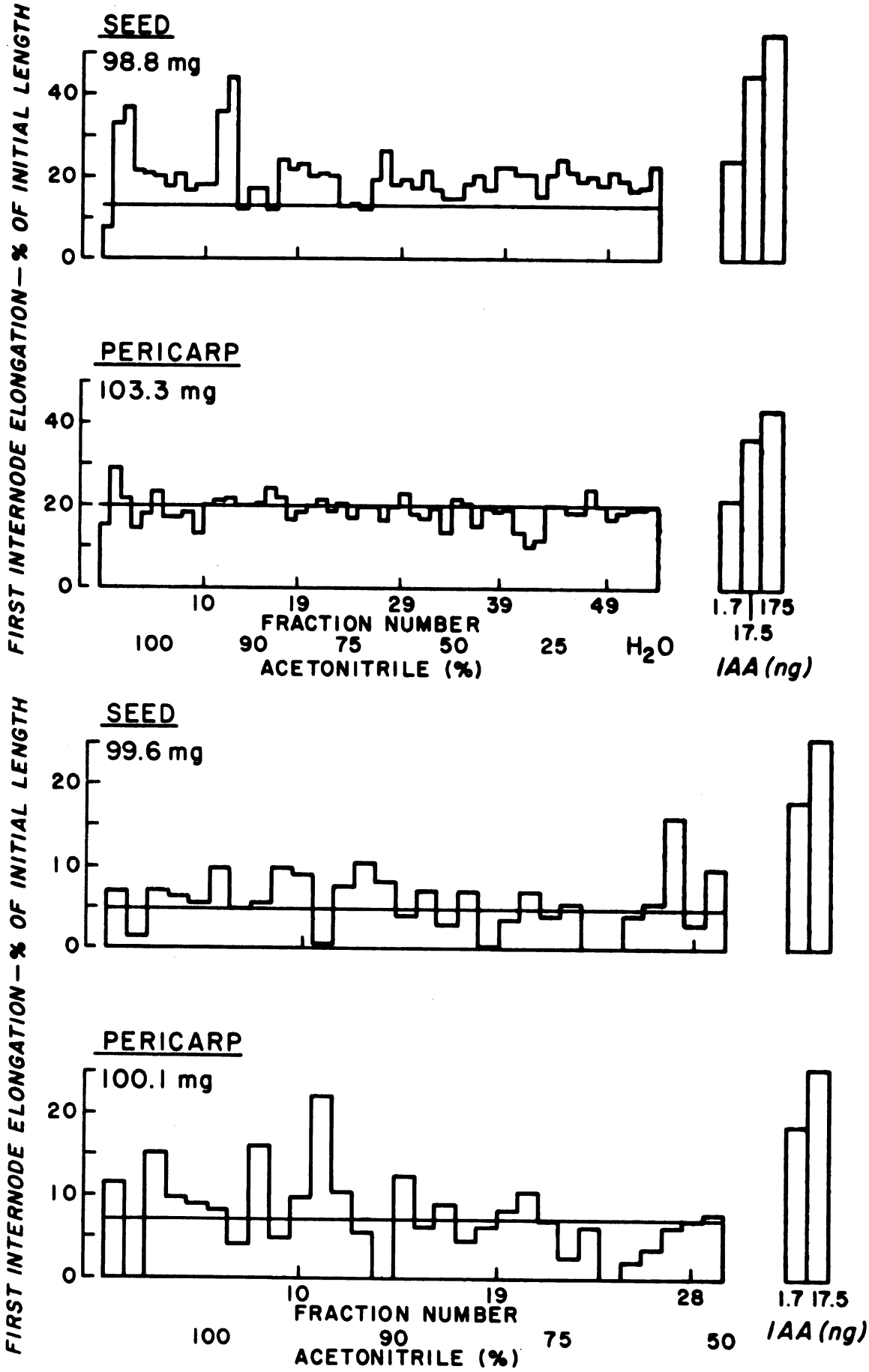

IAA
IAN
FIRST INTERNODE ELONGATION—
% OF INITIAL LENGTH
40
30
20
10
0
0
0.5
1.0
Rf
3.5
350
35
IAA (ng)

Figure 28.--Histograms depicting the Avena first internode bioassay response to water-soluble substances (seed and pericarp acidic aqueous fraction, 28 days after anthesis) fractionated by silicic acid column chromatography. Control value (——).

Figure 29.--Histograms depicting the Avena first internode bioassay response to water-soluble substances (seed and pericarp acidic aqueous fraction, 42 days after anthesis) fractionated by silicic acid column chromatography. Control value (——).

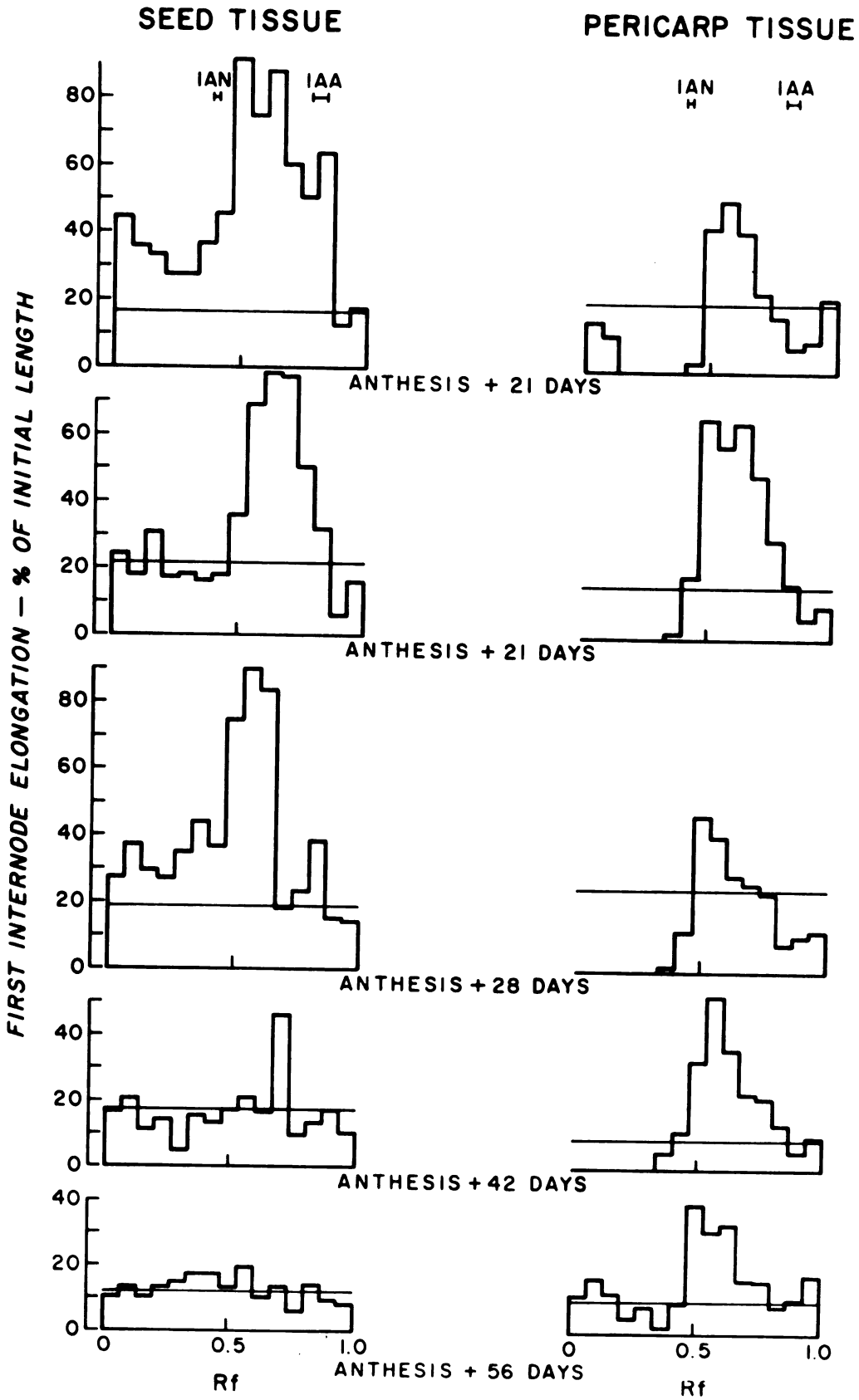

SEED
98.8 mg
PERICARP
103.3 mg
SEED
99.6 mg
PERICARP
100.1 mg
FIRST INTERNODE ELONGATION—% OF INITIAL LENGTH
FIRST INTERNODE ELONGATION—% OF INITIAL LENGTH
0
20
40
10
19
29
39
49
28
FRACTION NUMBER
100
90
75
50
25
H2O
ACETONITRILE (%)
1.7
175
17.5
IAA (ng)

acidic auxin resulting from incomplete partition chromatography. The other, and more active, substance (fraction numbers 12 and 13) was located in both seed (Figure 28) and pericarp (Figure 29) extracts. The possible identity of this second substance is unknown.

Changes in "bound" auxin levels during fruit development.--Chromatography and subsequent bioassay of ethyl ether-soluble substances released by base hydrolysis of the acidic aqueous phase (1971 harvests) revealed the presence of a highly active growth-promotive substance(s) (Rf 0.46 - 0.80, Figure 30) which did not chromatograph with either standard. Since the neutral auxins chromatograph with IAN in the solvent system used (water), this preliminary evidence suggests that the growth promoter(s) is different from those previously characterized. Moreover, seed promoter(s) levels exhibit dramatic quantitative changes with successive stages of fruit development. Similar changes are not so readily apparent in the pericarp fraction.

In addition to the zone of promotion, an intense zone of inhibition (Rf 0.0 - 0.33) was detected in pericarp fractions (21, 28, 42 days after anthesis). An equivalent zone of inhibition was not found in seed fractions nor in the pericarp fraction just prior to fruit maturity (56 days after anthesis).

Figure 30.--Histograms depicting the Avena first internode bioassay response to growth-promoting substances released by base hydrolysis of seed and pericarp aqueous phases (21, 28, 42, and 56 days after anthesis). 1.0 gram equivalents of each phase was chromatographed in water. Control value (——).


SEED TISSUE
PERICARP TISSUE
IAN
IAA
FIRST INTERNODE ELONGATION — % OF INITIAL LENGTH
ANTHESIS + 21 DAYS
ANTHESIS + 21 DAYS
ANTHESIS + 28 DAYS
ANTHESIS + 42 DAYS
ANTHESIS + 56 DAYS
Rf
Rf

In order to relate the levels of the "bound" growth promoter(s) to fruit growth, the peak area (Rf 0.46 - 0.80) above the upper 5% fiducial limit was converted to IAA equivalents by means of a standard curve and expressed as IAA equivalents per fruit.

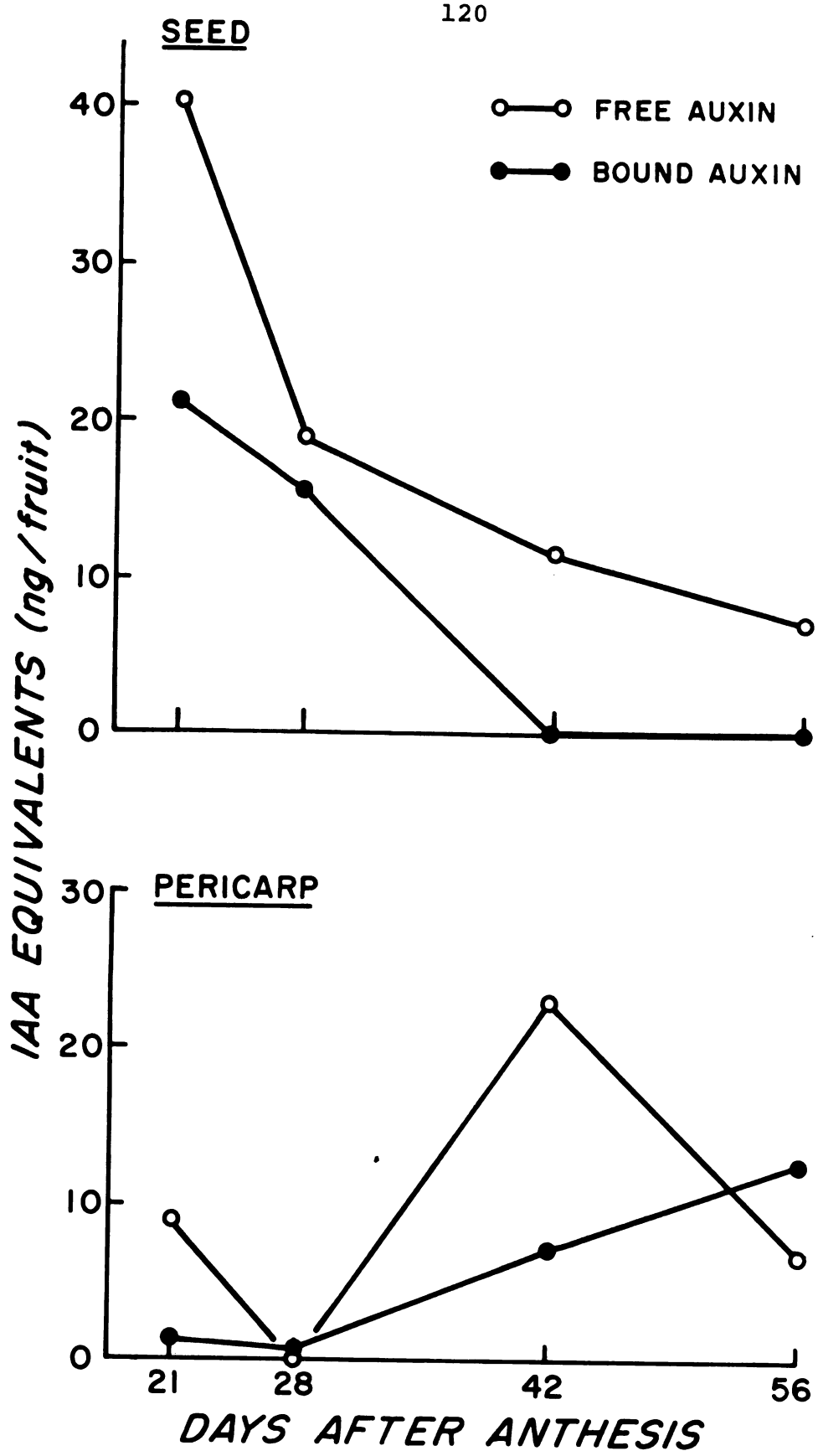
Comparison between auxins (free and bound) detected in seed and pericarp fractions (Figure 31) shows that during the fruit growth period 21-42 days after anthesis, the total quantities of free and bound auxin per seed decreased while the totals in the pericarp increased. Subsequent to this time, the levels of both free and bound auxins in the seed decreased further (Figure 31). The level of bound auxin in the pericarp, however, continued to increase even though the level of free auxin decreased.

Discussion

Seasonal Variation in Fruit Auxin Content

The seed auxin content (1969 fruit harvest), on the basis of ng IAA equivalents/gram dry weight, increased from an initially low value to a maximum 21 days after anthesis (Figure 4). This increase in seed auxin from day 14 to day 21 can be correlated with the rapid growth to full size of the nucellus and integuments (Inoue, 1970). It is unlikely that the endosperm contributed auxinic factors to this peak since, at this time, it has only attained 3% of its eventual

Figure 31.--Seasonal levels of free and bound seed and pericarp auxins. All free auxin values are totals of acidic, and neutral auxins obtained from fruit harvested 21, 28, 42, and 56 days after anthesis, 1971.


SEED
FREE AUXIN
BOUND AUXIN
40
30
20
10
0
PERICARP
30
20
10
0
21
28
42
56
IAA EQUIVALENTS (ng/fruit)
DAYS AFTER ANTHESIS

size. Subsequent to the completion of nucellus and integument growth, the seed auxin content fell rapidly to less than 1 ng/gram dry weight (Figure 4). A second peak of seed auxin occurred between 35 and 42 days after anthesis. The exact magnitude, and location, of this auxin peak is unknown because complete separation of all growth promoting substances was not achieved prior to bioassay (Figure 2, 3). However, both the endosperm and embryo undergo rapid development during the period in which the seed auxin content increased dramatically (Inoue, 1970). By 45 days from anthesis, the embryo had reached its maximum size. Yet a third, but considerably smaller peak of auxin activity was found 56 to 63 days after anthesis. Such a peak does not correlate with any macroscopic changes in the seed.

When similar determinations were made in 1971 a somewhat different result was obtained (Table 1). Although a major peak of seed auxin was shown to occur 21 days after anthesis, no dramatic decrease (day 28) and subsequent increase to a second peak (day 35 - 42) was found. Instead, the seed auxin content decreased to a minimum of 450 ng/gram dry weight and then slowly increased to a second maximum of 550 ng/gram dry weight 56 days after anthesis. These differences can largely be ascribed to differences between years in the onset of rapid endosperm and embryo growth

and to the differences in fractionation technique (individual chromatography of acidic and neutral auxins in a nitrogen atmosphere).

The pericarp auxin content, however, did not necessarily mirror the seasonal fluctuations found in the seed (Figure 4). For example, no evidence was found to suggest that the rapid loss of seed auxin (21 - 28 days after anthesis) was due to movement into the pericarp. Instead, the pericarp auxin content increased at an almost steady rate until 42 - 49 days after anthesis and subsequent to this time, declined. Thus the pericarp auxin content during the third stage of fruit growth appears to be inversely associated with rapid cell enlargement although this relationship is not tenable during the period of fruit maturation. Comparison of these results with those obtained in 1971 suggests that movement did occur from the seed to the fruit (Table 1), at least during the initial stages of fruit growth. Again, the total pericarp auxin content during the initial stages of the third growth stage (42 - 56 days after anthesis, Table 1) appeared to be inversely associated with rapid cell enlargement.

Although computing auxin content in concentration terms (ng/gram dry weight, Figure 4) is a valid means of comparing auxin levels at various growth stages within and between seasons, such computations invoke a "hidden"

dilution factor of increasing fruit dry weight with successive growth stages. Since cell division in the pericarp is complete by 21 days after anthesis, computation of auxin content in terms of ng IAA equivalents/fruit would be more meaningful physiologically. Similarly, initial growth of the seed is solely due to the enlargement of the nucellus and integuments (Tukey and Young, 1939; Inoue, 1970). Growth of the endosperm and embryo does not begin until the nucellus and integuments have reached their maximum size. Thus, expression of IAA equivalents/seed as a function of time from anthesis would more accurately reflect the auxin contribution from ovule tissues than would expression in terms of IAA equivalents/gram dry weight.

Comparison of seed and pericarp auxin levels obtained from two seasons of fruit growth (Table 2) showed that the nucellus and integument contribution (day 21) was appreciable in relation to that associated with the initial stages of endosperm and embryo development (day 28). Subsequent to this time the total seed auxin level increased (day 42, 1969) although the level detected from isopropanol : water (4:1) chromatograms is an underestimate (super-imposition of neutral auxin IIA and neutral auxin IIB, Figure 2). A similar increase was not found for seed tissue obtained from a second season of growth (1971).

The level of seed neutral auxin IIB (1971) declined from day 28 to day 56 at a slower rate than that found for either neutral auxin IIA or the acidic auxin. Since both neutral auxins were found to co-chromatograph in isopropanol : water (9:1) (Figure 17) and were poorly resolved in hexane : water (9:1) (Figure 3) no definitive evidence was obtained that would indicate a high level of seed neutral auxin IIB subsequent to day 42 (1969). However, a major peak of promotive activity was shown by histograms (56, 63 and 70 days after anthesis, Figure 3) that corresponds with the Rf value of neutral auxin IIB chromatographed in hexane : water (upper phase; Figure 16B). High levels of an unidentified neutral auxin have been found in peach seeds subsequent to the completion of embryo growth (Powell and Pratt, 1966).

The changes in total pericarp auxin levels (1971) reflect those found for the seed with the exception that the auxin level at day 28 was below the limit of bioassay detection. The level of acidic auxin decreased from a maximum at the onset of fruit growth stage II to a minimum at day 28. At the onset of growth stage III (day 42) the level of acidic auxin was considerably lower than that found for the neutral auxin IIB. Both auxins were detected at day 56 at which time the rate of cell enlargement in the pericarp had begun to decline. In contrast to the acidic

and neutral IIB auxins, no neutral auxin IIA was found at any stage of fruit growth.

Comparison of total free and bound auxins in seed and pericarp fractions (Figure 31) showed that the decrease in free seed auxin from 21 - 42 days after anthesis was paralleled by a decrease in bound auxin. If the bound auxin level is assumed to be a reflection of free auxin metabolism then an increase in bound auxin should have been detected at least during the period 28 - 42 days after anthesis. Such an increase was not found suggesting a loss of bound auxin from the seed fraction. The bound auxin level in the pericarp, however, increased from day 28 to day 56. This increase would be expected if the loss from the seed was due to movement to the pericarp. Furthermore, if the bound auxin is physiologically significant in fruit growth the observations by Tukey (1936b), that fruit growth was not impaired when seeds were destroyed after the onset of the third growth phase, could be explained.

Characterization of Seed and Pericarp Auxins

Paper chromatography of the acidic and neutral phases from seed tissues revealed one acidic and two neutral auxins (Figure 6, 7, 8, 13C, 14C and 16B). No evidence was obtained that the seed auxins were different from those detected in the pericarp (Figure 9, 15).

Schulte and Holm (1964) reported the isolation of one acidic, one neutral and one basic auxin from the developing seeds of sour cherry, although no data have ever been presented. In the persent case, the weak growth activity obtained in Avena first internode bioassays of the basic phase (Figure 25, 26, and 27) and the absence of a curvature response in the Avena curvature bioassay (Figure 25) were taken as evidence against the possibility of a basic growth promoter.

The Rf of the acidic auxin did not always coincide exactly with that of the applied standard IAA (Figure 6A, 6B, 7A, 7B, 8A, 8C, 9A, and 9B). However, separation of the growth promoting peak (Figure 7B) into two components, and re-chromatography of each component (Figure 7C, 7D) showed that the acidic auxin chromatographed with standard IAA. Moreover, application of Salkowski reagent always yielded a red-purple chromogenic reaction at the Rf of maximum biological activity.

IAA has been tentatively identified in seed extracts obtained from other Prunus sp. Stahley and Thompson (1959) extracted an acidic growth promoter from Halehaven peach seeds which possessed chromogenic (Salkowski and Ehrlich reagents) and Rf properties in four solvent systems similar to IAA. More convincing evidence has been presented by Powell and Pratt (1966) who prepared the methyl ester of

the acidic auxin found in extracts of Halehaven peach seeds and compared its fluorescence spectrum with that of authentic methyl indole-3-acetate.

Gas-liquid chromatography of the TFA - methyl ester of the acidic auxin gave retention times identical to those obtained for IAA (Figure 10). Co-chromatography of the endogenous acidic auxin and standard IAA, yielded a single, homologous, peak on two dissimilar columns (SE - 30, DC - 200). Unequivocal evidence that the acidic auxin is IAA was provided by mass spectrometry (Figure 11).

The neutral auxins, however, did not yield a chromogenic reaction with reagents specific for indoles (Salkowski, Ehrlich, 4-dimethylaminocinnamaldehyde) although both neutral auxins were active in the Avena first internode bioassay (Table 1, 2, Figure 13, 14, 15, 16, 17, 19 and 20). Since both neutral auxin IIA and neutral auxin IIB co-chromatographed in isopropanol : water and water alone, it is not known if both auxins were responsible for the Avena curvature response obtained (Figure 12A, 13A).

A zone of growth-promotive activity (Rf 0.7 - 0.9) has been demonstrated in extracts of peach, plum and sweet cherry chromatographed in isopropanol : water (4:1) (Stahley and Thompson, 1959; Ugolik and Nitsch, 1951; Pillay, 1965). This promotive zone resembles that found for sour cherry (Figure 2) although the latter was masked

by supraoptimal concentrations (Figure 12). Ugolik and Nitsch (1959) reported that this promotive activity was the only growth-promotive substance in the neutral ether phase. An analogous growth promoter was detected in eluates from silicic acid column chromatography of peach seed extracts (Powell and Pratt, 1966).

Gas-liquid chromatography of neutral auxin IIB, however, yielded 3 major and 2 minor components (Figure 21). Since a limited quantity of this auxin was available, the entire sample was subjected to combined gas-liquid chromatography-mass spectrometry rather than being purified further. The mass spectra obtained from each of the three major components indicated that all three were chemically similar, although at this time, a molecular structure cannot be proposed for any one compound.

THE CYTOKININS

Introduction

Cell division, both before and immediately after anthesis, accounts for much of the initial increase in fruit size in the sour cherry and many other fruits. This period of cell division is of limited duration and is subsequently followed by one or more periods of rapid cell enlargement. Study of the physiological mechanisms controlling this period of cell division led to the isolation of cell division stimulants (Goldacre and Bottomley, 1959; Letham and Bollard, 1961) and cell division inhibitors (Letham, 1963a). One of these stimulants was isolated from immature plum fruitlets and sweet corn seed (Zea mays L.) and identified as 6-(4 hydroxy-3 methyl but trans-2 enyl) amino purine (zeatin) (Letham, 1963a, 1963c). Subsequently, zeatin ribonucleoside and zeatin ribonucleotide were identified from immature sweet corn seed (Letham and Miller, 1965; Letham, 1966a, 1966b). A further cytokinin, (-)dihydrozeatin, was isolated in immature yellow lupin seeds (Koshimuzu, et al., 1967). Evidence for the occurrence of the ribonucleoside of this latter cytokinin has been recently presented by Krasnuk, et al.,(1971).

Although a considerable research effort has been expended on the isolation and identification of naturally occurring cytokinins, the determination of levels in relation to fruit growth has received only cursory examination. Perhaps the most comprehensive investigation of this latter aspect has been conducted by Blumenfeld and Gazit (1970) and Gazit and Blumenfeld (1970) on the avocado. Both seed coat and embryo tissue extracts were found to contain high levels of cytokinin during their initial growth stages although the level fell as the rate of fruit growth slowed. Comparable activity in the mesocarp was apparent only after acid hydrolysis. The level of bound cytokinin was positively correlated with the rate of mesocarp cell division, and the rate of fruit growth.

Materials and Methods

Source of Material

Fruit from mature, fifteen-year-old trees growing at the Horticulture Research Center in East Lansing were collected at weekly intervals from anthesis, quickly frozen in the field with dry ice, and stored at -25°C until lyophilized.

The General Method

Extraction

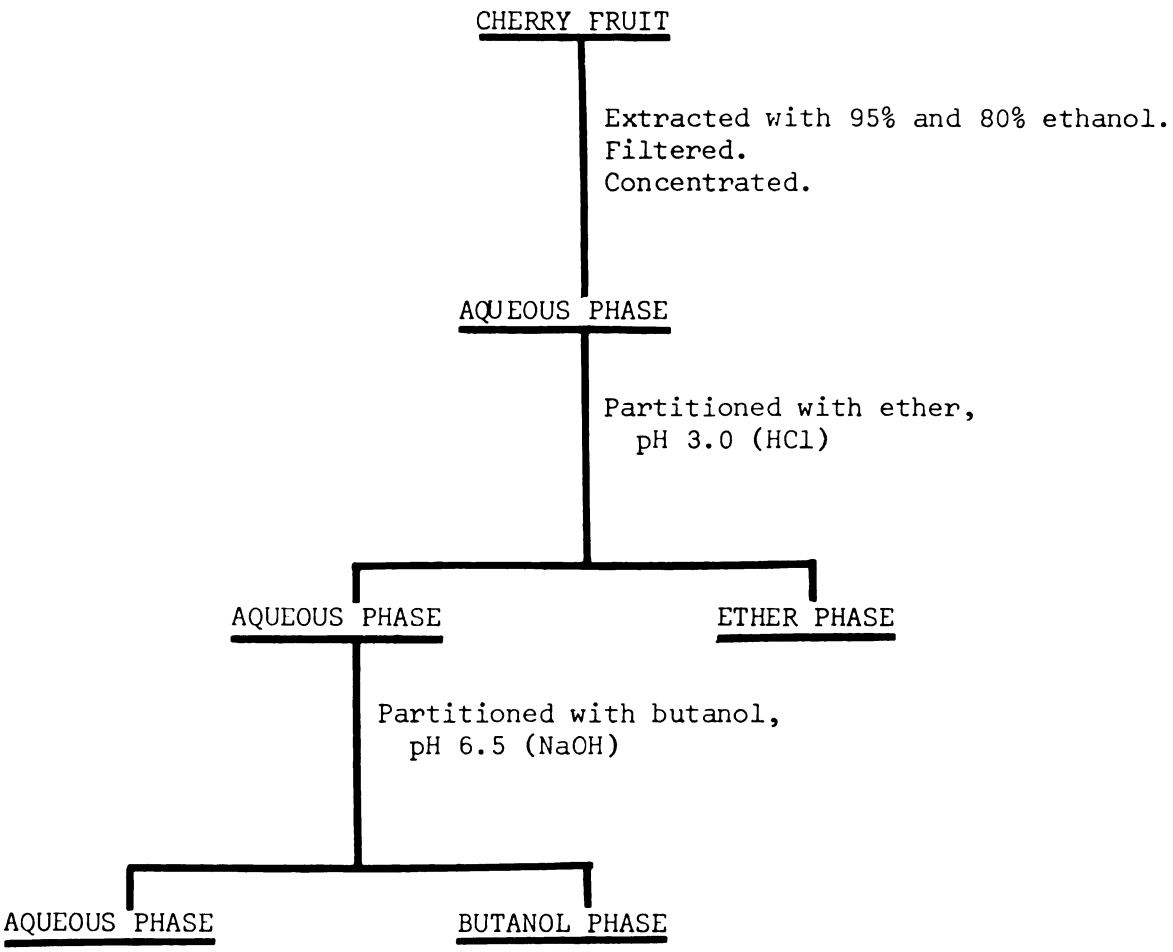
Lyophilized whole fruit were ground in a Wiley Mill to pass a 20 mesh screen. Ground material was extracted at 22°C with successive portions of 95% ethanol (30 minutes), 80% ethanol (30 minutes, 2 times); the extract being stirred constantly. Filtrates from each extraction were combined and stored in the dark at -25°C.

Fractionation

Partition chromatography.--The combined filtrates were evaporated to the aqueous phase under reduced pressure (flash evaporator, water bath temperature 30°C). The aqueous phase was adjusted to pH 3.0 and partitioned against ethyl ether until the aqueous phase was free of chlorophyll (Figure 32). The acidic aqueous phase was then adjusted to pH 6.5 and partitioned against 50 ml portions of butanol (4 times). The butanol was evaporated to near dryness under reduced pressure and the residue lyophilized to remove traces of butanol. The aqueous phase was similarly treated. Each lyophilized phase was solubilized in 95% ethanol and stored in the dark at -25°C.

Paper chromatography.--Extracted butanol-soluble and water-soluble substances were further fractionated by ascending paper chromatography. Aliquots of the extracted

Figure 32.--Flow diagram showing the procedure for extraction and fractionation of butanol-soluble and water-soluble cytokinins.


CHERRY FRUIT
Extracted with 95% and 80% ethanol.
Filtered.
Concentrated.
AQUEOUS PHASE
Partitioned with ether,
pH 3.0 (HCl)
AQUEOUS PHASE
ETHER PHASE
Partitioned with butanol,
pH 6.5 (NaOH)
AQUEOUS PHASE
BUTANOL PHASE

substances were streaked on Whatman No. 3 paper, equilibrated over the solvent, and the solvent front developed to a distance of 20 cm. All papers were pre-washed in the developing solvent to remove potential growth inhibitory substances (Burnett, et al., 1965). Developed chromatograms were dried in a cool air stream and stored in a nitrogen atmosphere at -25°C, in the dark.

Detection

The radish cotyledon bioassay.--Radish seed (Raphanus sativus L., cv. Vicks Scarlet Globe), screened for uniformity of size, were surface sterilized with 1% NaOCl for 10 minutes. Surface sterilized seed were washed with sterile distilled water (10 times) and sown on moistened filter paper in Petri dishes. All Petri dishes and filter paper had been previously autoclaved for 20 minutes at 15 lb/ square inch. Seeds were germinated, in the dark at 26°C (Letham, 1968) for 36 hours. At this time, etiolated cotyledon pairs had begun to separate. The smaller of the two cotyledons (the inner cotyledon) was excised, care being taken to remove all the hypocotyl. Cotyledons were held on sterile, moist, filter paper until their addition to test solutions.

Chromatograms to be tested (4 cm wide) were cut into 10 equally sized strips, each strip being placed into a Petri dish (4.5 cm). Control strips were taken from

the area below the start line. All strips were moistened with 1.0 - 1.5 ml of sterile distilled deionized water and 5 cotyledons of uniform size were laid on each paper strip. Known concentrations of kinetin were also bioassayed. In the latter case, 1.0 - 1.5 ml of standard was added to 2 x 4 cm strips of paper cut from developed, "blank," chromatograms. Petri dishes were randomly assigned to plastic trays lined with moist paper towels (and covered with clear plastic) and incubated at 25°C under 175 foot candles of continuous fluorescent light (Cool White). After 72 hours, cotyledons were removed from each Petri dish, blotted dry, and weighed. All treatment bioassays were replicated 3 times.

Computation of response.--The mean fresh weight was computed from the three replicates of each treatment and the results expressed in histogram form as a function of chromatogram Rf.

The soybean callus bioassay.--Zones of growth promotion, located on chromatograms of the aqueous and butanol phases by the radish cotyledon bioassay, were cut from duplicate chromatograms and prepared for the soybean (Glycine max [L.] Merrill, cv. Acme) callus bioassay. Each zone of growth promotion was subdivided on the basis of Rf, and the resulting 2 x 4 cm strips shredded by a paper cutter. Shredded strips were placed in Erlenmeyer

flasks (150 ml) and 50 ml of basal medium added (Miller, 1965). Standard kinetin solutions (1 x 10^{-5}M to 1 x 10^{-10}M, 10 ml) were added to 4 x 2 cm strips of "blank" developed chromatography paper, in 150 ml Erlenmeyer flasks, and taken to complete dryness by lyophilization. Subsequently, 50 ml of basal medium was added to each flask. Flasks were stoppered with cotton wool plugs, capped with aluminium foil, and autoclaved for 20 minutes at 15 lb/square inch. All treatments were replicated 3 times.

Three pieces of soybean callus (each weighing approximately 7 mg) were aseptically transferred to the surface of the cooled medium. Flasks were incubated at 26°C with intermittent fluorescent light flashes (Cool White) for 42 days. At the completion of the incubation period, the 3 callus pieces from each flask were blotted free of adhering medium and weighed.

Computation of response.--The mean fresh weight per flask was computed for each treatment. Treatment means were converted to kinetin equivalents by means of a standard curve.

Changes in Cytokinin Levels During Fruit Development

Ethanolic fruit extracts were fractionated into aqueous and butanol phases (Letham and Williams, 1969) and each phase chromatographed in isopropanol : water

(4:1) (Heide and Skoog, 1967). Developed chromatograms were bioassayed by the radish cotyledon test. Growth-promoting zones detected by this bioassay (Rf 0.7 - 0.9, butanol phase; Rf 0.0 - 0.3, aqueous phase) were also tested for substances that would promote cell division in the soybean callus bioassay.

Partial Characterization of Fruit Cytokinins

Ethanolic fruit extracts (21 days after anthesis) were fractionated into aqueous and butanol phases and each phase chromatographed in isopropanol : water (4:1). Zones of growth-promoting activity were eluted with 95% ethanol (2 times) and 80% ethanol (3 times) and eluates re-chromatographed in the following solvents (Nitsch, 1968; Letham and Williams, 1969):-

0.03 M boric acid adjusted to pH 8.4 with NaOH (Butanol I, II; Aqueous I)

Water, pH 6.0 (Aqueous I, II)

Ethyl methyl ketone saturated with 0.04 M boric acid (Butanol II)

After development, chromatograms were tested for growth promoting substances by the radish cotyledon bioassay.

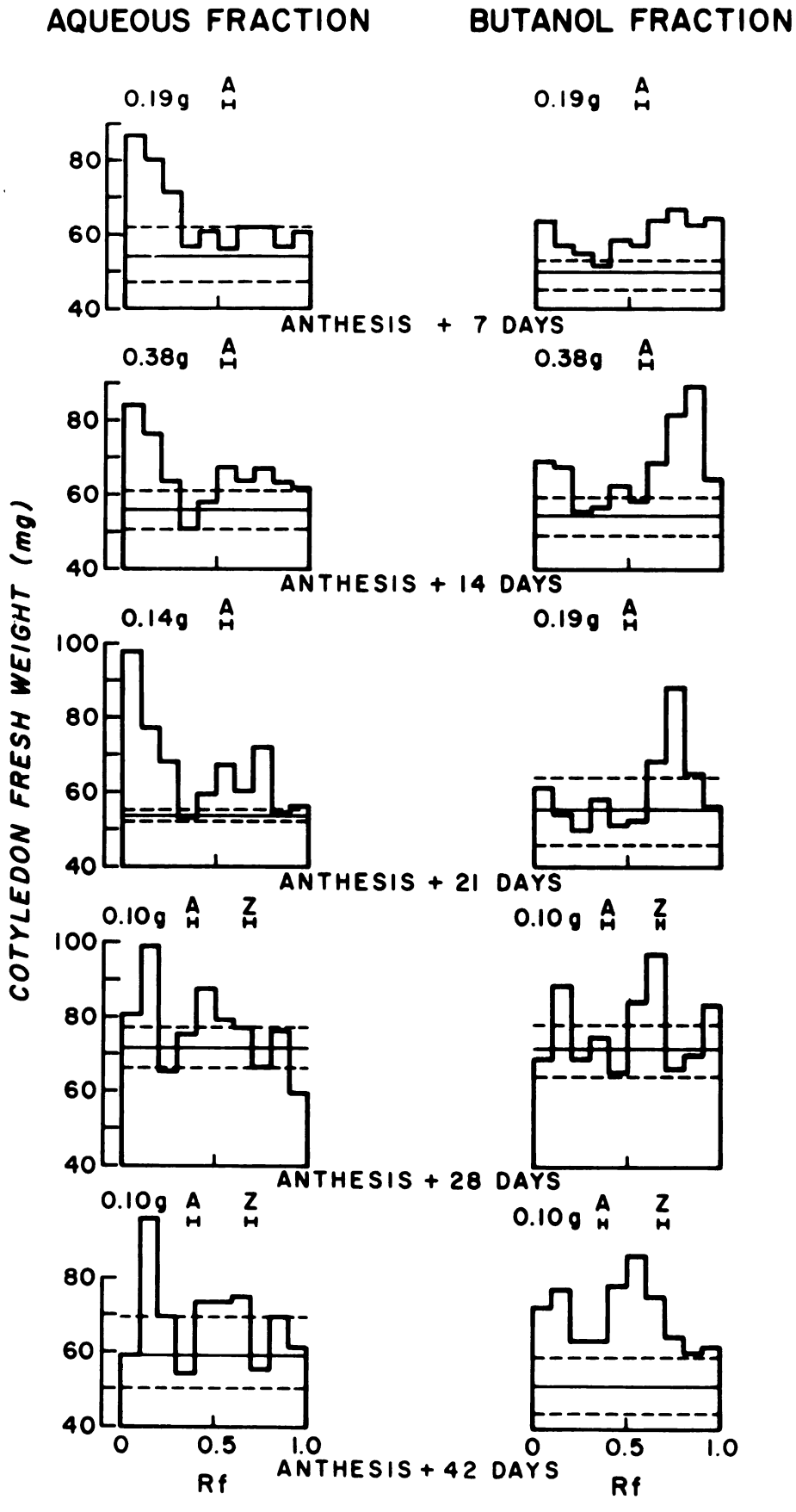
Results

Changes in Cytokinin Levels During Fruit Development

Chromatograms of the aqueous phase exhibited one major zone of growth promotion at Rf 0.0 - 0.3 (Figure 33).

Figure 33.--Histograms depicting the radish cotyledon bioassay response to growth-promoting substances detected in aqueous and butanol phases.

Solvent system : isopropanol : water (4:1).
Control value (——).
Upper and lower 5% fiducial limits (---).
A - Rf of adenine.
Z - Rf of zeatin.


AQUEOUS FRACTION
BUTANOL FRACTION
COTYLEDON FRESH WEIGHT (mg)
0.19g A H
0.19g A H
ANTHESIS + 7 DAYS
0.38g A H
0.38g A H
ANTHESIS + 14 DAYS
0.14g A H
0.19g A H
ANTHESIS + 21 DAYS
0.10g A H Z H
0.10g A H Z H
ANTHESIS + 28 DAYS
0.10g A H Z H
0.10g A H Z H
ANTHESIS + 42 DAYS
100
80
60
40
0
0.5
1.0
Rf

This promotive activity was found in all fruit extracts. Chromatograms of the butanol phase, however, exhibited a major zone of growth promotion at Rf 0.7 - 0.9 (Figure 33) for the early harvest dates (7, 14, and 21 days after anthesis) and a second major promotive zone (Rf 0.5 - 0.7) for all subsequent harvest dates. Expression of mobility of either promoter as a function of adenine mobility $\left[\frac{\text{Rf promoter}}{\text{Rf adenine}}\right]$ resulted in values in the range of 1.55 $\pm$ 0.05 for all chromatograms. The close similarities in relative Rf values strongly suggest that growth promotion was due to one substance. Minor growth-promotive activity was detected at Rf 0.4 - 0.7 (aqueous phase) and at Rf 0.0 - 0.2 (butanol phase).

Since gibberellin has been reported to cause slight increments in cotyledon weight over the concentration range 0.5 - 50 mg/liter (Letham, 1968), 4 levels of kinetin (0, 21.5, 215, 2150 ng/Petri dish) were combined with 4 levels of gibberellic acid (0, 3.46, 34.6, 346 ng/Petri dish) in a factorial design and treatment combinations subjected to the radish cotyledon bioassay (Figure 2, Appendix B). With the radish cultivar used in this study, an appreciable increase in cotyledon weight was found in the absence of kinetin. At low levels of kinetin (21.5 ng/Petri dish) all levels of gibberellin slightly increased the bioassay response although at higher levels of kinetin (215, 2150

ng/Petri dish) the higher concentrations of gibberellin markedly increased cotyledon weight.

Histogram peaks (Rf 0.0 - 0.3, aqueous phase; Rf 0.7 - 0.9, butanol phase 7-21 days after anthesis, Rf 0.5 - 0.7, butanol phase 28-42 days after anthesis) exceeding the upper 5% fiducial limit were converted to kinetin equivalents per 0.1 gram dry weight or per fruit by means of a standard curve (Figure 1, Appendix B). A new standard curve was errected for each bioassay. Similar computations were made using data from the soybean callus bioassay.

Comparison of kinetin equivalents detected from the same fruit extract, but by different bioassays (Table 3) showed that the cotyledon bioassay was more sensitive to the aqueous promoter (Rf 0.0 - 0.3, Figure 33) than was the callus bioassay. The opposite was true for the butanol promoter (Rf 0.8 $\pm$ 0.1, Figure 33).

The aqueous promoter (as detected by radish cotyledon bioassay) was initially at a high concentration (day 7) which declined (day 14) and then increased (day 21). The increase in aqueous promoter concentration at day 21 was not found for the soybean callus bioassay (Table 3). Subsequent to day 21, the concentration of aqueous promoter decreased (day 28) and then increased (day 42). The increase in butanol promoter concentration during the first three weeks of fruit growth (Table 3) was shown by

TABLE 3.--Comparison of cytokinin concentrations and levels detected in sour cherry fruits at different stages of fruit development.

Days After Anthesis	Kinetin Equivalents: Aqueous Promoter, Cotyledon Bioassay	Kinetin Equivalents: Aqueous Promoter, Callus Bioassay	Kinetin Equivalents: Butanol Promoter, Cotyledon Bioassay	Kinetin Equivalents: Butanol Promoter, Callus Bioassay
	ng/100 mg. dry wt.			
7	600.0	39.7	64.0	130.5
14	51.0	2.9	201.0	133.5
21	169.0	0	438.0	1795.0
28	68.0	-[1]	73.5	-[1]
42	370.0	-	765.0	-
	(ng/fruit)			
7	11.0	0.7	1.1	2.4
14	2.4	0.1	9.7	6.4
21	81.4	0	155.5	637.0
28	94.1	-[1]	101.5	-[1]
42	867.0	-	1790.0	-

[1]Promoter not bioassayed.

both bioassays. Subsequent to day 21 the concentration decreased (day 28) and then increased to a maximum (day 42).

Expression of aqueous and butanol promoter concentrations in terms of ng kinetin equivalents/fruit (Table 3) did not alter the direction of promoter change, only the magnitude. Peaks of promoter activity were located at day 42 (aqueous promoter) and days 21 and 42 (butanol promoter).

Partial Characterization of Fruit Cytokinins

The water-soluble cytokinins.--Chromatographic separation of the aqueous phase (21 days after anthesis) and subsequent detection by the radish cotyledon bioassay revealed a major zone of growth promotion (Rf 0.0 - 0.3, Aqueous I) and a minor zone of growth promotion at Rf 0.5 - 0.8 (Aqueous II) (Figure 33, 34A). Re-chromatography of Aqueous II, eluted from a duplicate chromatogram, resulted in further separation into two components (Rf 0.3, 0.8 - 1.0); (Figure 3A, Appendix B). Since Aqueous II could be due to butanol-soluble cytokinin remaining in the aqueous phase from incomplete partition chromatography, the Rf's of the two promotive components in Aqueous II were compared with reported Rf values for zeatin and zeatin ribonucleoside. The expected Rf of zeatin, based on that of adenine, would be Rf 0.59 (Heide and Skoog, 1967). No activity was located at this Rf (Figure 3A, Appendix B). The expected Rf of

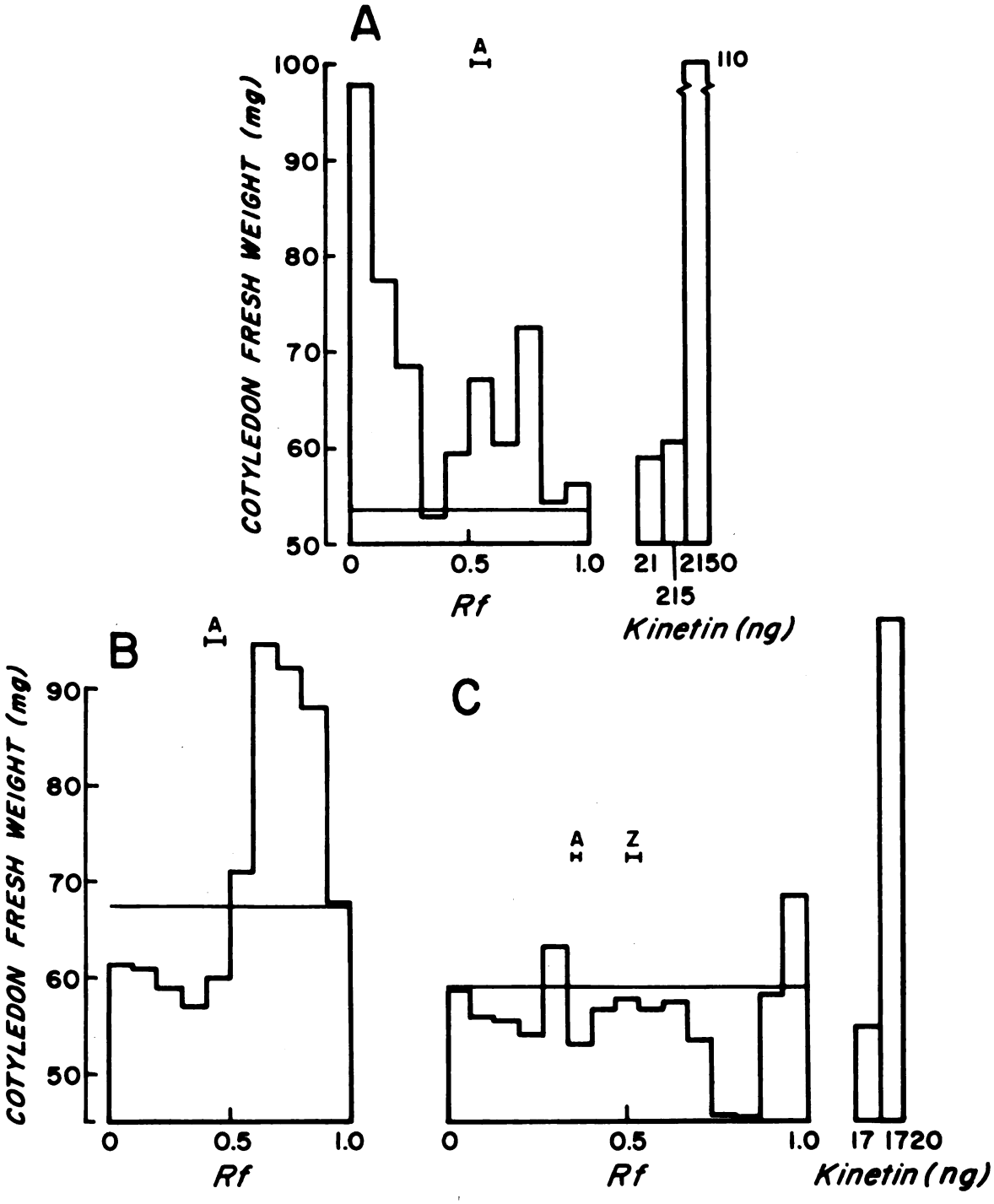
Figure 34.--Histograms depicting the radish cotyledon bioassay response to the growth promoters in the aqueous phase (21 days after anthesis).

A. Chromatography of 0.14 gram equivalents of the aqueous phase in isopropanol : water (4:1).

B. Re-chromatography of 0.18 gram equivalents of Aqueous I (Figure 34A, Rf 0.0 - 0.3). Solvent system:- water (pH 6.0).

C. Re-chromatography of 0.12 gram equivalents of Aqueous I (Figure 34A, Rf 0.0 - 0.3). Solvent system:- 0.03M boric acid adjusted to pH 8.4 with NaOH.

Control value (——).
A - Rf of adenine.
Z - Rf of zeatin.


A
B
C
COTYLEDON FRESH WEIGHT (mg)
100
90
80
70
60
50
0
0.5
1.0
Rf
A
I
110
21
2150
215
Kinetin (ng)
Z
17
1720

zeatin ribonucleoside would be Rf 0.75 (Miller, 1967; Gupta and Maheshwari, 1970) yet the observed promotive activity occurred at a higher Rf (0.8 - 1.0) (Figure 3A, Appendix B).

Re-chromatography of Aqueous I in water (Figure 34B) resulted in a single promoter peak at Rf 0.7 - 0.9. However, re-chromatography of Aqueous I in 0.03M boric acid (Figure 34C) yielded poor results with the largest peak of activity occurring at Rf 1.0. Zeatin ribonucleoside has a reported Rf of 0.86 - 0.91 in this solvent (Tegley, et al., 1971; Krasnuk, et al., 1971) but this cytokinin would be expected to partition into butanol (Letham and Williams, 1969) suggesting that the major aqueous cytokinin is zeatin ribonucleotide or some closely related compound.

The butanol-soluble cytokinins.--Fractionation of the butanol phase by ascending paper chromatography (7, 14, and 21 days after anthesis, Figure 33) revealed zones of weak growth promotion at Rf 0.0 - 0.2 (Butanol I), Rf 0.4 - 0.6 (Butanol II) and a major zone of growth promotion at Rf 0.7 - 0.9 (Butanol III). Re-chromatography of Butanol I (Figure 3B, Appendix B) and subsequent detection by the radish cotyledon bioassay revealed two weak promoter zones at Rf 0.3 and 1.0 similar to the results obtained when Aqueous I was chromatographed in the boric acid solvent (Figure 34C). Incomplete partitioning would account for residual zeatin ribonucleotide in the butanol

phase. Similar re-chromatography of Butanol II revealed a promoter peak at Rf 0.9 - 1.0. Such behavior is suggestive of zeatin ribonucleoside (Miller, 1967).

Re-chromatography of the major butanol phase component (Butanol III, Figure 35A) in the boric acid solvent system yielded several zones of activity between Rf 0.2 - 0.7 (Figure 35B). The reported Rf of zeatin in this solvent is 0.49 - 0.58 (Tegley, et al., 1971). More convincing evidence was obtained when Butanol III was chromatographed in methyl ethyl ketone saturated with boric acid (Figure 35C). In this instance, the major peak of activity chromatographed with standard zeatin. Zeatin ribonucleoside would be expected to move only slightly from the start line (Letham and Williams, 1969). Moreover, standard zeatin chromatographed with Butanol III in the isopropanol : water (4:1) solvent system (Figure 33). This evidence suggests that the major cytokinin in the butanol phase is zeatin or a closely related compound.

Discussion

Seasonal Variation in Fruit Cytokinin Content

The butanol-soluble cytokinin extracted from whole fruits (Figure 33) increased from an initially low value (day 7) to a peak of activity at 21 days after anthesis (Table 3). This change was detected by both cotyledon and

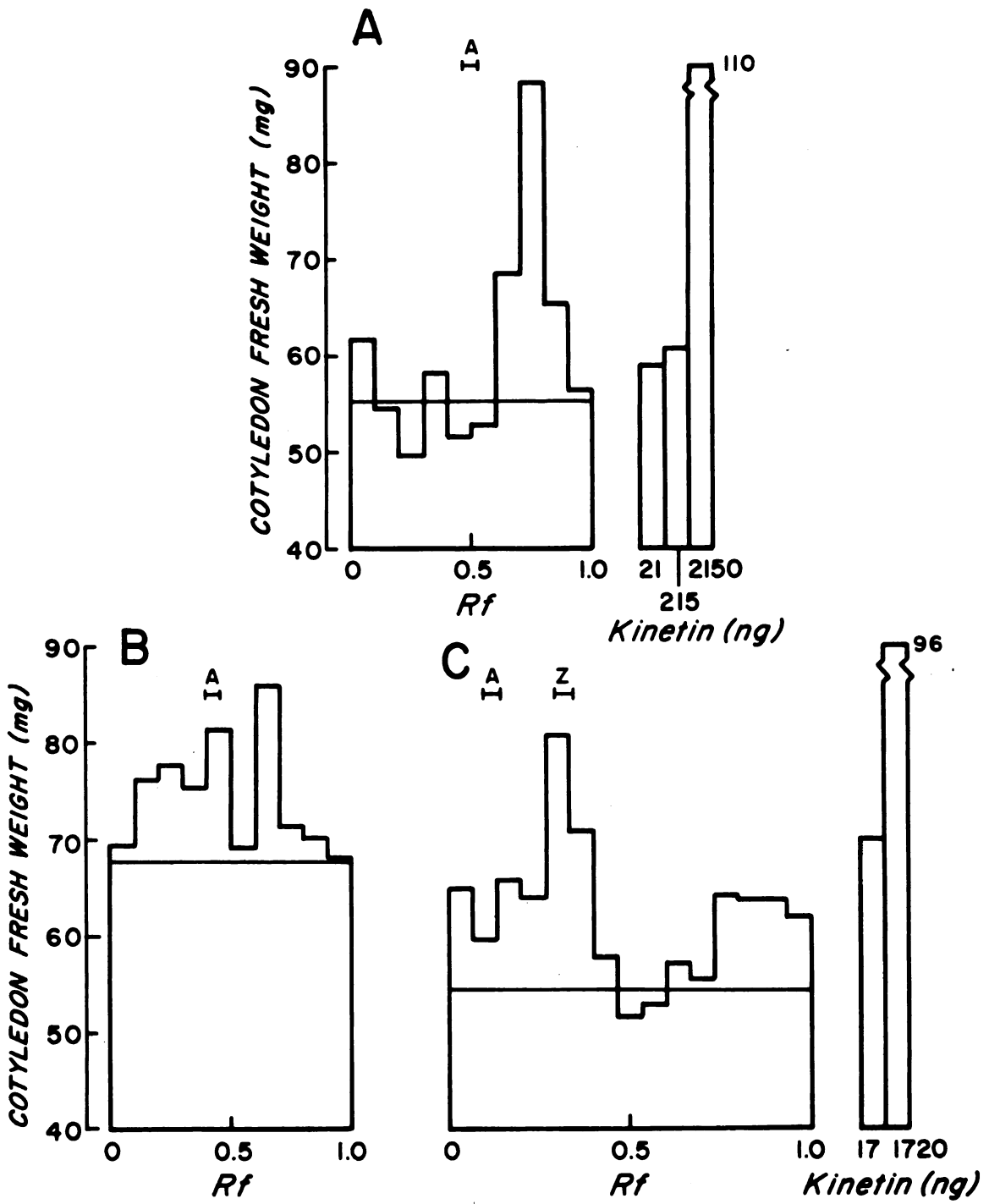
Figure 35.--Histograms depicting the radish cotyledon bioassay response to the growth promoters in the butanol phase (21 days after anthesis).

A. Chromatography of 0.19 gram equivalents of the butanol phase in isopropanol : water (4:1).

B. Re-chromatography of 0.37 gram equivalents of Butanol III (Figure 35A, Rf 0.7 - 0.9). Solvent system:- 0.03M boric acid adjusted to pH 8.4 with NaOH.

C. Re-chromatography of 0.25 gram equivalents of Butanol III (Figure 35A, Rf 0.7 - 0.9). Solvent system:- ethyl methyl ketone saturated with 0.04M boric acid.

Control value (——).
A - Rf of adenine.
Z - Rf of zeatin.


A
B
C
COTYLEDON FRESH WEIGHT (mg)
90
80
70
60
50
40
0
0.5
1.0
Rf
A
Z
110
96
21
2150
215
17
1720
Kinetin (ng)

callus bioassays. Moreover, the direction of the increase was not altered when data were expressed on a per fruit basis (Table 3). However, levels of water-soluble cytokinin (Figure 33), measured by the cotyledon bioassay did not correspond with those measured by the callus bioassay (Table 3). In this regard, no water-soluble cytokinin was found in fruit extracts (day 21) by the callus bioassay suggesting that either the cotyledon bioassay is more sensitive to water-soluble cytokinin, or that the chromatogram zone bioassayed contains further substances that would be promotive in the cotyledon bioassay, e.g., gibberellins (Figure 2, Appendix B), or inhibitory in the callus bioassay.

Gibberellin A_{32} has been shown to account for most of the gibberellin-like activity in seed and pericarp extracts of peach and apricot (Yamaguchi, et al., 1970; Coombe, 1971). Since the chromatographic location of this highly polar gibberellin would be close to the origin in isopropanol : water (4:1) gibberellin could account for much of the "aqueous cytokinin" activity at days 21, 28, and 42 in the cotyledon bioassay. However, re-chromatography of the water-soluble cytokinin (Figure 34B, 34C) revealed at least one inhibitor that co-chromatographed with the cytokinin in isopropanol : water (4:1). The relationships between the inhibitory substance and the callus bioassay

or between presumed GA_{32} and the cotyledon bioassay are unknown.

The butanol-soluble cytokinin decreased between days 21 and 28 and then increased (day 42) (Table 3). Since reasonable agreement was found between bioassays during the period 7-21 days after anthesis the fluctuations between days 21-42 are assumed to be representative of changes in cytokinin concentration, per se, and not due to gibberellin. Although cytokinins have been isolated from several Prunus sp, determination of seasonal variations has only been attempted for the plum. The level of cytokinins in plum fruitlets increased to a maximum 15 days after anthesis. This increase was correlated with the onset of rapid cell division (Letham, 1963a, 1964). Water-soluble and butanol-soluble cytokinins isolated from the endosperm and embryo of peach (Powell and Pratt, 1964) could be detected long after cell division in the pericarp had ceased. A similar cytokinin contribution from the developing seed would explain the increase in concentration, and level, from 28-42 days after anthesis (Table 3).

Partial Characterization of Fruit Cytokinins

Re-chromatography of the butanol-soluble sour cherry cytokinin (Figure 35) in two solvents which would separate zeatin from its ribonucleoside yielded a single peak of activity which chromatographed with authentic

zeatin. In this regard, the butanol-soluble cytokinin is either zeatin or some closely related compound. Evidence for a water-soluble cytokinin (Figure 34) is clouded by the possibility that gibberellin could have stimulated radish cotyledon growth.

GENERAL DISCUSSION

The development of the sour cherry fruit occurs in three distinct stages. Fruit growth during stage I is principally by cell division in the pericarp followed by a brief period of cell enlargement. The rate of cell enlargement, however, slows during the period 21-28 days after anthesis and continues at a low rate until the onset of growth stage III (about day 42 from anthesis). During growth stage III the rate of cell enlargement in the mesocarp dramatically increases and is maintained at almost a constant rate until just before the fruit matures (63-70 days after anthesis).

At anthesis, the number of cells in the radial direction through the fruit has been found to be in range of 14-17 for the endocarp and 22-24 for the mesocarp (Tukey and Young, 1939; Inoue, 1970). Subsequently, both tissues undergo a period of cell division (until 21 days after anthesis) which increases the cell number in the endocarp to 27-30 and 29-32 in the mesocarp. No cell division has been reported later than the end of growth stage I (Tukey and Young, 1939; Inoue, 1970).

The level of butanol-soluble cytokinin (Table 3) increased during the period of cell division and reached a maximum at the cessation of cell division (21 days after anthesis). The increase in this cytokinin paralleled the increase in pericarp cell division until day 21 and, as such, can be positively correlated with this period of cell division. However, the cessation of cell division does not appear to be related to a low level of butanol-soluble cytokinin at day 28 (Table 3).

Letham (1963a) reported that cell division inhibitors could be separated from cell division stimulants by partitioning aqueous fruit extracts against ethyl acetate or ethyl ether at low pH. The inhibitors partitioned into the organic phase. When the ethyl ether phase (Figure 32) from cherry fruits was chromatographed, (isopropanol : water [4:1]) and bioassayed (radish cotyledon) strong inhibitory activity was revealed at Rf 0.4 - 1.0 (21 days after anthesis). Insufficient evidence, however, was obtained to determine if this inhibitory activity was associated with the cessation in cell division. Expression of plum fruitlet inhibitor content in relation to fruit development showed that the level of inhibitors increased during the period of rapid fruitlet cell division and

reached a maximum at the time of cell division cessation (Letham, 1963a). The decrease in avocado mesocarp cell division has also been correlated with increased levels of an inhibitor (Gazit and Blumenfeld, 1970). Thus, the balance between promoter and inhibitor levels must be considered; high cytokinin levels in conjunction with high inhibitor levels could result in small increments of net growth, and thus account for the observed cessation of cell division when butanol-soluble cytokinin levels were high (day 21).

The level of water-soluble cytokinin (Table 3, callus bioassay) decreased during the period of cell division and, as such, probably plays no role in fruit cell division. Cell division in the avocado mesocarp has been positively correlated with the level of water-soluble, "bound" cytokinin (Gazit and Blumenfeld, 1970). This cytokinin was inactive in the soybean callus bioassay prior to acid hydrolysis. Since the water-soluble cytokinin from sour cherry fruits was active in the soybean callus bioassay, per se, it is unlikely that it is a similarly "bound" cytokinin. Furthermore, the magnitude of the decrease in water-soluble cytokinin (Table 3) and concomitant increase in butanol-soluble cytokinin suggests that the latter did not arise from the loss of the former.

Rapid cell enlargement in both the mesocarp and endocarp began 10 days after anthesis (Inoue, 1970) and ceased 18 days later. Both the concentration and level of fruit auxin was shown to be low during the period 7-21 days after anthesis (Table 1, 2) even though the level of seed auxin increased dramatically (day 21, Figure 2, 3). The low level of auxin detected in the pericarp during this initial period of cell enlargement could be the result of utilization.

Seed extracts from other Prunus sp. fruits have been shown to contain high levels of growth substances active in straight growth tests during the period of active nucellus and integument development (Stahly and Thompson, 1959; Ugolik and Nitsch, 1959; Powell and Pratt, 1966). Similar findings have been reported for black currant and grape fruits (Wright, 1956; Nitsch, et al., 1960). Yet in each of the above fruits the rate of cell enlargement in the pericarp began to decline about the time when the auxin associated with the development of the nucellus reached its maximum. In two fruits (black currant and grape) the rapid fall in the auxin content has been correlated with the onset of growth stage II (Wright, 1956; Nitsch, et al., 1960).

In the present study, the level of seed auxin associated with nucellus and integument development decreased from a maximum at day 21 to a non-detectable

level at day 28 (Figure 4, 5). In a second season of growth, the level of total auxin associated with nucellus and integument development also declined (Table 1, 2) and this decline was more dramatic for the acidic auxin than for either of the neutral auxins. It is improbable that the decrease in seed auxin content is associated with the onset of growth stage II since the level in the pericarp was found to increase throughout the period of reduced cell enlargement (Figure 4, 5; Table 1, 2). It is not known, however, if this increase in pericarp auxin level is due to movement from the seed. Rapid development of both the endosperm and embryo during growth stage II has been correlated with high auxin levels in a number of other fruits (Stahly and Thompson, 1959; Crane, et al., 1959; Pillay, 1966; Powell and Pratt, 1966) although studies on the fate of this auxin have never been attempted.

The control of cell enlargement during growth stage II could be due to one or more inhibitors specific to cell enlargement that conceivably would increase during growth stage I and be maintained at a high level during growth stage II. Subsequent to growth stage II the inhibitor(s) level would be expected to decrease. This presumed inhibition could be of the type shown by factorial combination of neutral auxins IIA and IIB (Figure 18, 19, 20). This hypothesis is not tenable

since neutral auxin IIA was not detected in any pericarp fraction investigated (Table 1, 2). However, no evidence was obtained on the interaction between neutral auxin IIB and acidic auxin I.

The presumed inhibition could also result from the interaction between one or more inhibitors and either acidic auxin I or neutral auxin IIB or from the interaction between inhibitor(s) and both auxins. However, no evidence was obtained that showed that ethyl ether-soluble inhibitors occurred in extracts of sour cherry pericarp tissue (Figure 2, 3, 9, 15, 27). However, these findings are tempered by the fact that the Avena first internode bioassay is less sensitive to inhibitory substances, such as abscisic acid, than is the Avena coleoptile bioassay (Nitsch and Nitsch, 1956) or the wheat coleoptile bioassay (Luckwill, 1952). Yet, when the pericarp aqueous phase was subjected to base hydrolysis a highly potent ether-soluble inhibitor(s) of the Avena first internode bioassay was released (Figure 30). No similar inhibitory activity was located from the seed hydrolysate. Furthermore, the inhibitor(s) was present in pericarp extracts during growth stage II until the onset of growth stage III. No inhibitory activity was found in pericarp extracts at day 56 suggesting that this inhibitor(s), if released by enzymatic hydrolysis during growth stage II, could account for the observed lack of mesocarp cell enlargement.

With the onset of growth stage III the seed auxins increased from a low level (Figure 5) and reached a maximum 56-63 days after anthesis. This increase was not observed in a second season of growth (Table 2). The level of total pericarp auxin, however, decreased from day 42 to day 56 (Figure 5, Table 2) and this decrease can be attributed to a fall in neutral auxin IIB (Table 2). Subsequent to day 56 the level of pericarp auxin increased (Figure 5) although confirmatory evidence of this increase was not obtained in a second season of growth (Table 2). Correlations between increased seed auxin levels and the period of rapid cell enlargement during growth stage III have been sought by several investigators. Such evidence has been found only for peach (Powell and Pratt, 1966). As a result of this considerable body of negative evidence, Crane (1964) concluded that "no relationship has been proven between the levels of growth substances in developing seeds and fruit growth."

However, the period of rapid cell enlargement immediately prior to fruit maturation has been shown to be independent of the seed and its attendant hormone levels for apple, peach, and sour cherry (Tukey, 1936b; Abbott, 1958; Southwick, et al., 1962). If the seed hormone content plays no part in cell enlargement during

growth stage III, then the pericarp auxin content subsequent to day 42 (Figure 5, Table 2) must have arisen either from translocation of auxin from vegetative organs to the fruit or from synthesis in the pericarp.

Meristematic tissue located in shoot and root apices and in lateral buds has long been associated with the synthesis of growth-promoting substances. Translocation of these substances to developing fruits could provide the necessary hormonal supply for continued cell enlargement during the period of final fruit swell. Examination of root exudates and xylem sap from a number of herbaceous plants has revealed the presence of cytokinins (Kende, 1964) gibberellins (Lang, 1970) and auxins (Nitsch and Nitsch, 1965). Similar investigations of xylem sap from fruit trees has not received commensurate attention although the xylem sap from apple branches has recently been shown to contain a cytokinin and an unidentified substance active in the Avena mesocotyl bioassay (Luckwill and Whyte, 1968). Examination of apple branch xylem sap at different times during the growth season showed that the levels of both substances was initially high during the spring and declined during the early summer to a low, but constant, level.

Evidence, albeit indirect, against the hypothesis that final fruit swell is dependent on growth-promoting substances translocated from vegetative organs to the

fruit has come from *in vitro* studies. Nitsch (1951) showed that tomato flowers, excised 2 days after pollination, could be grown to maturity under sterile conditions on a simple medium of mineral salts and sucrose. The validity of these experiments should be questioned since root formation on pedicels occurred. However, Nitsch (1967) reported that sweet cherry fruits grown on a medium containing mineral salts, sucrose, and two amino acids (L-glutamine, L-asparagine), did not reach a size comparable to those grown in the field, although they did undergo the normal color changes indicative of a maturing fruit.

Triiodobenzoic acid has been shown to inhibit polar auxin (IAA) transport in various coleoptile and herbaceous stem sections (McCready, 1966; Winter, 1968; Hejnowica and Tomaszewski, 1969). Application of TIBA in lanolin to sour cherry pedicels for various lengths of time during growth stage III (Figure 1, Appendix C) did not curtail cell enlargement in the mesocarp. Although these results could be taken as evidence that cell enlargement during growth stage III is independent of an external auxin source no experimental evidence as to the inhibitory effect of TIBA on auxin transport through phleom and xylem tissues is currently available.

The auxin extracted from pericarp tissues during growth stage III (Figure 4, 5; Table 1, 2) could have arisen either from synthesis or from hydrolysis of bound

auxin. No evidence has ever been presented for synthesis of auxin in pericarp tissues during the final stages of fruit growth. In contrast, bound auxins (Ueda and Bandurski, 1969), gibberellins (Lang, 1970), cytokinins (Gazit and Blumenfeld), and abscisic acid (Koshimizu, et al., 1968) have been detected in seed and fruit of a wide range of plant species. Hydrolysis of these bound hormones during final fruit swell could result in appreciable levels of the free hormone.

Comparison between the levels of free and bound auxin detected in sour cherry seed and pericarp tissue (Figure 31) showed that the level of free and bound auxin in the seed decreased as the level of free and bound auxin increased in the pericarp. The parallel decrease of free and bound auxin in the seed would argue against conversion of the free to the bound (Barendse, et al., 1968) unless the bound were lost from the seed. The increase in the level of pericarp bound auxin would suggest that this were the case. However, these results are confounded in so much as the auxin released by hydrolysis was chromatographically distinct from either of the neutral auxins or the acidic auxin (Figure 30). It is not known if this auxin was active in the Avena first internode bioassay per se, or was metabolised to an active form within the Avena sections. Since base hydrolysis of the bound auxin in corn kernels yielded free IAA (Ueda and Bandurski, 1969)

hydrolysis may have been incomplete. Thus, the bound auxin levels depicted in Figure 31 may be an underestimate of the total present in the sour cherry pericarp.

The inverse association found between the level of bound auxin in the pericarp and that in the seed suggests that the developing seed is the source of this pericarp auxin. Moreover, the level of bound auxin in the seed was found to be highest at a time when the levels of free auxin in the seed were at a maximum (day 21, Figure 31). This maximum in seed bound auxin content correlates with the development of the nucellus and integuments (Inoue, 1970). Destruction of specific seed tissues by application of maleic hydrazide to developing strawberry achenes showed that if application were delayed until the third day after anthesis, the receptacles were able to develop and ripen even though all achenes were devoid of viable embryos (Thompson, 1963). If the maleic hydrazide was applied prior to the third day from anthesis, however, development of the treated receptacles was prevented. This effect was shown to be due to the effect of maleic hydrazide on seed development, not to an effect of the chemical on receptacle development, *per se*. Treatment with 2-naphthoxyacetic acid partially overcame the effect of aborted achenes. Thompson (1963) concluded that the seed tissue directly controlling receptacle growth could be the nucellus,

since maleic hydrazide application inhibited the development of the nucellus when applied prior to the third day from anthesis but not when applied subsequently. Tukey (1936b) showed that mechanical destruction of developing sour cherry and peach seeds during growth stage I resulted in an abrupt check to fruit growth and eventual fruit abscission. This effect has also been observed following maleic hydrazide application to apricot fruits prior to pit hardening (Crane and Nelson, 1970), strongly suggesting that some factor associated with the developing nucellus and integuments plays a role in the prevention of premature fruit abscission. As seed destruction was successively delayed during growth stage II, higher percentages of treated fruits persisted and developed like untreated fruits (Tukey, 1936b; Crane and Nelson, 1970). Thus, development of fruits from various Prunus sp. is dependent on a factor associated with the development of the nucellus and integuments.

In the present study, the bound auxin located in seed tissues is suggested to result from metabolism of the free seed auxin. Furthermore, the maximum level of bound seed auxin has been correlated with the development of the nucellus and integuments. If this bound auxin moved from the seed to the pericarp and there was hydrolyzed to the free form during growth stage III, the low level of pericarp auxin found during this time

and the rapid rate of pericarp cell enlargement could be explained. Moreover, the finding that sour cherry fruit growth is dependent on the presence of a developing seed until the end of growth stage II (Tukey, 1936b) could also be explained, since the bound auxin must be mobilized from the seed to the pericarp.

In conclusion, correlations between the levels of any one free seed hormone and successive stages of fruit growth (Crane, 1964) should be questioned since the levels of free auxin in sour cherry pericarp tissue at different stages of fruit development bore no relation to concomitant levels found in the seed. A further factor is introduced when the presence of bound hormones were considered. Since Baldev, et al., (1965) showed that AMO-1618 application to pea pods growing on a defined medium inhibited seed gibberellin synthesis by 87% while only inhibiting seed weight by 16%, high seed hormone levels could be the result of seed development rather than its cause. If accumulating hormones were converted to bound forms as a de-toxification mechanism and then mobilized to the fruit, growth substances would be available for subsequent fruit growth. Such a hypothesis would explain many of the negative correlations found for seed hormone content and the later stages of fruit growth. Furthermore, if the auxins found immediately after anthesis in naturally

parthenocarpic fruits (Gustafson, 1939a; Nitsch, et al., 1960; Coombe, 1961) were incorporated into bound forms which underwent hydrolysis during later fruit development, fruit growth in the absence of a seed could be explained.

SUMMARY

Changes in seed and pericarp auxin content were determined in developing sour cherry (Prunus cerasus L.) fruits. Although changes in free seed auxin content were correlated with the development of the nucellus and integuments, endosperm, and embryo, no correlation was found between the changes in free seed auxin content and any stage of fruit development. The pericarp auxin content was found to be approximately two orders of magnitude lower than that found in the seed. No correlation was found between the level of free pericarp auxin and growth stage II or III, or with changes in the level of free seed auxin.

The level of bound seed auxin was found to parallel the changes in the level of free seed auxin. The level of pericarp bound auxin, however, was inversely associated with the levels of both free and bound auxin found in the seed.

Changes in fruit cytokinin content were also determined in developing sour cherry fruits. The period of rapid cell division in the pericarp could be correlated

with the level of butanol-soluble cytokinin. The cessation of cell division in the pericarp, however, could not be correlated with decreased levels of this cytokinin.

Characterization of free auxins in seed and pericarp tissues revealed both acidic and neutral substances that were highly active in the Avena first internode and curvature bioassays. The acidic auxin was chromatographically and chromogenically similar to indole-3-acetic acid. This tentative identification was confirmed by gas-liquid co-chromatography with standard IAA and by combined gas-liquid chromatography-mass spectrometry. Repeated chromatography of the neutral auxin resolved the growth promotive activity into two components (neutral auxins IIA and IIB). Neither of these auxins chromatographed with standard indole auxins nor did they yield chromogenic reactions with reagents specific for indolic compounds.

Avena first internode bioassay of these neutral auxins, either alone or in combination, showed that the concentration range over which they were stimulatory to cell enlargement was extremely narrow, suggesting that both auxins are non-indolic. Moreover, combination of low levels of neutral auxin IIA with all levels of neutral auxin IIB yielded a bioassay response that was significantly greater than the reciprocal combination of low levels of neutral auxin IIB with all levels of neutral auxin IIA. The bioassay response to all other combinations

of these neutral auxins was indicative of additivity of the one auxin with the other.

Gas-liquid chromatography of neutral auxin IIB resulted in the resolution of three compounds, none of which chromatographed with standard indole auxins. The non-indolic nature of these compounds was confirmed by combined gas-liquid chromatography-mass spectrometry. The mass spectra obtained from the three compounds exhibited many common fragment ions suggesting that all could be isomers. Insufficient evidence is presently available to assign a structure to these compounds.

No consistent growth-promoting activity was detected in basic ether fractions of either seed or pericarp tissues. A similar absence of growth-promoting substances was found for the aqueous fractions. Base hydrolysis of such aqueous fractions, however, resulted in the release of an ether-soluble auxin that was chromatographically distinct from either the acidic or neutral auxins.

Characterization of substances active in both the radish cotyledon and soybean callus bioassays revealed a butanol-soluble cytokinin that possessed chromatographic properties similar to that of zeatin. A further cytokinin, insoluble in butanol but soluble in water, was chromatographically similar to zeatin ribonucleotide.

The changes in levels of free auxins and cytokinins in seed and pericarp tissues were evaluated in relation to

fruit development. Although the changes in levels of these fruit hormones could be related to post-fertilization cell division and enlargement, it was concluded that the bound auxins were physiologically significant to cell enlargement during the later stages of fruit growth.

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APPENDICES

APPENDIX A

Figure 1.--*Avena* first internode bioassay of known concentrations of auxin (IAA). Each value plotted is the mean of all values derived from a number of similar bioassays conducted at intervals during the determination of endogenous auxin levels (1969).

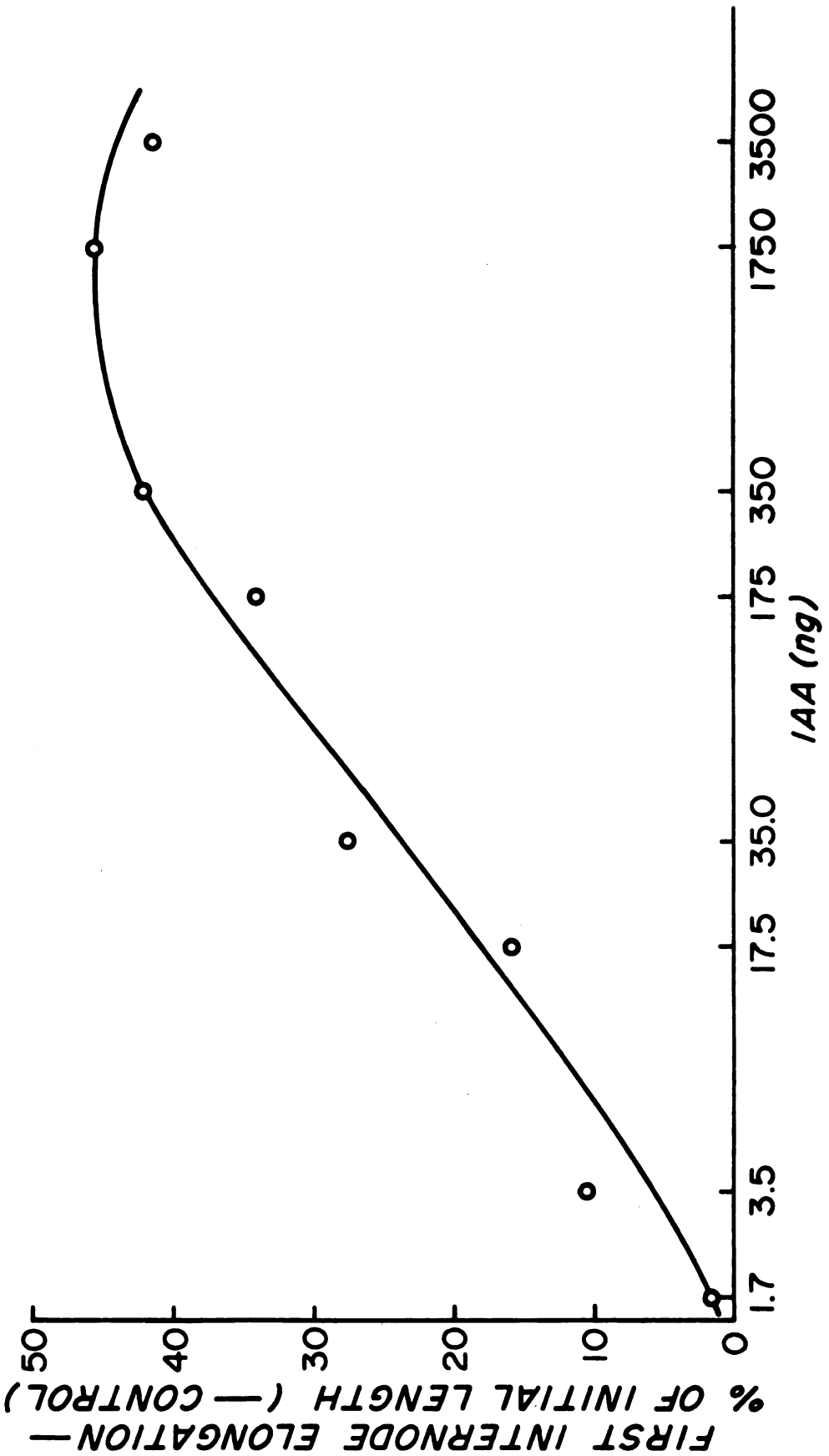

FIRST INTERNODE ELONGATION —
% OF INITIAL LENGTH (— CONTROL)
0
10
20
30
40
50
1.7
3.5
17.5
35.0
175
350
1750
3500
IAA (ng)

Figure 2.--The effect of organic solvent residues on the Avena first internode bioassay.

A. Residues from extraction and fractionation procedures chromatographed in isopropanol : water (4:1).

B. Residues from extraction and fractionation procedures chromatographed in hexane : water (9:1).

Control values (——).
Upper and lower 5% fiducial limits (---).

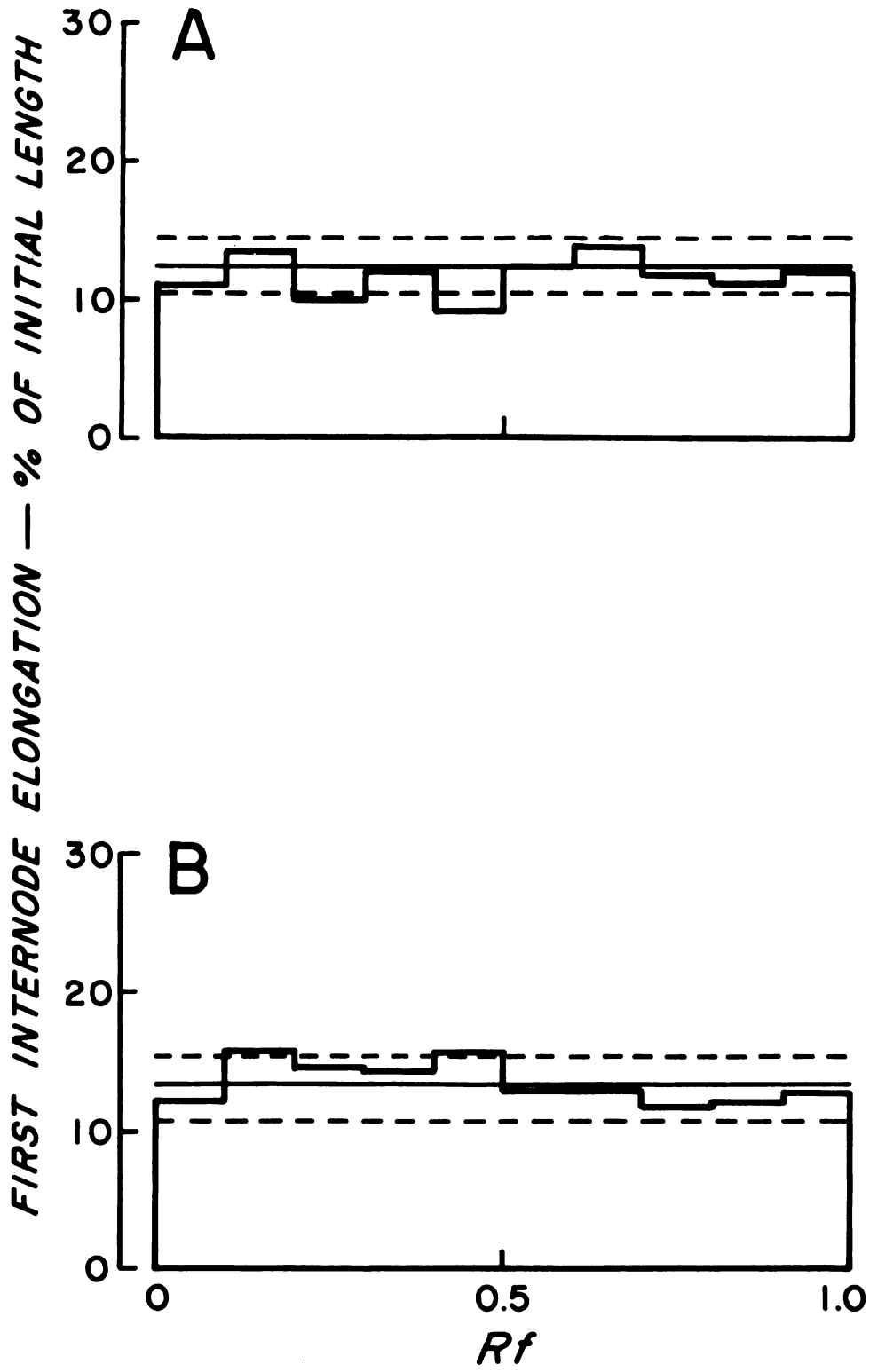

A
B
30
20
10
0
0
0.5
1.0
Rf
FIRST INTERNODE ELONGATION — % OF INITIAL LENGTH

Figure 3.--Histograms depicting the Avena first internode bioassay response to hexane and aqueous phases obtained from partition chromatography of seed and pericarp tissues (28 days after anthesis).

A. 0.3 gram equivalents of hexane phase developed in isopropanol : water (4:1).

B. 0.3 gram equivalents of acidic aqueous phase developed in isopropanol : water (4:1).

Control values (——).
Upper and lower 5% fiducial limits (---).

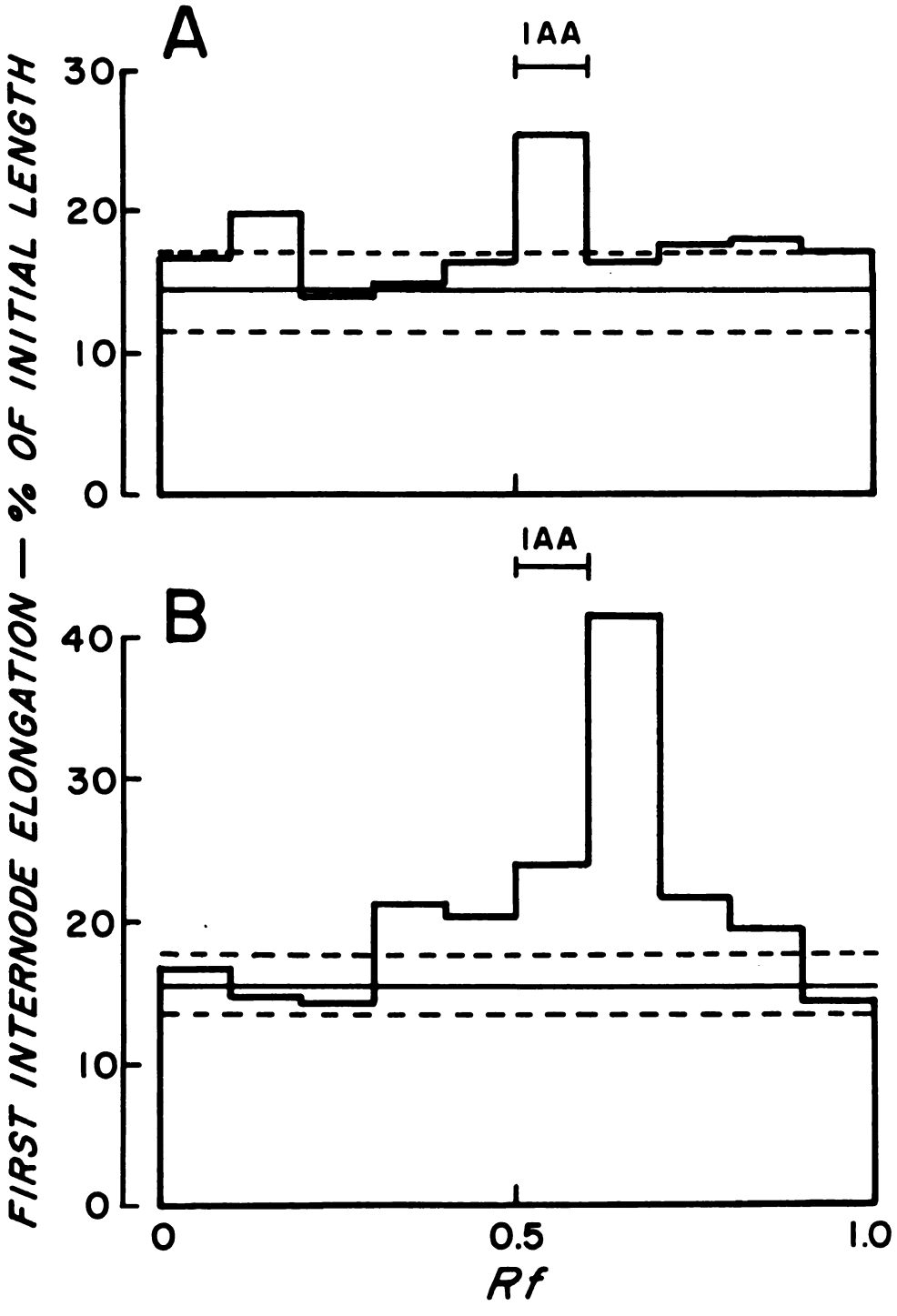

A
IAA
30
20
10
0
B
IAA
40
30
20
10
0
0
0.5
1.0
Rf
FIRST INTERNODE ELONGATION — % OF INITIAL LENGTH

Figure 4.--Histograms depicting the Avena first internode bioassay response to seed neutral auxins (42 days after anthesis). Each auxin was previously fractionated by chromatography in hexane : water (upper phase).

A. Re-chromatography of 0.5 gram equivalents of neutral auxin A (Figure 16B, Rf 0.0 - 0.2) in hexane : water (upper phase).

B. Re-chromatography of 0.5 gram equivalents of neutral auxin B (Figure 16B, Rf 0.5 - 0.7) in hexane : water (upper phase).

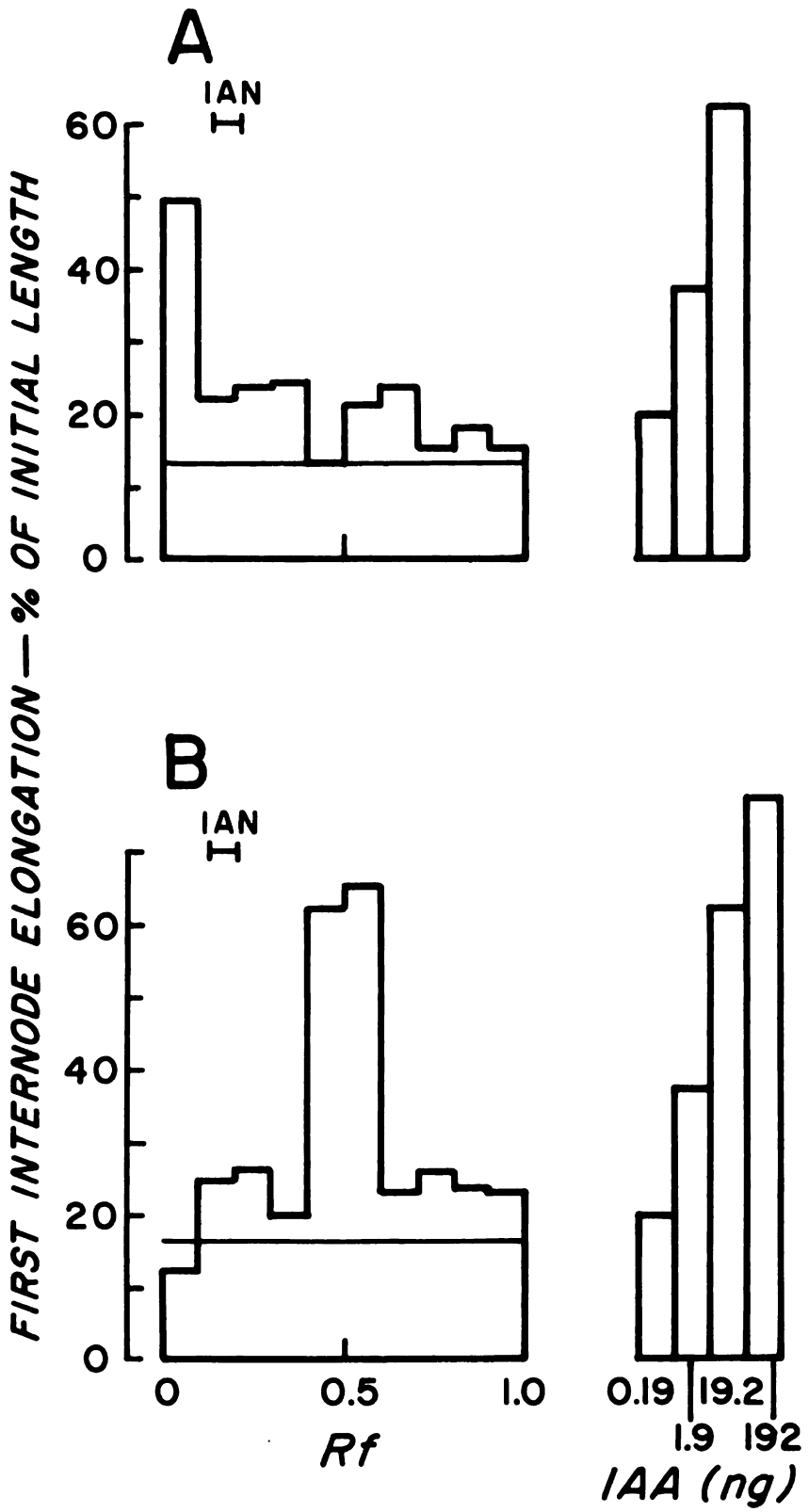

A
IAN
60
40
20
0
B
IAN
60
40
20
0
0
0.5
1.0
Rf
0.19
19.2
1.9
192
IAA (ng)
FIRST INTERNODE ELONGATION — % OF INITIAL LENGTH

Figure 5.--Factorial combination of 5 levels of neutral auxin IIA (0, 0.05, 0.1, 0.25, 0.5 gram equivalents) with 5 levels of neutral auxin IIB (0, 0.05, 0.1, 0.25, 0.5 gram equivalents) as determined by the Avena first internode bioassay. Growth responses are computed as a percentage of initial section length. Each value is the mean of 3 replicates.

Control value (---).

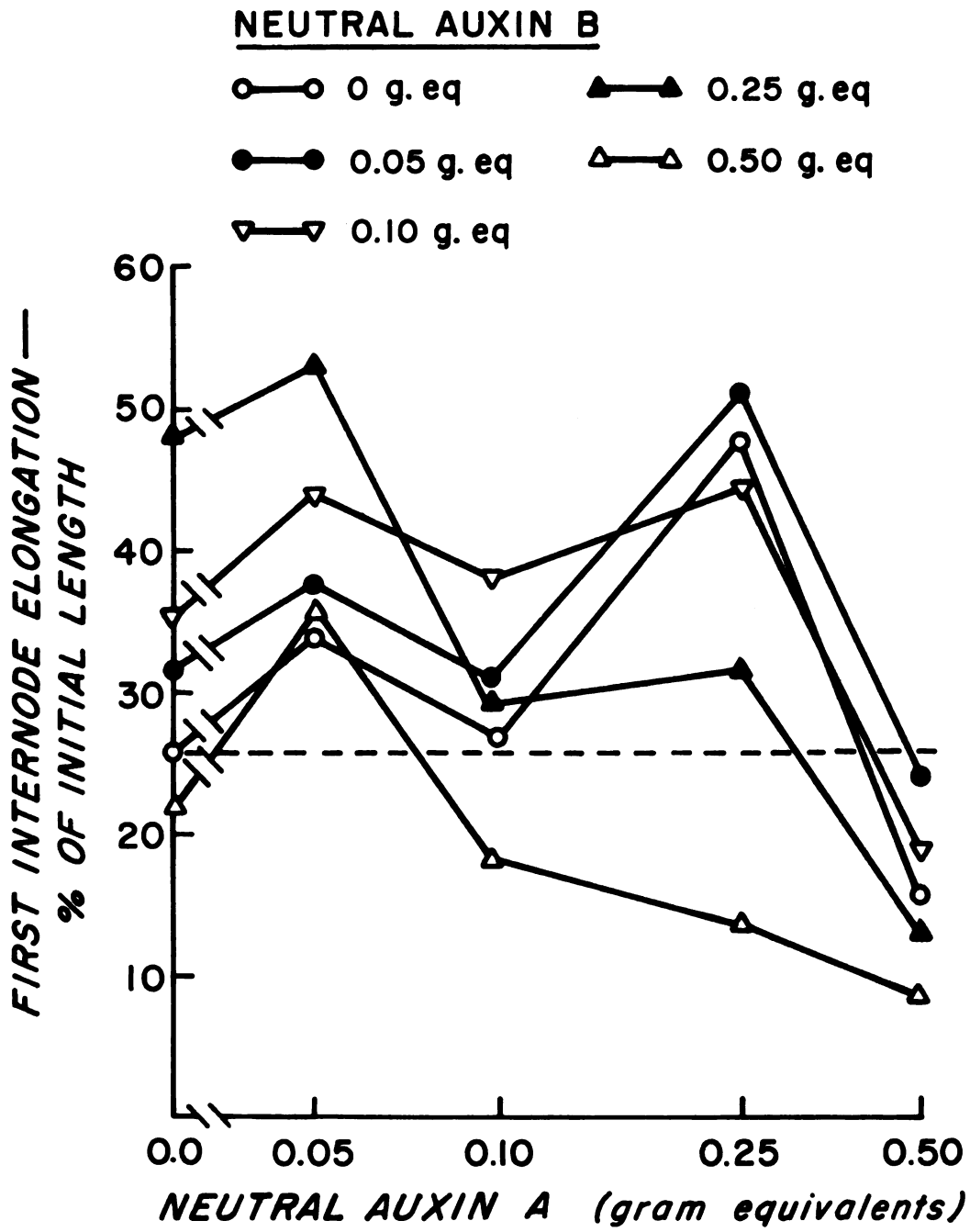
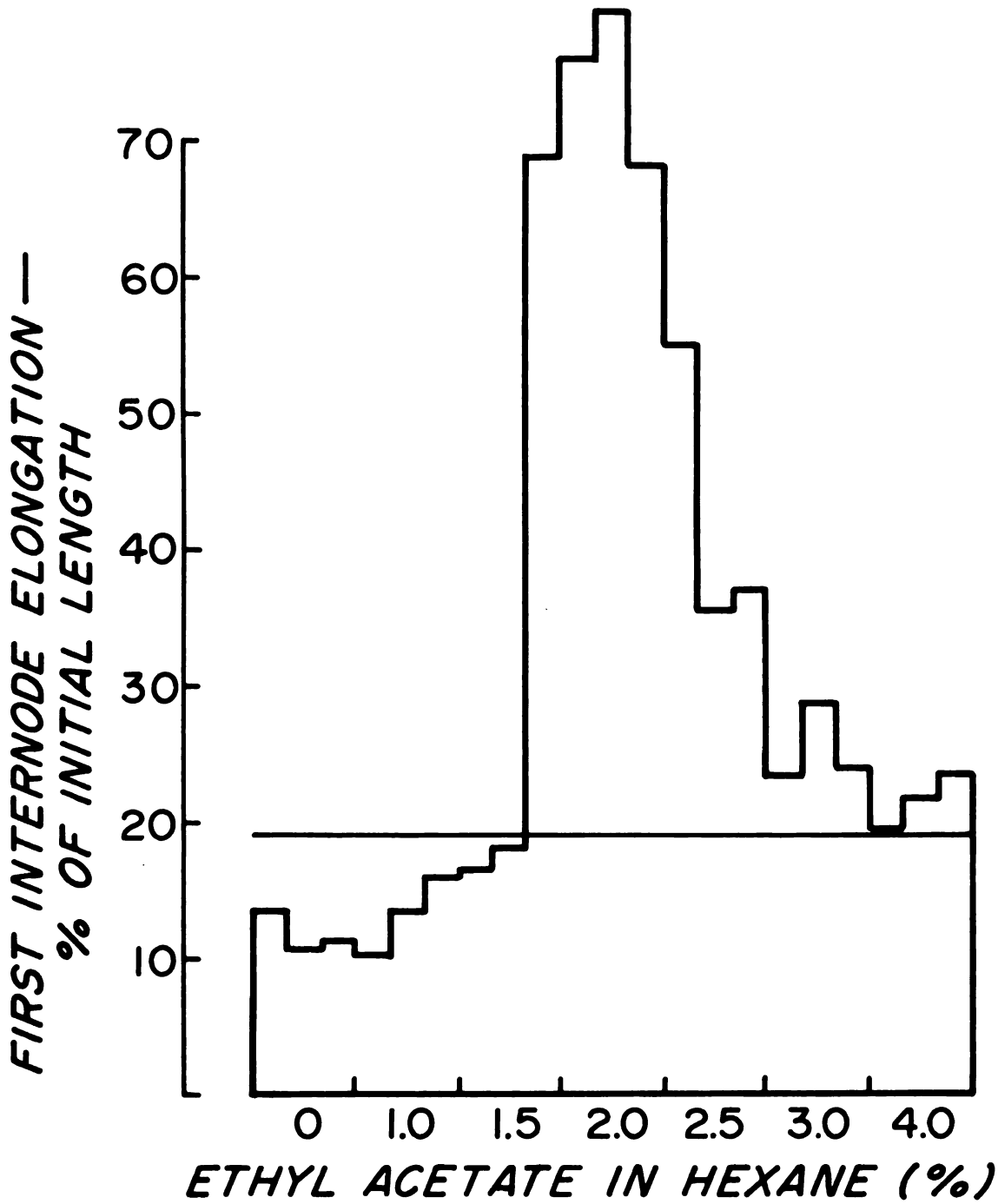

NEUTRAL AUXIN B
0 g. eq
0.05 g. eq
0.10 g. eq
0.25 g. eq
0.50 g. eq
FIRST INTERNODE ELONGATION —
% OF INITIAL LENGTH
60
50
40
30
20
10
0.0
0.05
0.10
0.25
0.50
NEUTRAL AUXIN A (gram equivalents)

Figure 6.--Histogram depicting the Avena first internode bioassay response to the seed neutral ether phase fractionated by silicic acid column chromatography.

Control value (——).


FIRST INTERNODE ELONGATION —
% OF INITIAL LENGTH
70
60
50
40
30
20
10
0
1.0
1.5
2.0
2.5
3.0
4.0
ETHYL ACETATE IN HEXANE (%)

APPENDIX A7.--Analysis of variance of factorial combination of neutral auxin IIA with neutral auxin IIB.

Analysis of Variance

Blocks		2	0.448	0.224	5.19
Treatments		24	16.952		
Source		d.f.	S.S.		F.
A	a_1	1	0.950	0.950	22.05**
	a_2	1	3.455	3.455	80.17**
	a_3	1	0.233	0.233	5.14*
	a_4	1	0.090	0.090	2.10N.S.
B	b_1	1	2.895	2.895	67.17**
	b_2	1	2.440	2.440	56.63**
	b_3	1	0.225	0.225	5.23*
	b_4	1	2.779	2.779	64.48**
AxB	a_1b_1	1	0.576	0.576	13.37**
	a_1b_2	1	0.174	0.174	4.04†
	a_1b_3	1	1.449	1.449	33.52**
	a_1b_4	1	0.145	0.145	3.36†
	a_2b_1	1	0.069	0.069	1.69N.S.
	a_2b_2	1	0.092	0.092	2.13N.S.
	a_2b_3	1	0.137	0.137	3.19†
	a_2b_4	1	<0.001	<0.001	<1.0 N.S.
	a_3b_1	1	0.834	0.834	19.35**
	a_3b_2	1	0.003	0.003	<1.0 N.S.
	a_3b_3	1	0.015	0.015	<1.0 N.S.
	a_3b_4	1	0.010	0.010	<1.0 N.S.
	a_4b_1	1	0.099	0.099	2.30N.S.
	a_4b_2	1	0.146	0.146	3.39†
	a_4b_3	1	<0.001	<0.001	<1.0 N.S.
	a_4b_4	1	0.186	0.186	4.32*
Error		48	2.070	0.043	
Total		74	19.471		

†P = 0.10

APPENDIX A8.--Retention times of components of the neutral auxin IIB complex fractionated by gas-liquid chromatography on different column packings.

	Retention Time (min.)			
	Column Packing			
	3% SE-30			3% DC-200
	Operating Temperature (°C)			
Peak Analyzed	120[1]	120[2]	130[2]	120[1]
1	3.10	3.4	2.3	3.35
2	4.50	5.9[3]	3.9[3]	4.85
3	6.10	7.2	4.3	6.65
4	8.50	8.6	5.2	8.80
5	9.45		10.5	9.15
6	9.95	17.7[3]	10.5[3]	10.87
7		20.4[3]	11.9	
TFA-Me-IAA	9.70			10.70

[1]Packard 7300.

[2]LBK 9000.

[3]Analyzed by gas-liquid chromatography-mass spectrometry.

APPENDIX B

Figure 1.--The response of radish cotyledons to known concentrations of kinetin. Values plotted are averages of 9 bioassays.

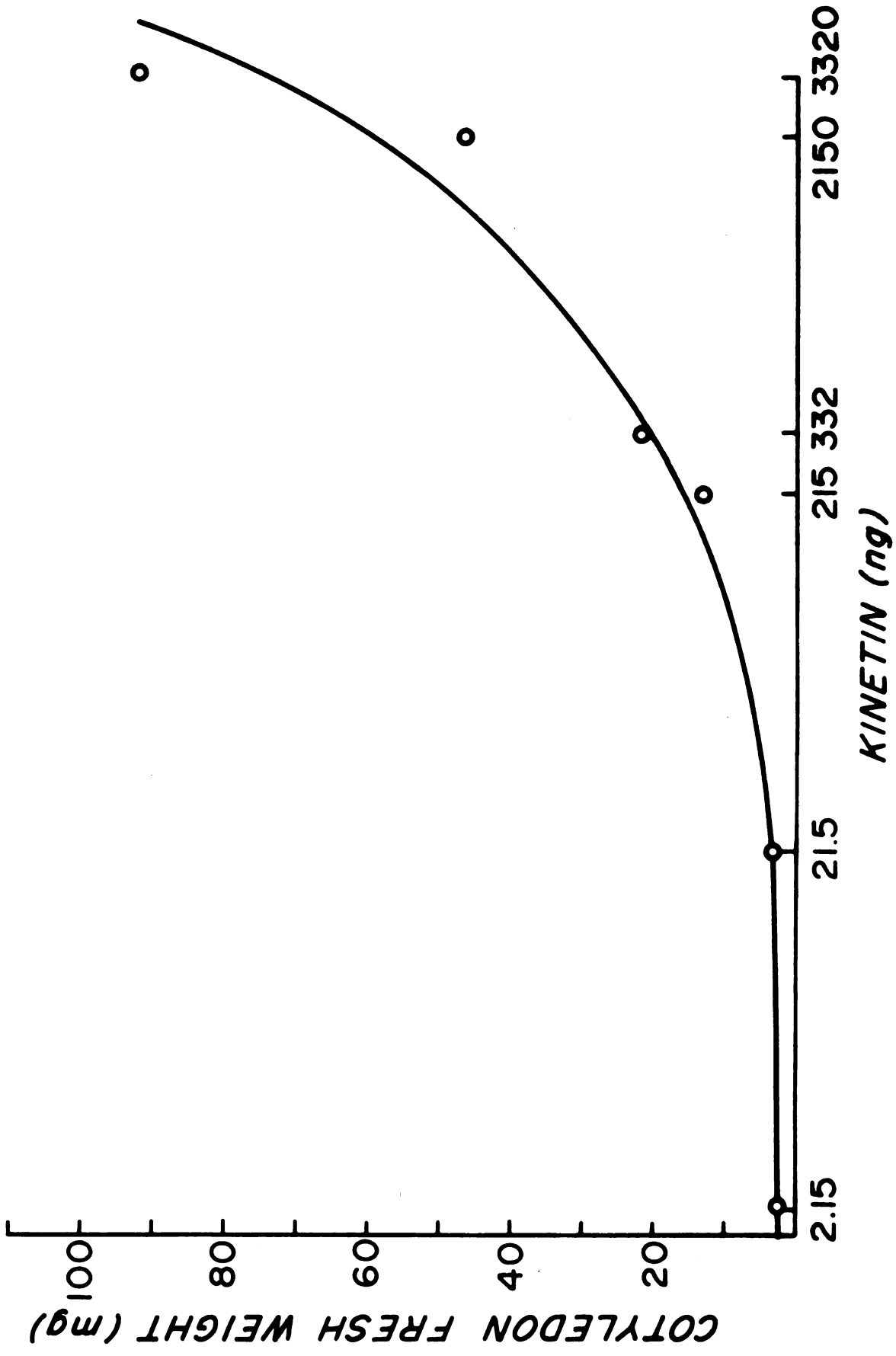

COTYLEDON FRESH WEIGHT (mg)
100
80
60
40
20
2.15
21.5
215
332
2150
3320
KINETIN (ng)

Figure 2.--Factorial combinations of 4 levels of kinetin (0, 21.5, 215, 2150 ng/Petri dish) with 4 levels of gibberellic acid (0, 3.46, 34.6, 346 ng/Petri dish) bioassayed by the radish cotyledon test.

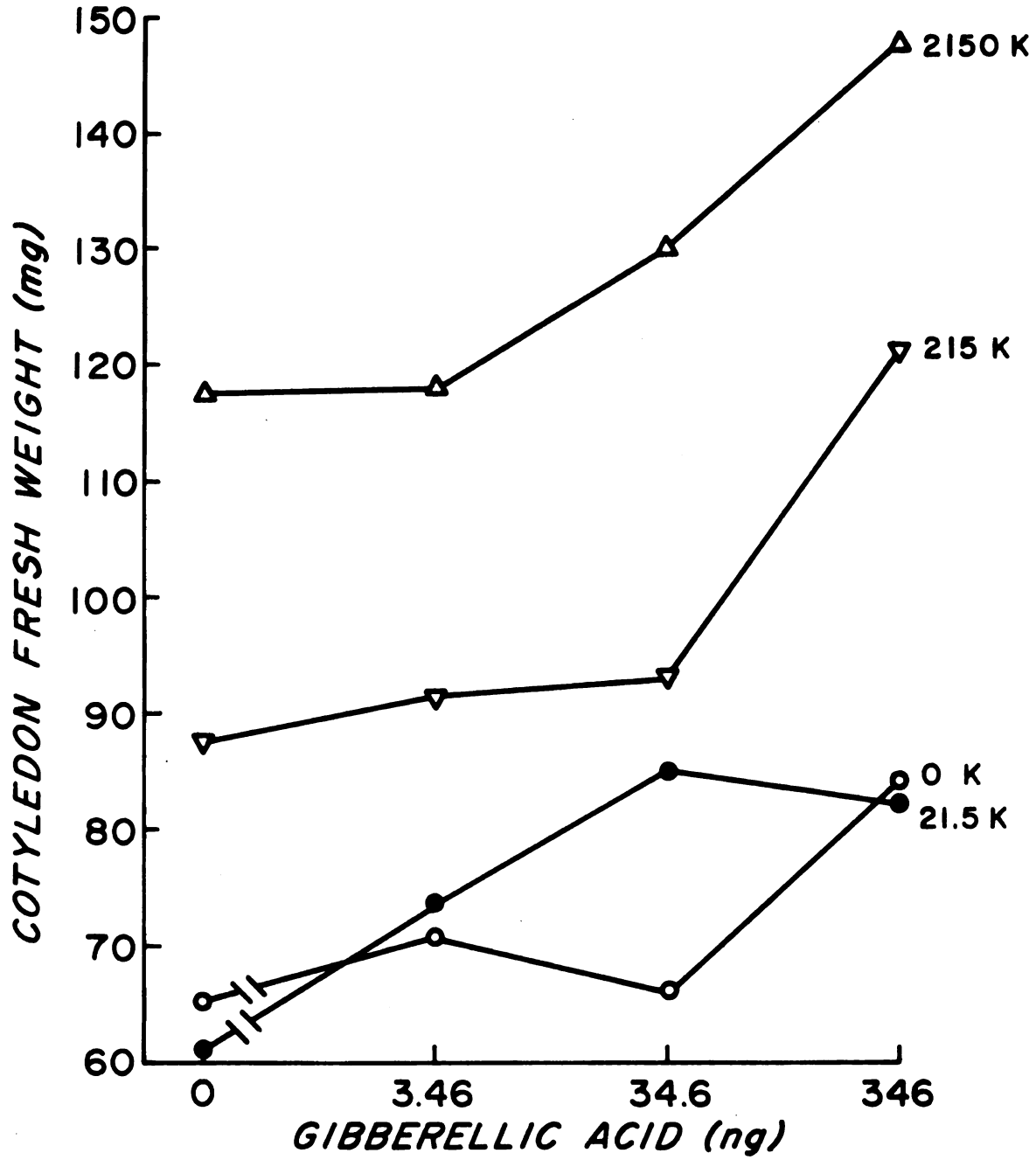

150
140
130
120
110
100
90
80
70
60
COTYLEDON FRESH WEIGHT (mg)
2150 K
215 K
0 K
21.5 K
0
3.46
34.6
346
GIBBERELLIC ACID (ng)

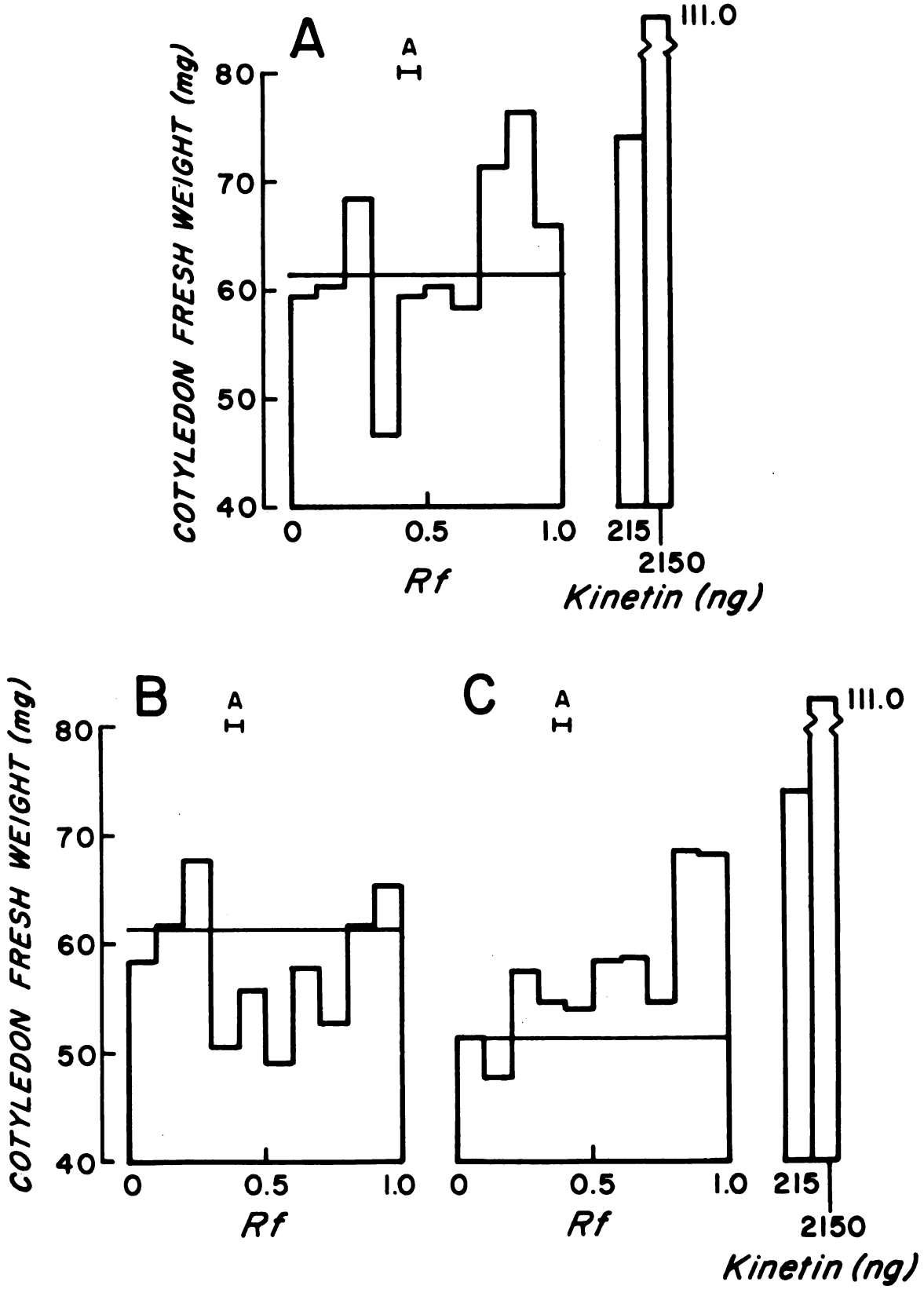
Figure 3.--Histograms depicting the radish cotyledon bioassay response to the growth promoters in the aqueous and butanol phases (21 days after anthesis).

A. Re-chromatography of 0.08 gram equivalents of Aqueous II (Figure 34A, Rf 0.5 - 0.8). Solvent system:- water (pH 6.0).

B. Re-chromatography of 0.17 gram equivalents of Butanol I (Figure 35A, Rf 0.0 - 0.2). Solvent system:- 0.03M boric acid adjusted to pH 8.4 with NaOH.

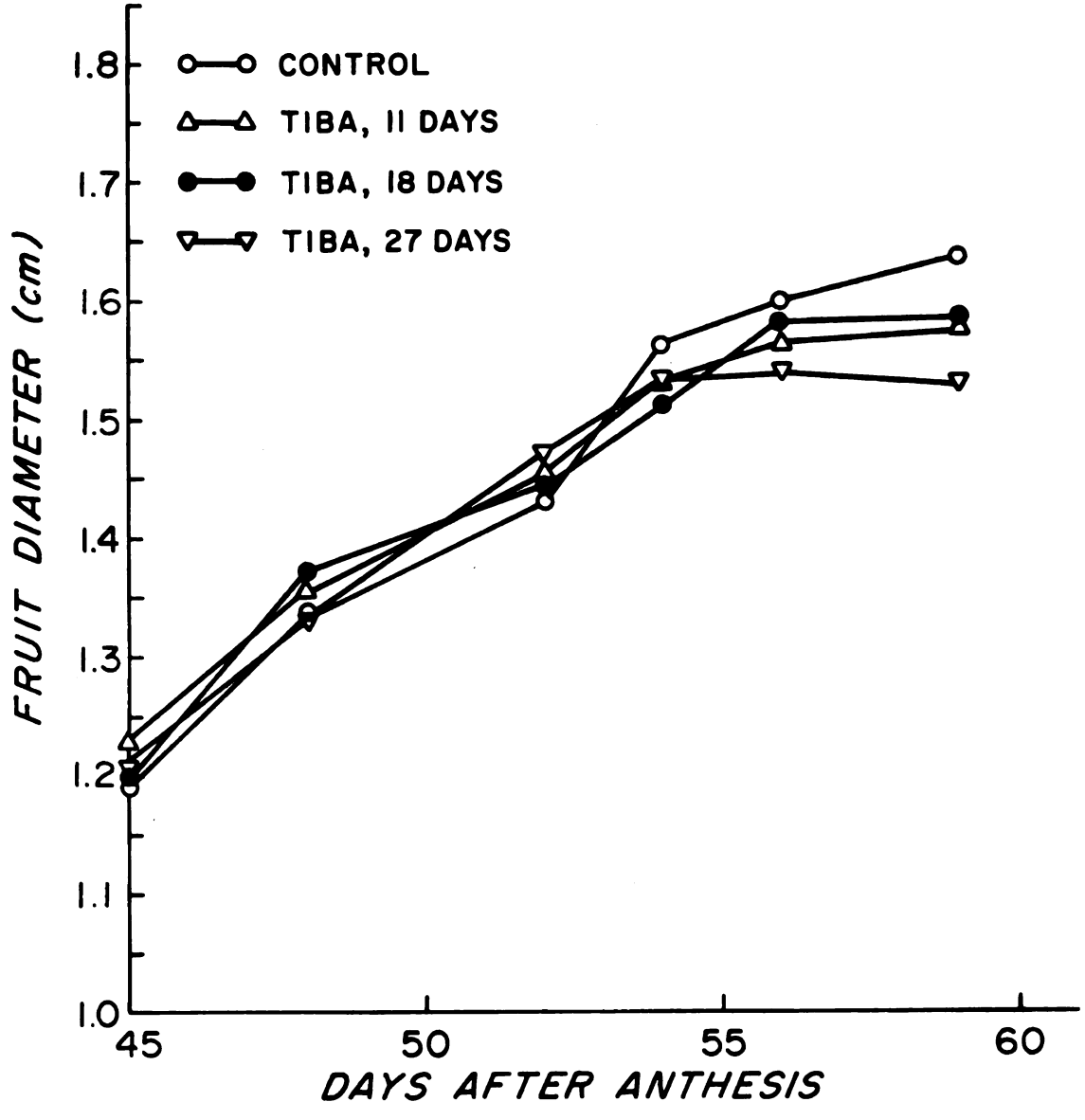
C. Re-chromatography of 0.17 gram equivalents of Butanol II (Figure 35A, Rf 0.4 - 0.6). Solvent system:- 0.03M boric acid adjusted to pH 8.4 with NaOH.

Control value (——).
A - Rf of adenine.


A
B
C
A
H
COTYLEDON FRESH WEIGHT (mg)
80
70
60
50
40
0
0.5
1.0
Rf
111.0
215
2150
Kinetin (ng)

APPENDIX C

Figure 1.--The effect of triiodobenzoic acid (TIBA) application (1% in lanolin) to sour cherry fruit pedicels 7 days before the onset of fruit growth stage III and maintained for 4 days after (Δ——Δ); 11 days after (●——●); and 15 days after (▽——▽) growth stage III had commenced. Fruit diameter (at right angles to the suture) of 10 randomly selected fruits from each treatment was measured at intervals during growth stage III and the mean diameter of each treatment expressed as a function of time after anthesis.


CONTROL
TIBA, 11 DAYS
TIBA, 18 DAYS
TIBA, 27 DAYS
FRUIT DIAMETER (cm)
1.8
1.7
1.6
1.5
1.4
1.3
1.2
1.1
1.0
45
50
55
60
DAYS AFTER ANTHESIS