THE BIOCHEMISTRY AND PHYSIOLOGICAL IMPLICATIONS OF INDOLE-3-ACETIC ACID IN THE DEVELOPING PEACH (PRUNUS PERSICA) SEED

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
Roger William Ritzert
1966

This is to certify that the

thesis entitled

THE BIOCHEMISTRY AND PHYSIOLOGICAL IMPLICATIONS OF INDOLE-3-ACETIC ACID IN THE DEVELOPING PEACH (PRUNUS PERSICA) SEED

presented by

Roger William Ritzert

has been accepted towards fulfillment of the requirements for

Ph.D. **degree in** Biochemistry

Harold M. Sell
Major professor

Date Feb 23, 1966

ABSTRACT

THE BIOCHEMISTRY AND PHYSIOLOGICAL IMPLICATIONS OF INDOLE-3-ACETIC ACID IN THE DEVELOPING PEACH (PRUNUS PERSICA) SEED

by Roger William Ritzert

Various phases of fruit growth and development have been suggested to be under the control of endogenous hormones. A previously unidentified auxin has been shown to be present in highest concentration during cytokinesis of the endosperm cells in immature peach (Prunus persica) seeds and thus has been implicated as being functional during this stage of development. The enhanced effect by naphthaleneacetic acid as a thinning agent during cytokinesis furnished further evidence that there was an increase in the endogenous auxin supply.

Previous work has suggested, but not confirmed, that indole-3-acetic acid is the auxin which may be implicated in cytokinesis. A mechanism for the control of its relative content was suggested from in vivo studies which showed a decrease in the ability of the immature peach seeds to decarboxylate indole-3-acetic acid during cytokinesis. Past knowledge of the indole-3-acetic acid oxidase system has shown that orthopolyphenolic compounds, which occur widely in plants, are inhibitors of the oxidative process. Regulation of possible indole-3-acetic acid destruction by inhibition of the enzyme was suggestive, but neither the enzyme nor an inhibitor had been previously demonstrated in homogenates of the immature seed tissue.

Roger William Ritzert

Indole-3-acetic acid was identified as the acidic auxin in the immature seeds of Redhaven peach fruit whose increase in concentration correlated with the time of cytokinesis of the seed endosperm. Identification of the auxin was made on the basis of its biological activity, mobility on paper and thin-layer chromatograms, and ultraviolet absorption spectra. An additional acidic auxin, not previously described as being present in the peach, was detected in the immature seed extracts. The compound had a R_f 0.80-1.00 in butanol-ammonia water (100:3:18) and displayed biological responses typical of auxins and colorimetric reactions typical of indoles. Evidence was also obtained for the presence of a neutral auxin.

An indole-3-acetic acid oxidase system was detected and characterized in the soluble fraction of peach seed extracts. Enzyme activity could be detected only after treatment of the extract with Tween 80 to disrupt possible protein-tannin complexes, and separation of the protein fraction by passage through Sephadex G-25. The enzyme had a pH optimum of 4.5 to 5.0 and required both Mn++ and 2,4-dichlorophenol for optimum activity. The product of the reaction was identified from the absorption spectrum of the reaction mixture as 3-methyleneoxindole. A polyphenol, catechin, was identified by chromatography in seed extracts. An authentic sample of catechin was shown to be a non-competitive inhibitor of the peach oxidase enzyme system.

The inhibitory action of catechin on indole-3-acetic acid oxidase is considered as a possible mechanism by which the concentration levels of indole-3-acetic acid may be adjusted during fruit

maturation. The possible complex nature of the unidentifed acidic auxin is also discussed.

THE BIOCHEMISTRY AND PHYSIOLOGICAL IMPLICATIONS OF INDOLE-3-ACETIC ACID IN THE DEVELOPING PEACH (*PRUNUS PERSICA*) SEED

By

Roger William Ritzert

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Biochemistry

1966

ACKNOWLEDGEMENTS

The encouragement and guidance of Dr. H. M. Sell throughout the author's graduate program is gratefully acknowledged. Words of appreciation are extended Dr. M. J. Bukovac for many stimulating discussions and use of laboratory facilities for portions of the study. The support and interest of the author's parents throughout his educational experience will always be remembered. The love, encouragement, and often sacrifice of the author's wife and children during the doctoral program has helped to make this a most rewarding experience.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION

INTRODUCTION

In addition to the many hormonal functions attributed to auxins in controlling plant growth, hormones are known to be functional in various phases of fruit growth and development. Auxins have been implicated in cytokinesis in immature peach seeds and generally occur in highest content during cytokinesis. Further, naphthaleneacetic acid is most effective in inducing fruit abscission during this critical time. No definite determination of the auxin which may be involved in this process has been reported (48, 52).

Although indole-3-acetic acid is generally implicated as the principal plant auxin, there are numerous reports that derivatives of indoleacetic acid are present in some plant tissues. Recent evidence has also indicated the occurrence of a non-indolic auxin in several plant species, including peach fruit. Thus, it was of interest to establish if there was a relationship between endogenous auxin and cytokinesis and to identify the auxin involved.

Assuming that indole-3-acetic acid is the auxin in question, a mechanism for the control of its relative content was suggested when *in vivo* studies showed a decrease in the ability of the immature peach seeds to decarboxylate indole-3-acetic acid during cytokinesis. Thus, a control of the rate of breakdown, not synthesis of the auxin, was suggested. The control mechanism could be manifested through regulation of indole-3-acetic acid oxidase enzyme synthesis or through control of enzyme activity by an inhibitor.

Inhibition of the enzyme is accomplished by orthopolyphenolic compounds, which are widely distributed in higher plants.

Two objectives were established, namely: To define the nature of the auxin or auxins present in the developing seed, and to describe biochemical mechanisms which may be responsible for regulating the levels of auxin present.

LITERATURE REVIEW

LITERATURE REVIEW

Role of Auxins in Fruit Development

Auxins are defined as "compounds characterized by their capacity to induce elongation in shoot cells" (93). There is much evidence for the participation of natural auxins in fruit development. The historical development of these concepts will be briefly outlined.

Nitsch describes a fruit as consisting "of the tissues which support the ovules and whose development is dependent upon the events occurring in these ovules" (64). The growth of the fruit can be summarized as two main growing periods: that portion before anthesis during which growth proceeds mostly by cell division, and a post-fertilization phase which is characterized essentially by cell enlargement. Post-fertilization, to which further discussion will be directed, is composed of three stages of growth as described by Connors (6) for the peach (Prunus persica). Stage I is characterized by rapid growth of all parts of the ovary and its contents, except for the endosperm and embryo. Stage II displays very little growth of the ovary, lignification of the endocarp, and rapid growth of the endosperm and embryo. Fruit maturity is achieved following rapid growth of the mesocarp during Stage III.

Since the increase in volume during the growth of fruits is mainly by cell enlargement and auxin is known to act by controlling cell enlargement in other tissues, it has often been assumed that such was the controlling factor in fruit growth (8). There appears

to be a correlation between seed development and distribution, and the ultimate size and shape of the fruit. Tukey (92), studying several species of Prunus including the peach, noted a relation between point of attachment of the ovule and development and size of the fruit. This would suggest the involvement of a stimulus passing from the developing ovule through the vascular system into the adjacent tissue. Additional indications that the seed is the source of auxin was presented by Nitsch (63) in studies with the strawberry. The weight of the fleshy receptacle was approximately proportional to the number of achenes (fertilized ovaries) contained thereon. Removal of the achenes from some areas resulted in failure of the receptable tissue to grow. The achenes were found to contain free auxin, while the receptacle tissue contained none. Gustafson (28) had dimilarly shown that the auxin content of ovules and developing seeds of the tomato fruit is much greater than that of other fruit parts. This led to his early suggestion that the seeds produce auxin which influence the growth of the surrounding tissue.

Further evidence for the involvement of auxins in fruit growth can be found in several instances where relationships between auxin content and growth of the fruit are reported. Relations of this type are based on the auxin content of extracts as measured by one of the common auxin assays, straight-growth or curvature. Luckwill (54, 55) demonstrated that the appearance of a hormone in the apple seed coincided with development of the endosperm. He further showed that natural fruit abscission could be correlated to low hormone content and that this occurred after developmental

stages which would require hormone. Wright (94) was able to show that high levels of an auxin in the currant coincided with cell division. Fluctuations in total auxin content were observed by Nitsch, et al. (70) in seeded grapes and were correlated with meristematic activity of the seed. In the seedless grape, there was a correlation with cell division. In studies on parthenocarpic and non-parthenocarpic figs, Crane et al. (9) did not observe a correlation between free auxin in the fruit and growth rate. A requirement for free auxin in leaves for cell division has been substantiated (34).

Stahly and Thompson (87), using ovules of the developing Halehaven peach, reported the existence of three auxins, each with its own pattern of fluctuation. During the periods when the two major auxins were at their highest levels, the fruit were in a period of suspended growth. These authors suggested that the high auxin content was acting to inhibit cell enlargement, and thus growth at this stage. Crane (8) has pointed out that this could not be true since parthenocarpic fruit exhibited the same growth pattern. Powell and Pratt (77) showed two distinct periods of high hormonal concentration during the maturation of three peach varieties. The first wave of auxin was correlated with the time of cytokinesis in the seed. Lombard and Mitchell (52) noted increases in levels of both an acidic and a neutral auxin 43 days after bloom in seeds of the Redhaven peach. According to extensive data compiled by Leuty (48), this period is most likely the time of cytokinesis. He also showed that the pericarp diameter measures 30 mm at this stage and that maximum fruit abscission was induced by exogenous auxin (NAA)

applied at this time. These above results point to the importance of some auxin or auxins during cytokinesis.

There is considerable evidence that indole-3-acetic acid (IAA) is the principal auxin in higher plants. Two excellent reviews by Bentley (1, 2) list numerous species and the nature of the auxins which have been detected in them. The auxins present in some fruit tissues will be discussed further because of their pertinence to the subject. A notable flaw in relating these auxins to fruit development is the failure by most workers to utilize bioassays which are as close as possible to the type of growth being studied (45). In most instances, straight-growth assays of paper chromatogram sections accompanied by chromatographic characteristics have been used for identification of the auxins, usually indolic in nature.

Luckwill (56) identified three acidic auxins in the leaves and fruits of the apple, but none of the auxins appeared to be the same as the common indole compounds, particularly IAA. The absence of IAA and the presence of a non-indole auxin was shown by Luckwill and Powell (57). Other reports (14, 15, 36, 80, 90) indicate that IAA and several of its derivatives are present in some varieties of apple. The ethyl ester of IAA (EIA) was extracted by Teubner (90) from apple endosperm using ethanol, so the compound might have been IAA. There is also evidence that IAA is present in the currant (94) and grape (70). Stahly and Thompson (87) showed the apparent presence of IAA and two other auxins which are probably indolic. It should be noted that their detection by chromogenic reagents was

transient, but this may very well be due to low levels of the compound.

Khalifah, et al. (41) have recently reported the presence of a non-indolic auxin in young citrus fruit whose presence can be correlated with the development of the orange (40). These workers have also noted the presence of "citrus auxin" in the young fruit of the date, fig, plum, and peach (49). The physiological role of this auxin, especially in tissues where IAA has been detected is not immediately clear.

Other Growth Regulators in Fruit Development

In addition to auxins, there is evidence that both kinins and gibberellins are involved in regulation of plant growth. The general aspects of these two substances will be reviewed only as they relate to fruit growth and development. Thorough reviews on gibberellins (74) and kinins (58) have appeared recently.

Gibberellins are known for their ability to stimulate cell division and cell elongation. There is some evidence to implicate a requirement for gibberellins in fruit growth. One line of evidence is the ability of gibberellic acid (GA_3), in particular to set parthenocarpic fruits (8), including the peach (10). Gibberellins A_3 and A_4 have been shown by Bukovac (4) to set parthenocarpic fruit of the apple. Phinney et al. (75) have indicated that young seeds, including almond, apricot, and plum seeds, are rich sources of gibberellin-like activity. Apple endosperm has been shown by Nitsch (65) to contain gibberellin-like substances. Three gibberellin-like

substances, two of which appear to be A_1 and A_9, have recently been detected in citrus fruits (40). Although increased levels of gibberellin-like substances seem to accompany growth of the seed, no relationship has yet been established between fruit growth and levels of these substances as there exists for auxins (8).

These findings, when considered with the knowledge we have of auxins in fruit growth, led Bukovac (4) to suggest that no single stimulus can be considered by itself in relation to fruit growth and that several factors alone or interacting might be responsible.

Kuraishi and Muir (42, 43) have shown that treatment with gibberellic acid causes an increase in diffusible auxin from stem apices. Other work demonstrates that gibberellin treated tomato ovaries produce the same quantity of diffusible auxin as pollinated ovaries (86). The fact that both auxins and gibberellins are probably in the seeds of most fruits may suggest an interrelationship of the two, with gibberellin perhaps being the controlling factor of auxin synthesis. Evidence for this control system has been suggested by Sastry and Muir (85), who found that gibberellic acid stimulated the conversion of tryptophan and other IAA precursors to IAA in apical Avena segments.

The work with kinetin, a synthetic kinin, exhibited that kinin-induced cell division takes place only in the presence of IAA (58). Very little is known about the natural kinins in fruit tissue except from the scattered reports of kinin-like activity in extracts of these tissue. The first observation of a kinin in young apple fruits (later demonstrated to be similar to that found in coconut

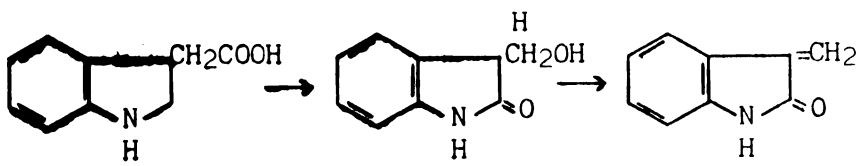
milk [98]), was made by Goldacre and Bottomley (22). Likewise, plum fruitlets (46, 47) yielded a kinin whose properties appear to be similar to an unknown purine crystallized from an extract of sweet corn (46). Lavee (44) noted kinin-like activity in extracts of peach fruitlets. Kinins were found by Powell and Pratt (73) in extracts of the embryo and endosperm of the immature peach seed. The kinin activity which requires IAA, may be more abundant in the endosperm than in the embryo suggesting a possible interaction in cytokinesis. Nitsch and Nitsch (68) have also shown kinin-like activity in tomato fruit.

Most interesting results have been obtained recently by Crane and van Overbeek (11) in inducing the parthenocarpic growth of fig fruit with a synthetic kinin. A possible interrelationship of auxins, gibberellins, and kinins gains momentum from this experiment since identical parthenocarpic fruit can be set with either auxin or gibberellin. As stated earlier, in all cases reported, IAA or natural auxin are necessary for kinin-induced cell division.

Indole-3-Acetic Acid Oxidase

The number of reports concerning the systems for destroying IAA in plant tissue are voluminous. Reviews by Ray (81) and Hare (29) will serve as a broad background on this subject for those unfamiliar. It will suffice to say that IAA can be rapidly destroyed by an oxidase-peroxidase system extracted from various tissue. The course of disappearance of IAA may be followed manometrically (O_2 or CO_2), colormetrically (84), or spectrophotometrically (82). The enzyme has

been suggested by Ray (83) to be the same as the peroxidase of Omphalia flavida. Horseradish peroxidase will also carry out the destruction of IAA (32, 33). It has recently been shown that the horseradish peroxidase system oxidizes IAA (I) to oxindole-3-carbinol (II) and then to 3-methyleneoxindole (III). The same products were detected from a riboflavin-catalyzed photooxidation of IAA (18). 3-Methyleneoxindole is known to be a potent growth inhibitor of higher plants and microorganisms (18, 88). Inhibition of growth may be due to the binding of 3-methyleneoxindole to the sulfhydryl groups of enzymes (88).



I II III

Monophenols are known to be catalytic to the destruction process (29, 81), with 2,4-dichlorophenol commonly being used to assay the enzyme (23). The metallic requirement is satisfied by manganous ion (31). Several naturally occurring monophenols have been shown to serve as cofactors in both in vitro and in vivo (96) systems.

Most polyphenols with adjacent phenolic groups are inhibitors of the IAA oxidase system (29, 81). Naturally occurring inhibitors have been isolated from tissues known to possess IAA oxidase systems. Ferulic acid was shown by Gortner and Kent (26) to be a naturally occurring inhibitor of the pineapple oxidase system. An inhibitor of

the oxidase in light-grown cotton has been demonstrated, but not characterized (59, 60). Mumford et al. (61) have isolated a kaemferol derivative from the pea plant which acts as an inhibitor of the oxidase. This inhibitor, however, does not have the expected adjacent phenolic groups. A synergistic effect by chlorogenic acid, an inhibitor of the oxidase, was noted on IAA induced curvature in Avena coleoptiles (91). The same was not true for indole-3-butyric acid and naphthaleneacetic acid. Momophenols (cofactors for IAA oxidase) had the reverse effect.

Galston (19) has related inhibitor levels to the photomorphogenesis of the pea plant. Thus, a direct relationship between inhibitor levels and quality and quantity of light is suggested. In addition, there appears to be an effect of light quality on free IAA content in bean plants (17). Inhibitor levels may be controlled by light, thus affecting IAA content and ultimately growth. Donoho et al. (13) have detected IAA oxidase activity in Redhaven peach seeds using in vivo techniques. The decarboxylation of carboxyl labeled IAA was used as a measure of activity. The oxidase activity at the time of cytokinesis was lower than other periods suggesting the possibility of a control mechanism for IAA. Kefford et al. (35) have suggested that IAA oxidation may play a role in IAA growth regulating functions.

Biochemical Aspects of Auxin Action

Our present level of knowledge in the area of molecular biology has opened the road for severalllaboratories to apply the methodology to the biochemical aspects of auxin-induced growth.

Evidence that auxin-induced cell enlargement is accompanied by protein synthesis was reported by Nooden and Thimann (71, 72) and confirmed by Key (37). The uptake of glycine, presumably into protein, is enhanced by IAA in an *in vitro* system (16).

The synthesis of DNA-like RNA, probably messenger RNA, is necessary for the growth of excised plant tissue (20, 37, 38). Further, the synthesis of RNA is enhanced by auxin (39). These results suggest that perhaps the auxin induced response controls synthesis of the message for protein synthesis. Whether this is a specific protein or not is not known. Some possible significance may be found in the observation that IAA can be bound to the RNA fraction, but only in the presence of peroxidase (20, 35). IAA can be conjugated to a protein (95).

METHODS AND MATERIALS

METHODS AND MATERIALS

Identification of Auxins

Peach fruit (Prunus persica, cv. Redhaven) were collected at periodic intervals between fertilization and pit hardening. The seeds were removed from the fruit immediately after harvest and cropped into containers packed in dry ice. Additional fruits were placed in plastic bags and stored at -20°. The frozen seeds were lyophillized on a Virtis freeze-drier and then ground using a Virtis homogenizer. The ground seeds were stored at -10° in closed containers.

Extraction and purification

Preliminary experiments were designed for the purpose of detecting IAA in the seed extracts. Five grams of dried, ground seeds were covered with 125 ml of dry peroxide-free ethyl ester and kept at 4° for 1 hour. The mixture was filtered and the above extraction repeated twice. The combined filtrates were evaporated to dryness for further purification by one of the following methods.

The residue was taken up in 50 ml of ethanol and passed through a column of acid activated alumina (51) (0.9 cm x 15 cm) to adsorb the acidic components. The column was washed with 50 ml of ethanol and eluted with 25 ml of 0.1 N KOH. The basic solution was acidified with 1 N H_2SO_4 to pH 2.8 and the acids extracted from the aqueous solution with ether. The concentrated ether extract was used for identification by chromatographic means.

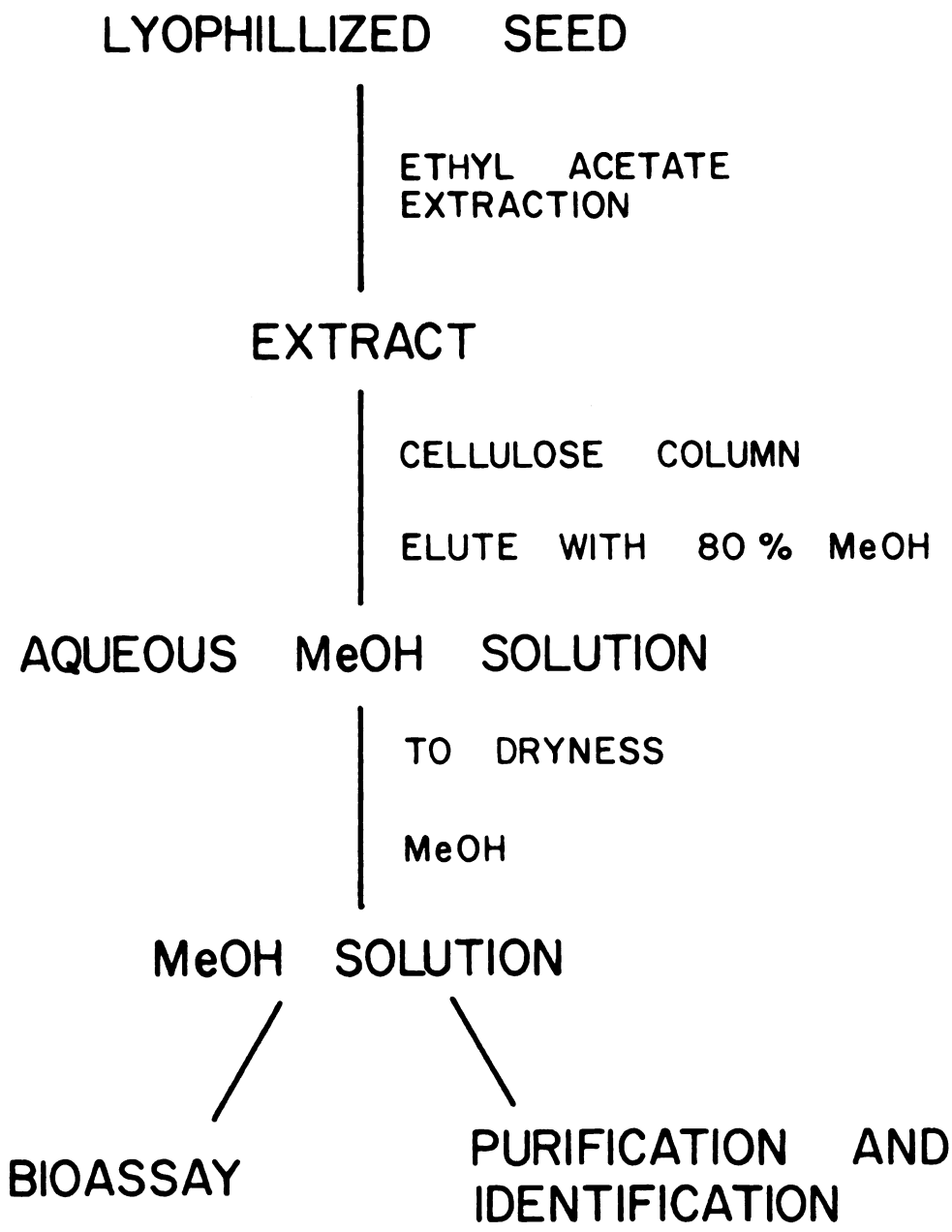
The other method used is essentially that described by Nitsch (66). The residue was taken up in 25 ml of dry acetonitrile and extracted with dry hexane until the hexane remained colorless. The acetonitrile fraction was taken to dryness and the residue dissolved in 25 ml of ether. The ether solution was extracted three times with 10 ml of 5 per cent $NaHCO_3$. The remaining ether fraction constitutes the non-acidic components. The $NaHCO_3$ solution was acidified to pH 2.8 with 6 N HCl. Extraction of the acidified solution with three portions of ether gave the acid fraction which was used for chromatography.

Both of the above clean-up procedures are designed to remove lipids which interfere with bioassays and consistent mobility of the auxins on paper. These were useful for detecting and identifying IAA in the extracts, but did not offer consistent quantitative results as measured by the straight-growth assay. A method which offered the following advantages was thus developed: (1) remove lipid material, (2) preserve extracted auxins unaltered, (3) give qualitative and quantitative reproducibility, and (4) speed. For these reasons, the procedures as outlined in figure 1 were utilized for the major portion of the experiments.

One or two grams of dried seeds were extracted with 25 ml of ethyl acetate at 4° with constant stirring for 20 hours. The mixture was filtered through a sintered glass filter and the residue extracted two more times for 1 hour. The combined extracts were reduced in volume to 3-4 ml and mixed with 0.5 g of powdered cellulose. The solvent was completely removed under a stream of N_2. The cellulose

Figure 1

Scheme used for extraction of auxins from peach seeds. Details are given in text.


LYOPHILLIZED SEED
ETHYL ACETATE EXTRACTION
EXTRACT
CELLULOSE COLUMN
ELUTE WITH 80 % MeOH
AQUEOUS MeOH SOLUTION
TO DRYNESS
MeOH
MeOH SOLUTION
BIOASSAY
PURIFICATION AND IDENTIFICATION

with sample was placed on a column of powdered cellulose (0.9 cm x 10 cm) and the column eluted with 50 ml of 80 per cent methanol. The total eluent was taken to dryness and the residue taken up in 1 ml of methanol. Portions of this solution were used for bioassay, and for purification and identification.

Chromatography

Paper chromatography was carried out on Whatman 1 mm paper (9-1/8 in. x 24 in.) using the Drummond paper chromatography apparatus. The paper was developed using the ascending technique after equilibration with the solvent. Auxins were detected using the Avena coleoptile straight-growth assay on sections of chromatograms on which extracts of 50 mg of seeds were separated. Separations using thin-layer chromatography were effected using 0.25 mm layers of silica gel G or H. The chambers were kept saturated prior to and during development of the layers using paper dipped in the solvent and adhering to the side of the chamber. The chromogenic reagents used on various occasions are listed in Table I. The DMAC reagent was used most frequently because of its stability and sensitivity. The Prochazka reagent was useful for detecting low levels of indoles on thin layers.

Preparative thin-layer chromatography

Portions of the stock solution of the extract representing about 0.5 g of dry seeds were spotted across a thin layer plate. The layer was developed with either chloroform-acetic acid (95:5 v/v) or isopropyl ether-acetic acid (95:5 v/v) using IAA as a marker. After

Table I. Chromogenic Reagents for Detection of Indoles

Reagent	Reference	Preparation	Comments
p-Dimethylamino-cinnamaldehyde (DMAC)	30	1 g p-dimethylamino-cinnamaldehyde, 50 ml ethanol, 50 ml 6 N HCl	Sensitive, turns red 12 hours after application
p-Dimethylamino-benzaldehyde (Ehrlich)	3	0.5 g p-dimethyl-amino-benzaldehyde, 1 ml 12 N HCl, 100 ml ethanol	Good, less sensitive
$FeCl_3$-$HClO_4$ (Salkowski)	25	1 ml 0.5 M $FeCl_3$, 50 ml 35% $HClO_4$, 50 ml ethanol	Most sensitive for IAA over other indoles
Prochazka	5	10 ml 35% formalde-hyde, 10 ml 12 N HCl, 20 ml ethanol	Heat 5 minutes at 100°; faint colors become fluorescent under long wave U.V. lamp, very sensitive

development, the layer was covered except for that portion with the marker, and sprayed with DMAC reagent or Prochazka reagent. That portion of the layer correlating to IAA was removed and eluted with methanol. The eluent was reduced to 0.1 ml and 0.01 ml chromatographed on thin layers of silica gel G or H for characterization. Additional areas producing indole-positive colors were removed and handled similarly.

Coleoptile straight-growth assay

The assay used is essentially that described by Nitsch and Nitsch (69). The green light source was supplied by green fluorescent tubes wrapped with two layers each of green and amber acetate sheets. Avena seeds, cv. Torch, were soaked in tap water for 2 hours in the dark. The seeds were placed embryo up on moist filter paper spread over a porcelain plate. The plate was laid in a culture dish in sufficient glass-distilled water to keep the paper moist and covered. After germination at 25° for 24 hours, the seeds were exposed to 2 hours of red light (photographic safe-light) to inhibit elongation of the first internode and returned to the dark. The coleoptiles were harvested when they were 20-25 mm in length, usually 72 hours after planting. In later experiments, the seeds were spread at random after soaking on a layer of moist vermiculite, germinated under red light at 25° for 18 hours, covered with 1/4-inch of vermiculite, and placed in the dark at 25°. The coleoptiles were harvested as described before.

A 4.5 mm section of the coleoptile was removed 3-4 mm below the apex. Sections were floated on $MnSO_4$ (1 mg/l) dissolved in

glass-distilled water for 3 hours. Test solutions were made up in a pH 5.0 phosphate-citrate buffer (1.794 g K_2HPO_4 and 1.019 g citric acid monohydrate) containing 2 per cent sucrose and 0.1 per cent Tween 80. Bioassays of paper strips were made by adding 1 ml of buffer to a tube with a portion of the paper representing a 0.1 R_f unit. Sections were taken from -0.2 R_f to 1.0 R_f. The -0.2 to 0 R_f section served as the control. Ten coleoptile sections were added to 1 ml of solution in an 18 mm x 150 mm tube. The tubes were rotated on a horizontal axis at 1 rpm in the dark at 25° for 20 hours. The sections were then measured to the nearest 0.2 mm using a photographic enlarger.

Identification of Catechin

Ten grams of ground, dried seeds collected on June 12, June 20, and July 1, 1962, were extracted with 200 ml of 50 per cent acetone at 4° for 24 hours. The mixture was filtered and the residue reextracted for 24 hours. A drop of the combined extracts spotted on paper produced a green color when sprayed with the DMAC reagent. The acetone was removed *in vacuo* and the remaining aqueous solution saturated with NaCl. The NaCl saturated solution was extracted twice with 100 ml of acetone and the combined acetone extracts taken to dryness. The residue was dissolved in water and layered on a column of Sephadex G-25 (2.5 cm x 25 cm). The column was eluted with water, collecting 5 ml fractions. The fractions producing a green color also showed maximum absorption at 279 mμ. These tubes were pooled, reduced in volume, and the extract subjected to preparative

thin-layer chromatography on a layer of cellulose using the upper phase of a n-butanol-acetic acid-water (4:1:5 v/v) mixture. The component was eluted from the layer and the eluent used for identification with commercial d-catechin (Calbiochem) used for comparison.

Indole-3-Acetic Acid Oxidase

The seeds used for this study were taken from fruit which had been stored frozen. Protein determinations were according to the method of Lowry et al. (53).

Preparation of soluble enzyme fraction

Forty grams of seeds taken from immature peach fruit which had been previously frozen were homogenized in 100 ml of 0.1 per cent Tween 80 solution in glass-distilled water. The detergent was used to eliminate formulation of protein-tannin complexes as described by Goldstein and Swain (24). The homogenate was centrifuged at 2000 x g at 4° for 20 minutes to remove cell wall debris. The supernatant was centrifuged at 22,000 x g for 30 minutes to give a mitochondria-free preparation. The preparation failed to show any IAA oxidase activity.

The supernatant was freeze-dried, taken up in 2-3 ml of glass-distilled water, and placed on a column of Sephadex G-25 (2.5 cm x 30 cm) packed in a column specially made for gel filtration (Pharmacia Fine Chemicals, Inc.). Elution of the column was accomplished with glass-distilled water. Three milliliter fractions were collected and their absorption at 280 mμ read on the Beckman DK-2 Ratio Recording Spectrophotometer. Alternate tubes of the

first peak were checked for IAA oxidase activity. Tubes with enzyme activity were pooled and used for further studies.

Enzyme assay

Incubation mixtures were made up as follows: 1 ml of 1 x 10^{-3}M $MnCl_2$ (57), 1 ml of 1 x 10^{-3}M 2,4-dichlorophenol (31), 0.1 ml of enzyme solution, 1 ml of 1 x 10^{-3} IAA, and buffer to total 10 ml. The reaction was initiated by the addition of substrate. Incubations were carried out at 25°. The progress of the reaction was followed by measuring the change in the absorption spectrum of the reaction mixture as described by Ray (82), by following the change in absorption at 253 mμ which measures oxindole formation (33), or by measuring IAA remaining using a colorimetric determination (76). For the latter, 4 ml of a modified Salkowski reagent (3 ml of 1.5 M $FeCl_3$, 60 ml of H_2SO_4, and 100 ml of H_2O) was added to 1 ml of the incubation mixture and the color read at 530 mμ after 15 minutes on the Bausch and Lomb Spectronic 20.

RESULTS

RESULTS

Auxin Identification

Chemical

Immature peach seeds collected during the years 1961 and 1962 were used in preliminary experiments to detect and identify indoles present in the tissue. In all seeds tested from these years, IAA was detected by paper chromatography using isopropanol-water as developing solvent (Table II). IAA was detected upon chromatography of the entire acid fractions. The use of isopropanol-water as developing solvent did not give the low mobility reported in the literature (67, 87). IAA could be detected upon chromatography of the entire acid fraction of the extract of 4 g of seeds. The neutral fraction obtained from the fractionation did not produce indole positive areas upon chromatography. This may have been a result of low concentrations of such auxins in the seed.

The chemical detection of much lower levels of indole compounds in seed extracts was possible by thin-layer chromatography. The sensitivity range using thin-layer techniques approaches that for the bioassay. Extracts of seeds equivalent to 50 mg dry weight were routinely chromatographed on thin layers after partial purification of the extract by the cellulose column procedure. Three DMAC-positive spots were detected upon separation of extracts from seeds collected before, during, and after cytokinesis. The qualitative evidence for three indoles is represented by chromatographic separation of an extract of seeds collected during cytokinesis

Table II. Characteristics of Auxin Detected in Immature Peach Seeds

Characteristic	Indole-3-acetic acid	Indole-3-propionic acid	Indole-3-butyric acid	Peach auxin
R_{fI}[1] (Paper)	0.74	--	--	0.75
R_{fII} (Paper)	0.33	--	--	0.32
R_{fIII} (TLC)[2]	0.43	0.52	0.58	0.43
R_{fIV} (TLC)	0.52	0.57	0.59	0.55
R_{fV} (TLC)	0.39	0.45	0.49	0.38
Color with DMAC reagent	Violet	Purple	Purple	Violet
Color with $FeCl_3$-$HClO_4$ reagent	Pink	--	--	Pink
Absorption maxima	280, 288 mμ	--	--	280, 288 mμ

[1]The solvents used for chromatography were: R_{fI} - isopropanol, H_2O (4:1); R_{fII} - n-butanol, NH_3, H_2O (100:3:18); R_{fIII} - chloroform, acetic acid (95:5); R_{fIV} - chloroform, ethyl acetate, formic acid (50:40:10); R_{fV} - isopropanol, ethyl acetate, NH_3 (45:35:20).

[2]TLC - Thin-layer chromatography on silica gel G or H.

(Table III). Further experiments supported the identification of compound A as being IAA. In addition to a positive chromogenic reaction, B was active in the straight-growth assay and has absorption maxima typical for the indole nucleus, 280 and 288 mμ. Rechromatography and comparison of its mobility to ethyl indole-3-acetate, indole-3-acetonitrile, indole-3-acetaldehyde did not provide conclusive evidence as to the identification of B. Compound C did not move similarly to any known indole. The possible nature of these two unknowns will be presented in the discussion.

Table III. Properties of DMAC-Positive Components in Seed Extract

Compounds	R_f[1]	Color with DMAC	Straight-growth activity
Known IAA	0.43	Violet	+
A	0.42	Violet	+
B	0.57	Pink	+
C	0.87	Pink	--

[1]Chromatography on thin layer of silica gel G or H using chloroform-acetic acid (95:5 v/v).

Evidence that compound A is IAA is summarized in Table II. The chromatographic results from the preliminary experiments are those represented for R_{fI}. The other data represents typical results obtained upon rechromatography of the eluent from preparative scale separations of the extracts. The R_f values of known IAA in all five solvents agree well with those of the unknown acid. The

characteristic colors obtained with chromogenic reagents and the ultraviolet absorption maxima also support the assignment of the auxin as being IAA.

Bioassay

The biological activity represented by the effect on growth of Avena coleoptile sections by areas of the paper on which seed extracts were chromatographically separated offered a qualitative and quantitative means for detecting auxins in the seeds. The resulting auxin-like activity patterns of the chromatograms will be referred to as biograms. In the preliminary work, acid fractions were chromatographed on paper using isopropanol-water (4:1 v/v) as the developing solvent. A representative biogram from the separation of an extract from 50 mg of seeds collected at the time of cytokinesis is presented in figure 2. The resulting activity corresponded to the R_f of IAA, as well as the R_f's of other indolic auxins. Thus separation of the possible auxins was not possible with this solvent. On the basis of estimates of the quantities detected chemically, it was apparent that IAA was not the only auxin present. This supposition was supported after assaying sections of paper developed in butanol-ammonia-water.

The biograms obtained after separation of 50 mg dry weight equivalent extracts of seeds collected before, during, and after cytokinesis, respectively, are illustrated in figure 3. Authentic IAA has an R_f of 0.33 in the solvent system. The activity between 0.3 and 0.4 R_f is thus representative of IAA in the seeds. The presence of IAA in these extracts was confirmed by thin-layer

Figure 2

Biogram resulting from separation of extract of 50 mg of seeds collected on June 11, 1964, (cytokinesis) with isopropanol-water

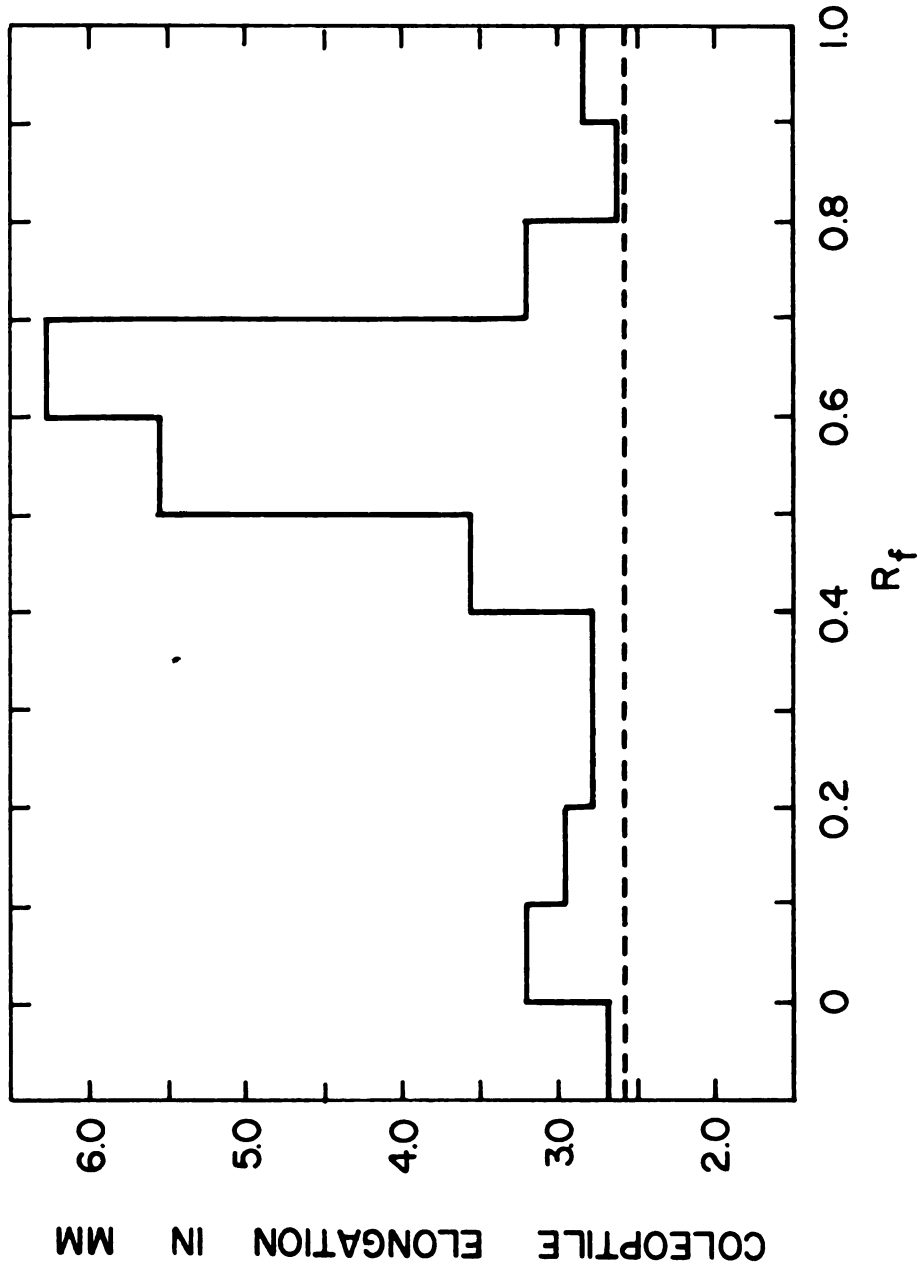
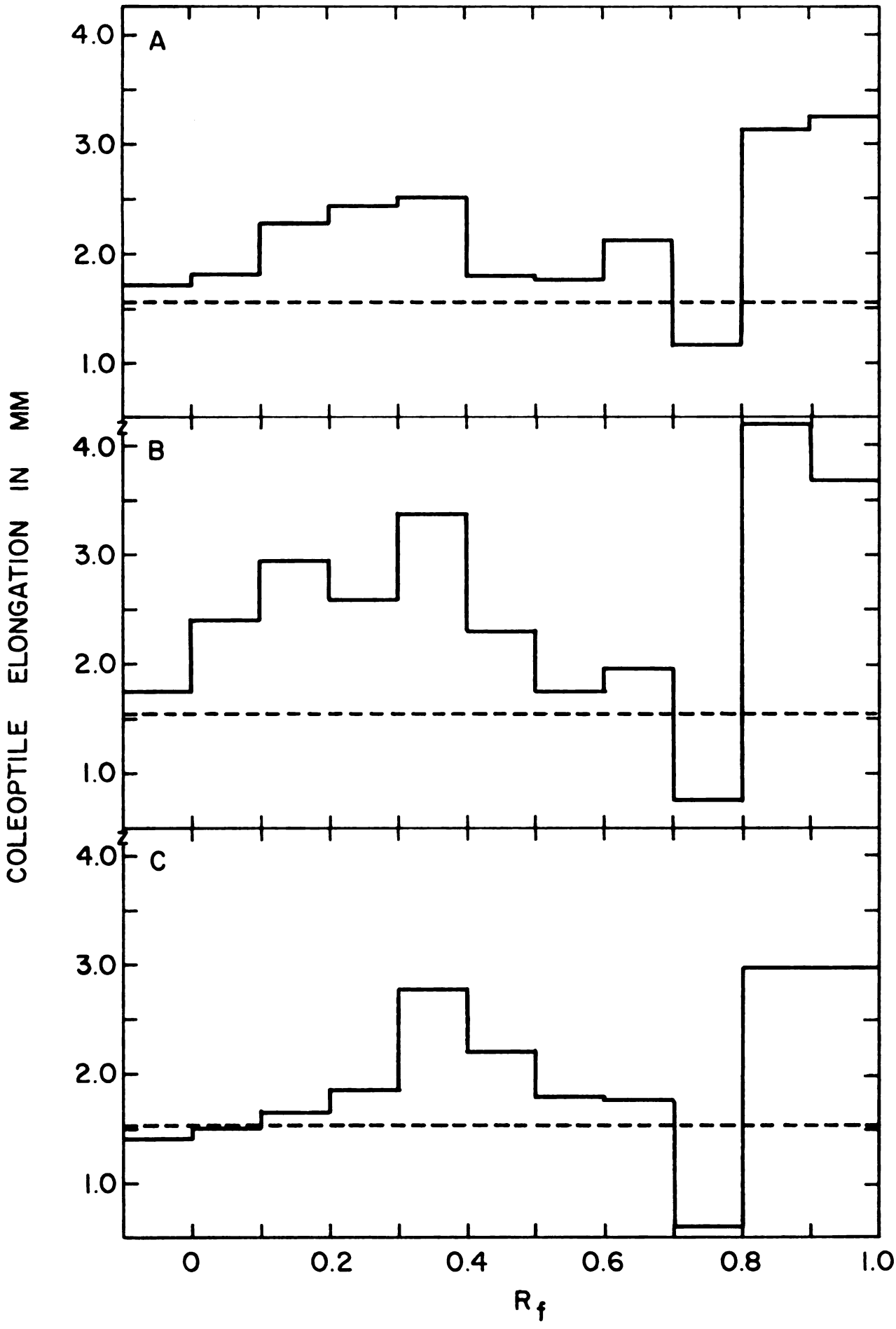

COLEOPTILE ELONGATION IN MM
6.0
5.0
4.0
3.0
2.0
0
0.2
0.4
0.6
0.8
1.0
R_f

Figure 3

Biograms resulting from separation of extracts of 50 mg of seeds collected (A) before, (B) during, and (C) after cytokinesis with butanol-ammonia-water.


A
B
C
4.0
3.0
2.0
1.0
COLEOPTILE ELONGATION IN MM
0
0.2
0.4
0.6
0.8
1.0
R_f

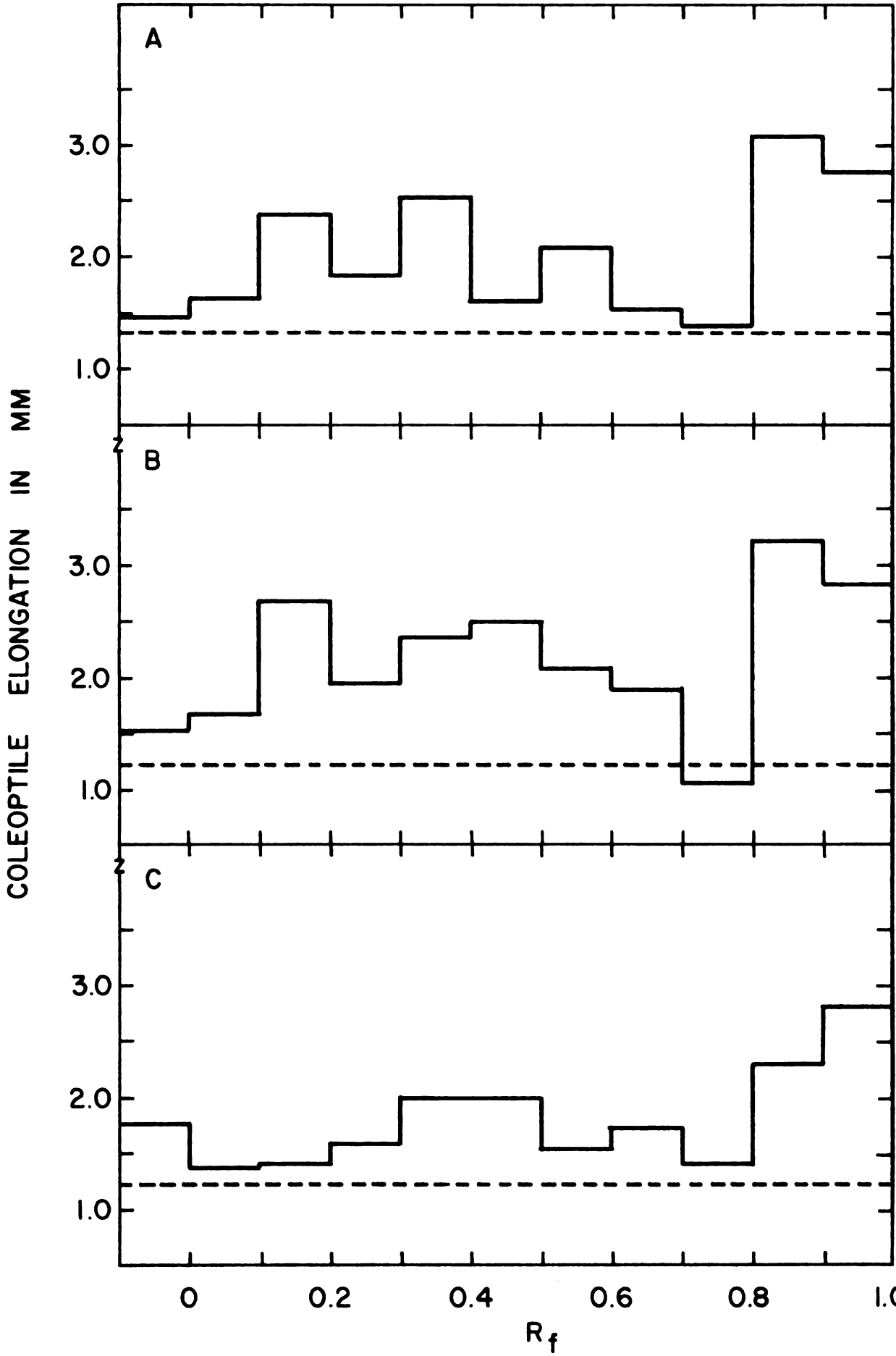
chromatography. In each of the biograms, auxin activity also appeared between 0.8 and 1.0 R_f units. The lower level of activity in this region for sample C resulted from inhibitory concentrations evidenced by the unusual curling of the coleoptile sections. Possible identification of the auxins detected in this area will be presented later. Auxin activity between 0.1 and 0.2 R_f was also apparent in extracts of A and B. An inhibitor between 0.7 and 0.8 R_f increased in concentration with seed maturity.

Thin-layer chromatography on silica gel H with 2-butanone-hexane (18:82 v/v) as developing solvent separates indoles which are non-acidic and leaves the acidic compounds at the origin (73). Extracts equivalent to 50 mg of seeds were chromatographed in this solvent system and the origins, which are minus the non-acidic auxins, were eluted and chromatographed on paper. The biograms of the acidic auxin fractions are shown in figure 4. The zones of activity at 0.1 to 0.2 R_f and 0.3 to 0.4 R_f resulted as before. In addition, activity was still observed at 0.8 to 1.0 R_f. Simple neutral indole auxins chromatograph in this area, but they would have been removed by the thin layer separation. The compound, which developed a pink color after spraying with DMAC reagent must thus be a new acidic auxin. No single auxin with these properties has been described in the literature. Identification of this acidic component apparently the same as compound C must await isolation of larger quantities.

Chromatography of the original extracts on paper and development with hexand-chloroform-water (75:15:10 v/v, upper phase)

Figure 4

Biograms resulting from separation of extracts of 50 mg of seeds collected (A) before, (B) during, and (C) after cytokinesis after separation of non-acidic components. Method used is given in text.

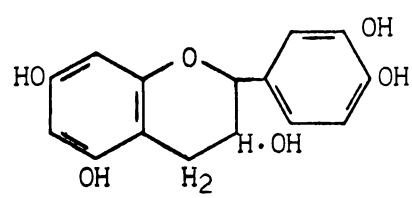

A
B
C
3.0
2.0
1.0
COLEOPTILE ELONGATION IN MM
0
0.2
0.4
0.6
0.8
1.0
R_f

resulted in high activity at the origin (acidic components) and at the solvent front where ethyl indole-3-acetate, indole-3-acetonitrile, and indole-3-acetaldehyde also chromatographed. This was additional evidence that a neutral auxin was present, but as with the chemical experiments, identification was not possible.

By using the straight-growth assay to measure the IAA on chromatograms of extracts, the auxin levels in the seeds before, during, and after cytokinesis could be compared as shown in figure 5. The time of cytokinesis was determined according to Leuty (48) as that period of growth at which the pericarp length reached 30 mm. The IAA level was highest during cytokinesis, which occurred in this case on June 11. Lombard and Mitchell (52) noted the increase of an acidic auxin, but were unable to demonstrate that it was IAA. These results show that IAA is present at an elevated level during cytokinesis.

Catechin Identification

Evidence that the compound in the acetone-water extracts was catechin (IV) is presented in Table IV. Catechin has been previously



IV

detected in mature peach fruit (7), but there was no previous evidence for its presence in immature seed tissue.

Figure 5

Comparison of IAA content to fruit growth and especially cytokinesis. Fruit growth was measured by pericarp diameter and IAA content by bioassay of duplicate biogram sections of 0.3 to 0.4 R_f.

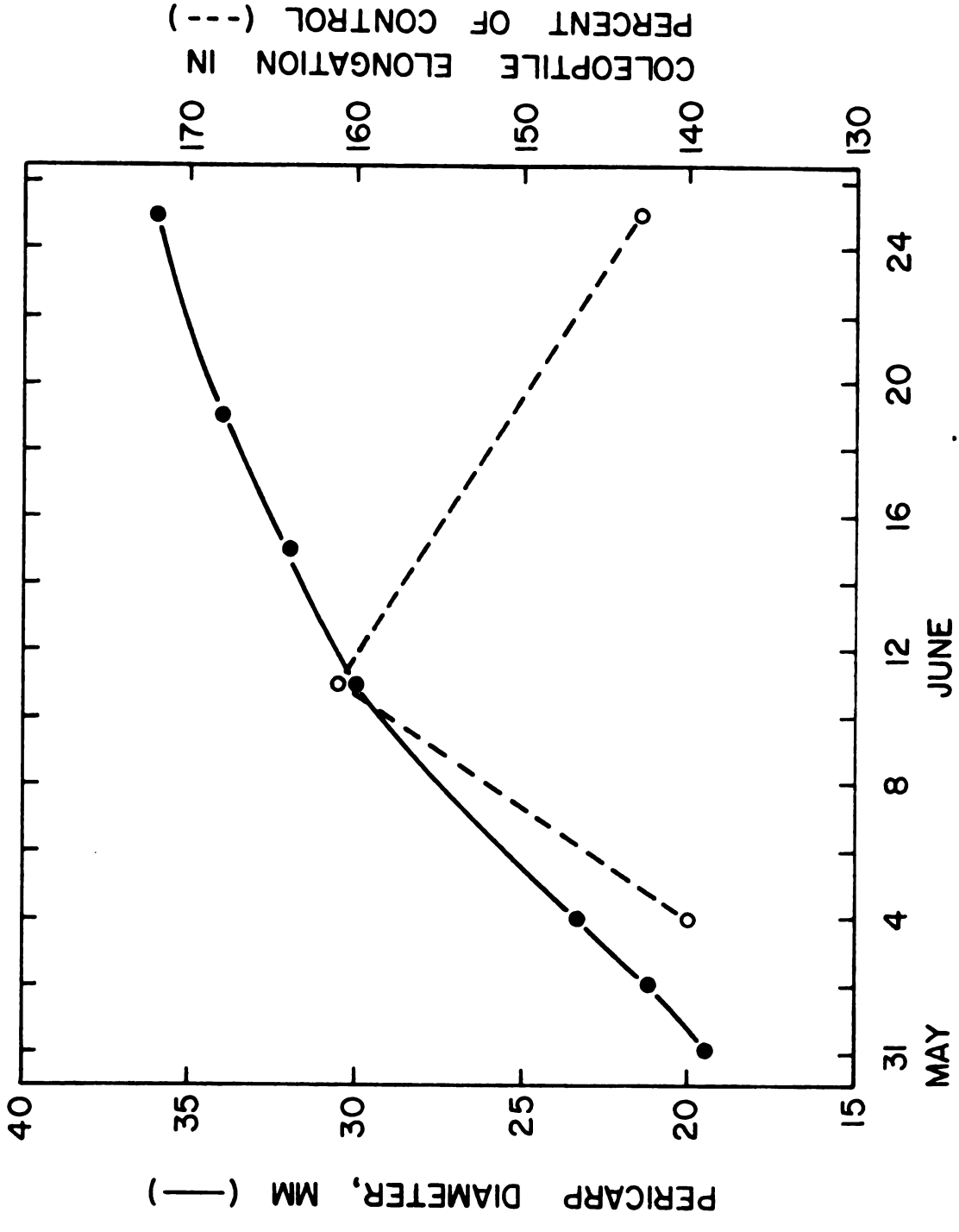

PERICARP DIAMETER, MM (——)
40
35
30
25
20
15
31
MAY
4
8
12
JUNE
16
20
24
COLEOPTILE ELONGATION IN
PERCENT OF CONTROL (---)
170
160
150
140
130

Table IV. Evidence for Catechin in Immature Peach Seeds

Compound	R_f[1]	Color with DMAC	Absorption (mμ) max.	Absorption (mμ) min.	IAA Oxidase[2]	Effect on IAA Induced Growth[3]
Catechin	0.51	Green	278	252	Inhibits	Synergistic
Peach Seed Component	0.51	Green	279	252	Inhibits	Synergistic

[1]Isopropanol-water (4:1 v/v) on paper.

[2]Measured spectrophotometrically using preparation from bean roots.

[3]Avena coleoptile straight-growth assay using 1 x 10^{-7} M IAA.

It was noted that an aliquot of the purified preparation increased the growth-inducing effect of 1 x 10^{-7} M IAA in the Avena coleoptile straight-growth assay. This prompted the experiment shown in figure 6, which demonstrated the effect of catechin on IAA induced growth. Catechin at 1 x 10^{-3} M completely eliminated the induction of 1 x 10^{-6} M to 1 x 10^{-4} M IAA on growth and it appeared that some reduction of IAA induced growth at these concentrations prevailed at all concentrations of catechin. Levels of catechin less than 1 x 10^{-5} M increased growth induced by 1 x 10^{-7} M IAA. It has been previously reported (97) that 1 x 10^{-3} M catechin inhibited growth induced by 1 x 10^{-5} M IAA.

In view of a report by Letham and Bollard (47) that catechin retarded the growth of carrot tissue, it was of interest to note its effect on buckwheat root growth (figure 7). Inhibition of root

Figure 6

Effect of catechin on IAA induced growth of Avena coleoptiles. Molar concentrations of catechin are designated for the respective curves.

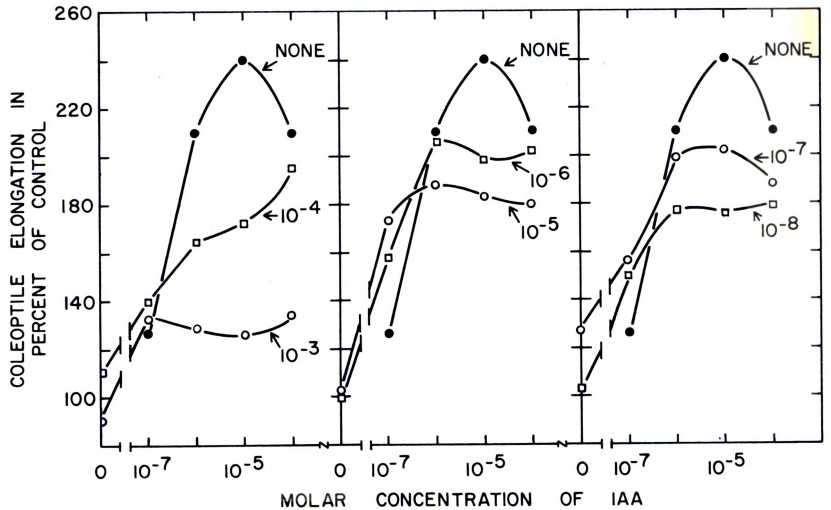
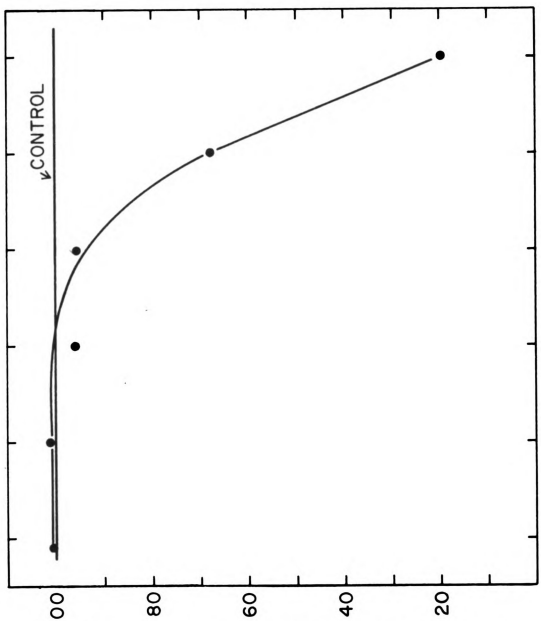

260
220
180
140
100
COLEOPTILE ELONGATION IN PERCENT OF CONTROL
NONE
10-4
10-3
NONE
10-6
10-5
NONE
10-7
10-8
0
10-7
10-5
MOLAR CONCENTRATION OF IAA

Figure 7

Effect of catechin on buckwheat root growth.


ROOT GROWTH IN PERCENT OF CONTROL
100
80
60
40
20
CONTROL
10-7
10-6
10-5
10-4
10-3
10-2
MOLAR CONCENTRATION

growth was observed with 1 x 10^{-3} M and 1 x 10^{-2} M catechin, with the greater inhibition at 1 x 10^{-2} M.

Indole-3-Acetic Acid Oxidase

The elution of the 280 mμ absorbing material off the Sephadex G-25 column is illustrated in figure 8. Enzyme activity correlated with the 280 mμ abosrption of the protein peak. The 280 mμ absorbing material coming off the column later was not protein and apparently included materials which prevented IAA oxidase activity before the Sephadex treatment. In experiments where the seeds were not homogenized in 0.1 per cent Tween 80, but rather in water alone, lower yields of protein were obtained. Since tannins are present in the seed tissue, the formation of protein-tannin complexes would be expected to precipitate (24) and thus reduce the yield of protein. The detergent increased the yield of soluble protein.

The pH optimum for the peach IAA oxidase system was found to be between 4.5 and 5.0 (figure 9). A 0.05 M phosphate buffer, pH 5.0, was used for subsequent enzyme assays. Buffers containing citrate or acetate inhibited the destruction activity.

Spectrum 3, figure 10, shows that the product of IAA oxidation catalyzed by the peach enzyme system is 3-methyleneoxindole (III) (33). The enzyme requires both Mn^{++} and 2,4-dichlorophenol for optimum activity (figure 10). These two cofactors were included at 1 x 10^{-4} M concentration.

Catechin inhibited the rate of methyleneoxindole formation and thus represents inhibition of IAA oxidation (figure 11). IAA had

Figure 8

Elution of soluble protein extract and IAA oxidase activity from Sephadex G-25 column. Oxidase activity measured colorimetrically as moles $x10^{7}$ destroyed /ml/ 30 minutes.

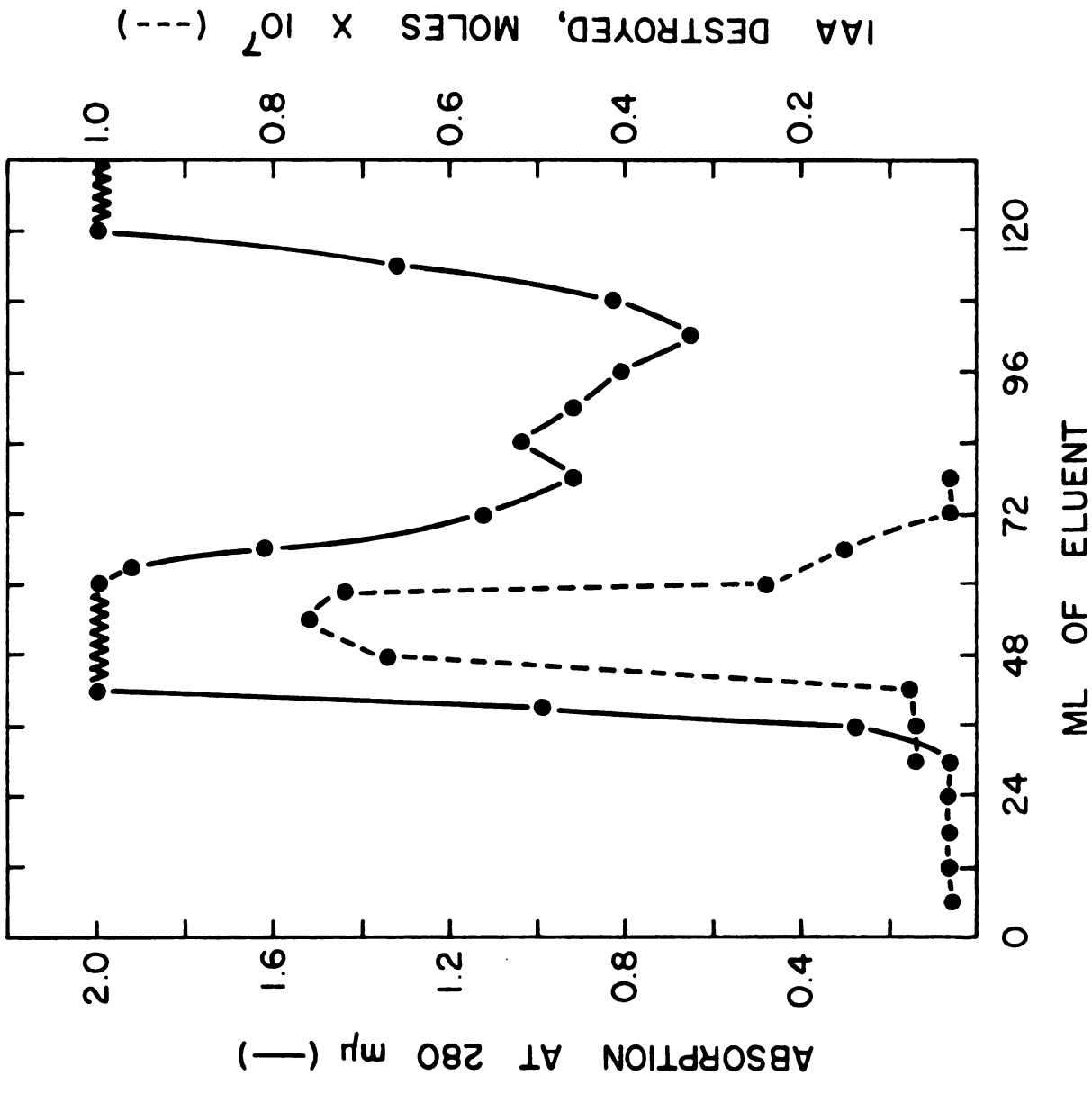

IAA DESTROYED, MOLES X 10^7 (---)
1.0
0.8
0.6
0.4
0.2
ABSORPTION AT 280 mμ (——)
2.0
1.6
1.2
0.8
0.4
0
24
48
72
96
120
ML OF ELUENT

Figure 9

Optimum pH conditions for IAA oxidase activity. IAA destroyed /ml was measured after 2, 4, and 6 hours.

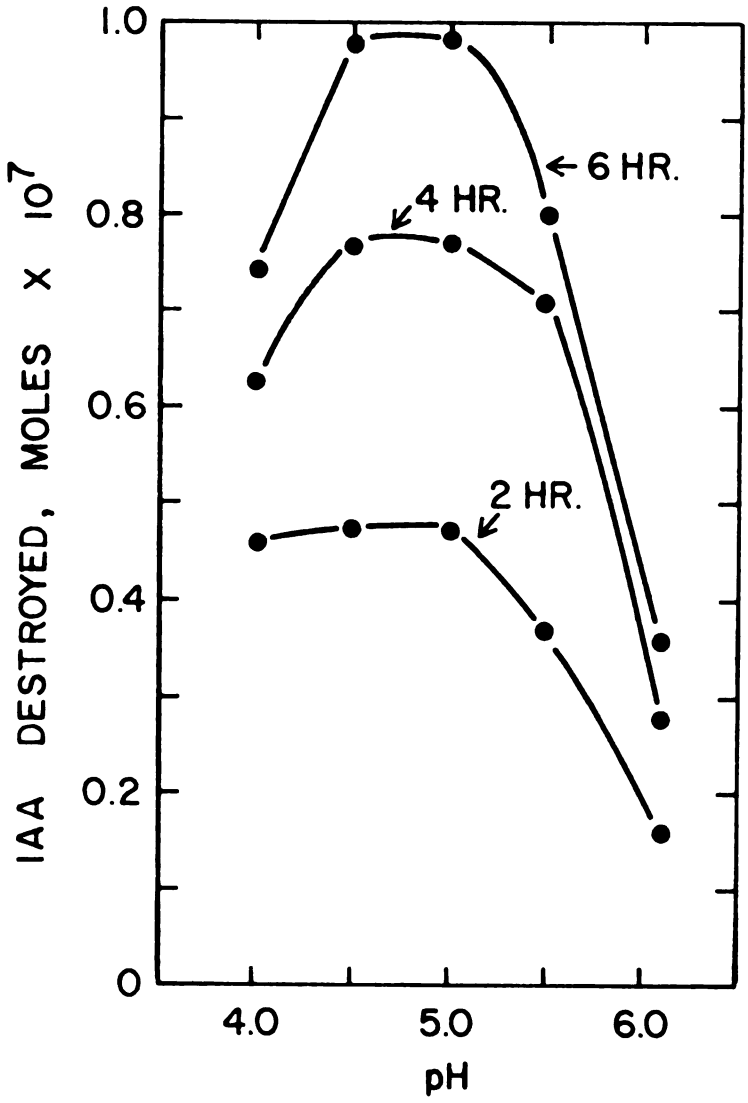

1.0
0.8
0.6
0.4
0.2
0
IAA DESTROYED, MOLES X 10^7
6 HR.
4 HR.
2 HR.
4.0
5.0
6.0
pH

Figure 10

Ultraviolet absorption spectra of reaction mixtures showing cofactor requirements for the peach IAA oxidase. Curve 1, complete reaction mixture after 1 minute; curve 2, same after 40 minutes; curve 3, same after 10 hours; curve 4, same minus 1 x 10^{-4} M $MnCl_2$ after 10 hours; and curve 5, same minus 1 x 10^{-4} M 2,4-dichlorophenol after 10 hours.

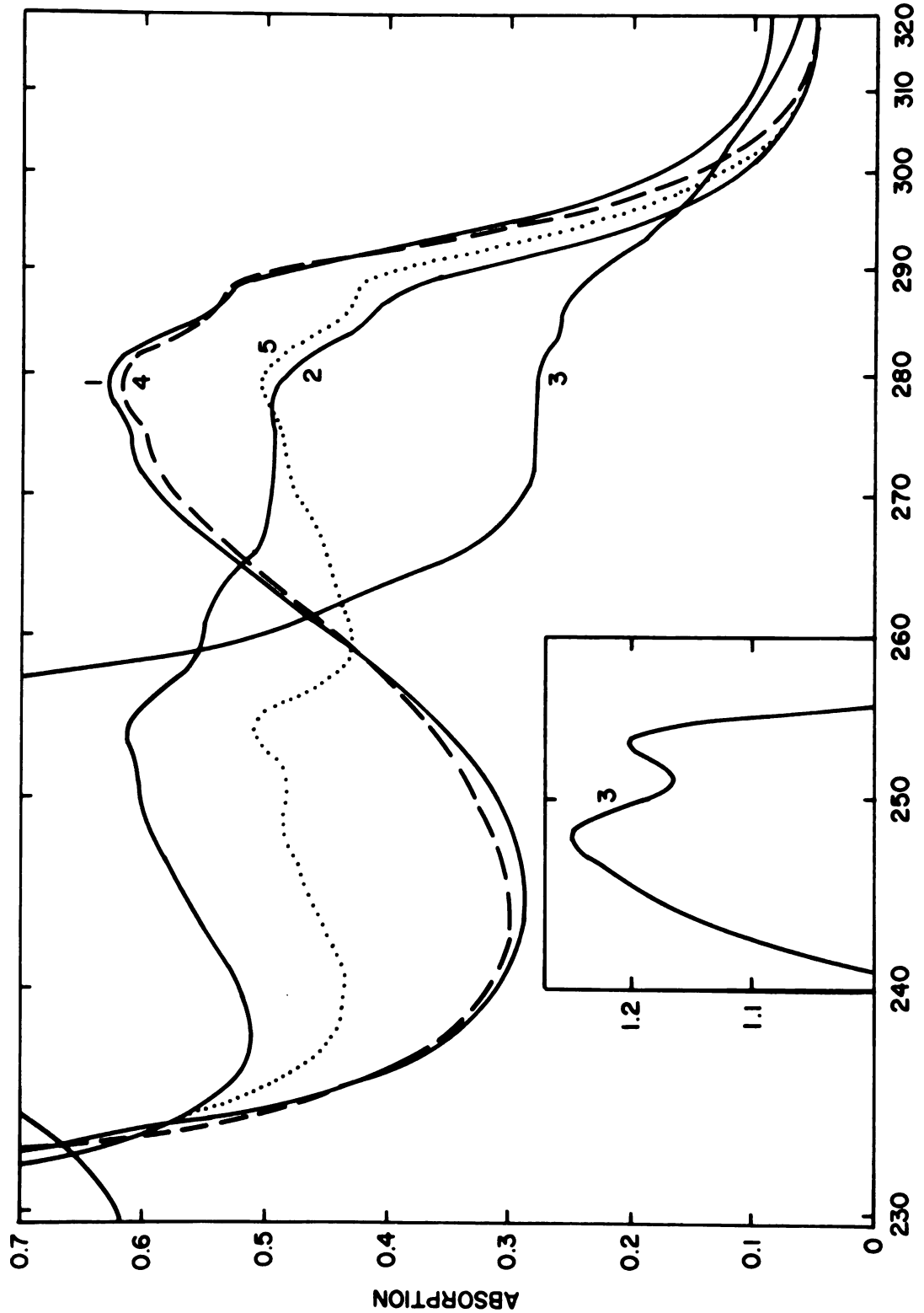
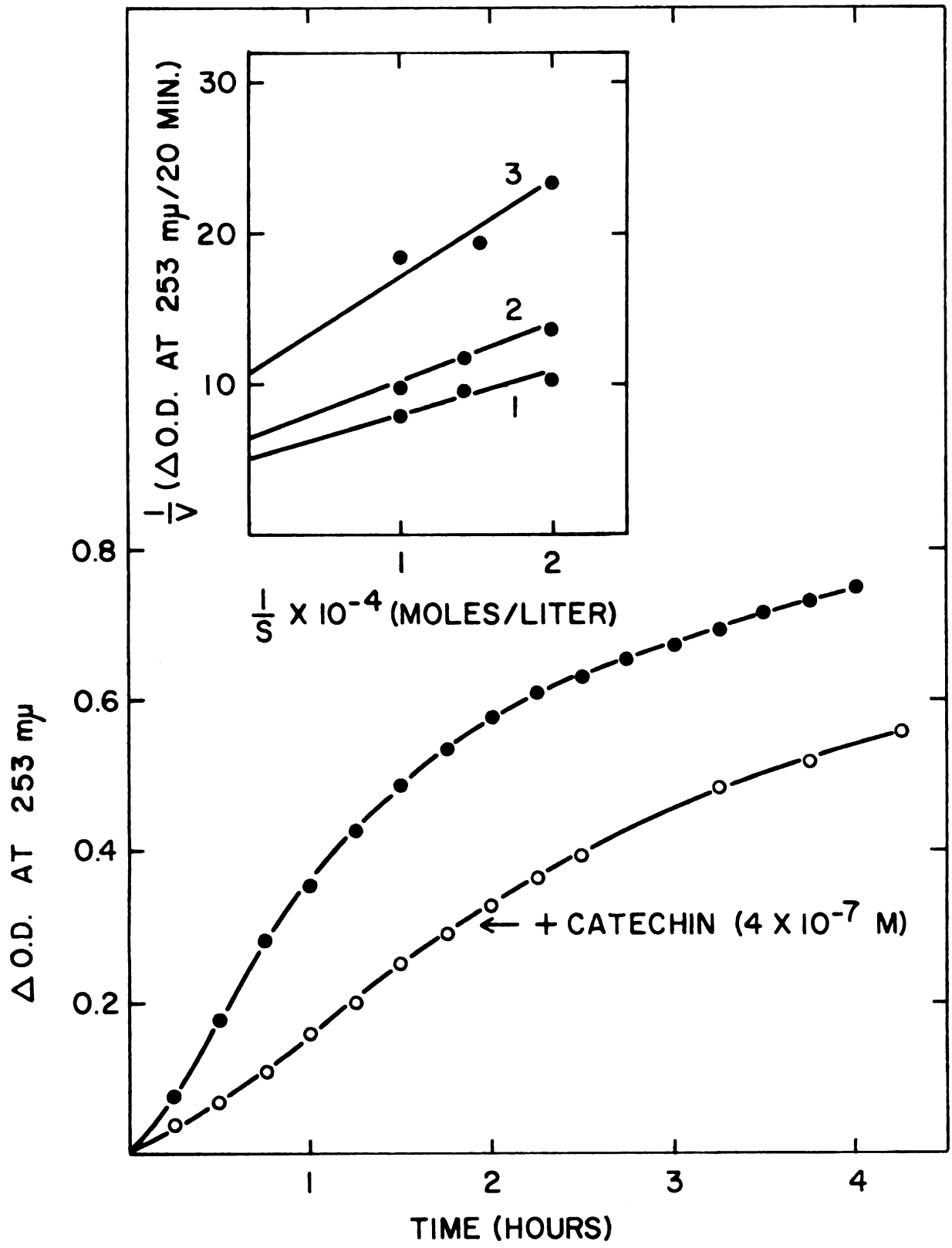

ABSORPTION
WAVELENGTH, mμ
0
0.1
0.2
0.3
0.4
0.5
0.6
0.7
230
240
250
260
270
280
290
300
310
320
1
2
3
4
5
1.1
1.2

Figure 11

Rate of 3-methyleneoxindole formation catalyzed by peach IAA oxidase and its inhibition by catechin. The Lineweaver-Burke plot (inset) was prepared utilizing (1) no catechin, (2) 2×10^{-7} M catechin, and (3) 4×10^{-7} M catechin.


30
20
10
3
2
1
1/V (ΔO.D. AT 253 mμ/20 MIN.)
1
2
1/S X 10⁻⁴ (MOLES/LITER)
0.8
0.6
0.4
0.2
ΔO.D. AT 253 mμ
+ CATECHIN (4 X 10⁻⁷ M)
1
2
3
4
TIME (HOURS)

almost completely disappeared after 2 hours in the absence of catechin as measured with the Salkowski reagent. Attempts to follow IAA destruction in the presence of catechin with the Salkowski reagent were unsuccessful. It appeared from the rate curve that catechin affected IAA oxidation in a non-competitive manner. The Lineweaver-Burke plot (12) in figure 11 substantiated the non-competitive manner of catechin inhibition.

DISCUSSION

DISCUSSION

In view of reports by others (52, 87) the presence of free IAA in immature peach seeds was not unexpected. Evidence heretofore for the presence of IAA in the tissue was accumulated from biological activity. The utilization of thin-layer chromatography combined with the bioassay made it possible to detect and identify low levels of the auxin. Even during cytokinesis, the level of IAA estimated on the basis of the limits of detection used, may not be greater than 4 or 5 mg per gram of dry weight. Stahly and Thompson (87) found from quantitative bioassays that the highest level of auxin in the seed of Halehaven peaches which they tentatively identified as IAA is 15 µg per gram dry weight. The levels of IAA for corresponding periods of development would not be expected to be consistent from year to year nor from cultivar to cultivar. Lewis et al. (49) have found that the uncharacterized "citrus" auxin is also present in peach fruit and questioned whether the transient spots obtained by Stahly and Thompson (87) were really IAA. The experiments presented herein establish the identification. No evidence was obtained in this work to suggest the presence of "citrus auxin" in the immature peach seed.

The requirement for auxin in conjunction with a kinin for promoting cell division has been well established (58). Recently reported evidence has shown that kinin activity is abundant in the peach seed endosperm (78). One the basis of the increase in IAA at cytokinesis, it may well be required for the rapid cell division in endosperm. No data is yet available on concentrations of the kinin in these tissues relative to maturation of the fruit.

Since the IAA level apparently fluctuates, biochemical processes which may regulate its content should be considered. The data suggest that a system which regulates the rate of the oxidative destruction of IAA may be functional for this purpose. The process would comprise the non-competitive inhibition by catechin of the IAA oxidase from peach seeds reported here. The non-competitive mode of inhibition suggests that the inhibitor is binding at a site removed from the active site of the enzyme (12). In the *in vitro* system, the substrate (IAA) is protected from oxidation by the presence of the inhibitor (catechin) and the rate of IAA destruction is dependent upon the level of the inhibitor. Chlorogenic acid, a competitive inhibitor of pea IAA oxidase (79), is also present in peach tissue and may also function as an inhibitor of the peach enzyme also.

A physiological interrelationship between IAA, catechin, and IAA oxidase may be functional during maturation of the peach seed and fruit. Biological analysis of the IAA content showed that it increased during cytokinesis and then decreased after completion of cell division. Donoho *et al.* (13) demonstrated that the rate of IAA destruction *in vivo* was considerably less during cytokinesis. The present work suggests that this fluctuation in oxidase activity may be manifested by the non-competitive inhibitory action of catechin. The rate of IAA oxidase activity would thus be dependent on the amount of catechin bound to the enzyme at a particular time. When higher levels of IAA are observed, higher quantities of catechin would be bound to the enzyme, invoking a decreased rate of oxidation.

Because of probable localization in the tissue and cells of both enzyme and inhibitor, this hypothesis could not be clarified in this study.

The end-product of the in vitro oxidation of IAA as catalyzed by the peach enzyme system was shown to be 3-methyleneoxindole. In view of recent observations (18) that this compound inhibits the growth of higher plants, considerations as to its effects on fruit growth and development would be in order. When IAA oxidase is most active, and therefore the IAA level is decreased, the concentration level of this inhibitory compound would be increased. The IAA oxidase system could thus be responsible for both a growth promoting compound (IAA) and a growth inhibiting compound (3-methyleneoxindole) and both may be physiologically important.

In addition to IAA, other auxins were detected but not identified. One of these is non-acidic and is probably equivalent to auxin B mentioned earlier. Preliminary evidence suggests that it is either an ester of IAA, indole-3-acetonitrile, or indole-3-acetaldehyde. The physiological function of auxin B is not evident at this time.

It is possible that the acidic auxin (auxin C) which traveled to the higher R_f regions on paper in butanol-ammonia-water may be representative of a new class of auxins recently described as being present in the culture medium of excised tomato roots (89). The auxin from tomato roots was complex and included 5-hydroxyindole-3-acetic acid, 5-hydroxytryptophan and several amino acids. The complex auxin was soluble in ethyl acetate, had a high R_f value in

solvents containing ammonia, and gave a pink color with Ehrlich's reagent. The complex was reported to have activity in the straight-growth assay. Compounds which were equally complex were also detected in other plant roots. Although characterization of the peach seed component with similar properties has not been attempted, it is nevertheless tempting to speculate that the acidic auxin observed at the high R_f region by bioassay is perhaps a complex of the type described in tomato roots. If true, this is the first instance known to the author that a compound of this type has been described in immature peach seeds. No data is yet available which might explain a role for this complex in peach fruit maturation.

SUMMARY

SUMMARY

An auxin, which was correlated by its relative concentration in the seed tissue with cytokinesis of the endosperm of the seed of immature peach fruit (Prunus persica, cv. Redhaven), was identified by its biological activity, paper and thin-layer chromatography, and ultraviolet absorption as indole-3-acetic acid. Evidence was obtained for the presence of another acidic auxin in extracts of the immature seeds. This compound had a Rf of 0.80-1.00 in butanol-ammonia-water (100:3:18) and displayed typical auxin responses as well as colorimetric reactions typical of indoles. An indole-3-acetic acid oxidase system was detected and characterized in the soluble fraction of peach seed extracts. The enzyme had a pH optimum of 4.5 to 5.0 and required both Mn++ and 2,4-dichlorophenol for activity. The product of the reaction was shown to be 3-methyleneoxindole. A polyphenol, catechin, was identified in the seed by chromatography. The compound was shown to be a non-competitive inhibitor of the oxidase enzyme.

The inhibitory effect of catechin on the indoleacetic acid oxidase is discussed as a possible mechanism by which the concentration levels of indoleacetic acid may be controlled during fruit maturation. A discussion is presented as to the possible complex nature of the unknown acidic auxin detected.

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Bentley, J. A., Ann. Rev. Plant Physiol. 9, 47 (1958).

2. Bentley, J. A., Encyclopedia of Plant Physiol. 14, 609 (1961).

3. Block, R. J., Durrum, E. L., and Zweig, G., in Paper Chromatography and Paper Electrophoresis, Academic Press, New York, (1958), P. 305.

4. Bukovac, M. J., Bot. Gaz. 124, 191 (1963).

5. Collett, G., Dubouchet, J., and Pilet, P. E., Physiol. Veg. 2, 157 (1964).

6. Connors, C. H., New Jersey Agr. Exp. Sta. Rep. 40, 82 (1919).

7. Craft, C. C., Proc. Amer. Soc. Hort. Sci. 78, 119 (1961).

8. Crane, J. C., Ann. Rev. Plant Physiol. 15, 303 (1964).

9. Crane, J. C., Bradley, M. V., and Luckwill, L. C., J. Hort. Sci. 34, 142 (1959).

10. Crane, J. C., Rebeiz, C. A., and Campbell, R. C., Proc. Amer. Soc. Hort. Sci. 78, 111 (1961).

11. Crane, J. C., and van Overbeek, J., Science 147, 1468 (1965).

12. Dixon, M., and Webb, E. C., in Enzymes, Academic Press, Inc. New York (1964), p. 329.

13. Donoho, C. W., Jr., Mitchell, A. E., and Sell, H. M., Proc. Amer. Soc. Hort. Sci. 80, 43 (1962).

14. Durkee, A. B., and Poapst, P. A., Agr. Food Chem. 13, 135 (1965).

15. Durkee, A. B., and Poapst, P. A., Nature 193, 273 (1962).

16. Fang, S. C., and Yu, T. C., Plant Physiol. 40, 299 (1965).

17. Fletcher, R. A., and Zalik, S., Plant Physiol. 39, 328 (1964).

18. Fukuyama, T. T., and Moyed, H. S., J. Biol. Chem. 239, 2392 (1964).

19. Galston, A. W., in Photoperiodism and Related Phenomena in Plants and Animals, AAAS (1959), pp. 137-157.

20. Galston, A. W., Jackson, P., Kaur-Sawhney, R., Kefford, N. P., and Meudt, W. J., in Regulateurs Naturels de la Croissance Vegetale, Centre National de la Recherche Scientifique (1964), pp. 251-264.

21. Geissman, T. A., in The Chemistry of Flavonoid Compounds, The MacMillan Co., New York (1962), p. 528.

22. Goldacre, P. L., and Bottomley, W., Nature 184, 555 (1959).

23. Goldacre, P. L., Galston, A. W., and Weintraub, R. L., Arch. Biochem. Biophys. 43, 358 (1953).

24. Goldstein, J., and Swain, T., Phytochemistry 4, 185 (1965).

25. Gordon, S. A., and Weber, R. P., Plant Physiol. 26, 192 (1951).

26. Gortner, W. A., and Kent, M., J. Biol. Chem. 204, 593 (1953).

27. Gortner, W. A., and Kent, M. J., J. Biol. Chem. 233, 731 (1958).

28. Gustafson, F. G., Am. J. Botany 26, 189 (1939).

29. Hare, R. C., Bot. Rev. 30, 129 (1964).

30. Harley-Mason, J., and Archer, A. A. P. G., Biochem. Jour. 69, 60P (1958).

31. Hillman, W. S., and Galson, A. W., Physiol. Plantarum 9, 230 (1956).

32. Hinman, R. L., and Frost, P., in Plant Growth Regulation, Iowa State University Press (1961), pp. 205-216.

33. Hinman, R. L., and Lang, J., Biochemistry 4, 144 (1965).

34. Humphries, E. C., and Wheeler, A. W., in Regulaterus Naturels de la Croissance Vegetale, Centre National de la Recherche Scientifique, (1964), pp. 505-515.

35. Kefford, N. P., Kaur-Sawhney, R., and Galston, A. W., Acta Chem. Scand. 17(Suppl.), 313 (1963).

36. Kenworthy, A. L., and Harris, N., Mich. St. Univ. Agr. Exp. Sta., Quart. Bull. 44, 67 (1961).

37. Key, J. L., Plant Physiol. 39, 365 (1964).

38. Key, J. L., and Ingle, J., Proc. Nat. Acad. Sci. U.S. 52, 1382 (1964).

39. Key, J. L., and Shannon, J. C., Plant Physiol. 39, 360 (1964).

40. Khalifah, R. A., Lewis, L. N., and Coggins, C. W., Jr., Plant Physiol. 40, 441 (1965).

41. Khalifah, R. A., Lewis, L. N., and Coggins, C. W., Jr., Science 142, 399 (1963).

42. Kuraishi, S., and Muir, R. M., Plant and Cell Physiol. 5, 61 (1964).

43. Kuraishi, S., and Muir, R. M., Science 137, 760 (1962).

44. Lavee, S., Science 142, 583 (1963).

45. Leopold, A. C., Can. J. Bot. 40, 745 (1962).

46. Letham, D. S., in Regulateurs Naturels de la Croissance Vegetale, Centre National de la Recherche Scientifique, Paris, (1964), pp. 109-117.

47. Letham, D. S., and Bollard, E. G., Nature 191, 1119 (1961).

48. Leuty, S. J., Ph.D. Thesis, Michigan State University (1964).

49. Lewis, L. N., Khalifah, R. A., and Coggins, C. W., Jr., Phytochem. 4, 203 (1965).

50. Lewis, L. N. Khalifah, R. A., and Coggins, C. W., Jr., Plant Physiol. 40, 500 (1965).

51. Linser, H., and Maschek, F., Planta 41, 567 (1953).

52. Lombard, P. B., and Mitchell, A. E., Proc. Amer. Soc. Hort. Sci. 80, 163 (1962).

53. Lowry, O. H., Rosenbrough, N. J., Farr, A. L., and Randall, R. J., J. Biol. Chem. 193, 265 (1951).

54. Luckwill, L. C., J. Hort. Sci. 24, 32 (1948).

55. Luckwill, L. C., J. Hort. Sci. 28, 14 (1953).

56. Luckwill, L. C., J. Hort. Sci. 32, 18 (1957).

57. Luckwill, L. C., and Powell, L. E., Science 123, 225 (1956).

58. Miller, C. O., Ann. Rev. Plant Physiol. 12, 395 (1961).

59. Morgan, P. W., Plant Physiol. 39, 741 (1964).

60. Morgan, P. W., and Hall, W. C., Plant Physiol. 38, 365 (1963).

61. Mumford, F. E., Smith, D. H., and Castle, J. E., Plant Physiol. 36, 752 (1961).

62. Mumford, F. E., Stark, H. H., and Smith, D. H., Phytochemistry 2, 215 (1963).

63. Nitsch, J. P., Am. J. Botany 37, 211 (1950).

64. Nitsch, J. P., Ann. Rev. Plant Physiol. 4, 199 (1953).

65. Nitsch, J. P., Bull. Soc. Bot. France 105, 479 (1958).

66. Nitsch, J. P., in The Chemistry and Mode of Action of Plant Growth Substances, Wain, R. L., and Wightman, F. eds., Butterworth Scientific Publications, London (1956), pp. 3-31.

67. Nitsch, J. P., and Nitsch, C., Beit. Biol. Pflanz. 31, 387 (1955).

68. Nitsch, J. P., and Nitsch, C., in Plant Growth Regulation, Klein, R. M., ed., Iowa St. Univer. Press (1961), pp. 687-707.

69. Nitsch, J. P. and Nitsch, C., Plant Physiol. 31 94 (1956).

70. Nitsch, J. P., Pratt, C., Nitsch, C., and Shaulis, N. J., Am. J. Botany 47, 566 (1960).

71. Nooden, L. D., and Thimann, K. V., Plant Physiol. 40, 193 (1965).

72. Nooden, L. D. and Thimann, K. V., Proc. Nat. Acad. Sci. 50, 194 (1963).

73. Obreiter, J. B. and Stowe, B. B., J. Chromatog. 16, 226 (1964).

74. Paleg, L. G., Ann. Rev. Plant Physiol. 16, 291 (1965).

75. Phinney, B. O., West, C. A., Ritzel, M., and Neely, P. M., Proc. Natl. Acad. Sci. U.S. 43, 398 (1957).

76. Pilet, P. E. and Collet, G., in Methode d'Analyse du Catabolisme Auxinique, Ch. Zwalen, Lausanne (1962).

77. Powell, L. E. and Pratt, C., Farm Res. 26, No. 3, 11 (1960).

78. Powell, L. E. and Pratt, C., Nature 204, 602 (1964).

79. Rabin, R. S. and Klein, R. M., Arch. Biochem. Biophys. 70, 11 (1957).

80. Raussendorf-Bargen, Gisela, Planta 58, 471 (1962).

81. Ray, P. M., Ann. Rev. Plant Physiol. 9, 81 (1958).

82. Ray, P. M., Arch. Biochem. Biophys. 64, 193 (1956).

83. Ray, P. M., Arch. Biochem. Biophys. 87, 19 (1960).

84. Ray, P. M. and Thimann, K. V., Arch. Biochem. Biophys. 64, 175 (1956).

85. Sastry, K. K. S. and Muir, R. M., Plant Physiol. 40, 294 (1965).

86. Sastry, K. K. S. and Muir, R. M., Science 140, 494 (1963).

87. Stahly, E. A. and Thompson, A. H., Univ. Md. Agr. Exp. Sta. Bull. A-104.

88. Still, C. C., Fukuyama, T. T., and Moyed, H. S., J. Biol. Chem. 240, 2612 (1965).

89. Street, H. E., Butcher, D. N., Handoll, C., and Winter, A., in Regulateurs Naturels de la Croissance Vegetale, Centre National de la Recherche Scientifique, Paris (1964) p. 529.

90. Teubner, F. G., Science 118, 418 (1953).

91. Tomaszewski, M., in Regulateurs Naturels de la Croissance Vegetale, Centre National de la Recherche Scientifique (1964), pp. 335-351.

92. Tukey, H. B., Science 84, 513 (1936).

93. Tukey, H. B., Went, F. W., Muir, R. M., and van Oberbeek, J., Plant Physiol. 29, 307 (1954).

94. Wright, S. T. C., J. Hort. Sci. 31, 196 (1956).

95. Zenk, M. H., in Regulateurs Naturels de la Croissance Vegetale, Centre National de la Recherche Scientifique, (1964) pp. 241-249.

96. Zenk, M. H. and Muller, G., Nature 200, 761 (1963).

97. Zinsmeister, H. D. and Hoellmueller, W., Plant 63, 133 (1964).

98. Zwar, J. A., Bruce, M. I., Bottomley, W., and Kefford, N. P., in Regulateurs Naturels de la Croissance Vegetale, Centre National de la Recherche Scientifique, Paris (1964), pp. 123-130.