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BARD

FINAL REPORT

PROJECT No. I-5-79

Concentration and Metabolism of Indolyc Growth Hormones in Fruit Trees

E. Epstein, R.S. Bandurski

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1983

1862

18-2-1982

Standard BARD Cover Page for Technical Reports

Date 20/1/82

BARD P.O.B. 6
Bet Dagan ISRAEL

BARD No. Of Proposal: I-5-79

Title of Proposed Research:

Concentration and Metabolism of Indolyl Growth Hormones in
Fruit Trees.

Name(s) of Investigator(s):

Principal Investigator: Ephraim Epstein

Cooperating Investigator: Robert S. Bandurski

Name and Address of Affiliated Institution (s)

Grantee Institution: Institute of Horticulture, Agricultural
Research Organization, The Volcani Center, Bet Dagan, Israel

Cooperating Institution: Dept. of Botany and Plant Pathology,
Michigan State University, E. Lansing, Michigan 48824 USA.

Project's starting date:

Type of Report:

Initial

Comprehensive

Final

Signature

Principal Investigator

Institution's Authorizing Official

E. Epstein

Evel Eshel

State of Israel
Ministry of Agriculture
AGRICULTURAL RESEARCH ORGANIZATION
The Volcani Center Bet-Dagan

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- 1) Fruit trees
- 2) Plants - Melibolism
- 3) Plant hormones
- 4) Plant growing media
- 5) Growth (Plants)

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SUMMARY

A microscale procedure was developed for the isolation, purification and quantitation of the plant hormone indole-3-acetic acid (IAA). This procedure allows assays of picogram quantities of endogenous IAA. Quantitation is done by the conversion of IAA to its pentafluorobenzyl ester and employment of electron capture gas chromatography detection and isotope dilution analysis. Greater sensitivity and selectivity can be attained by negative ion chemical-ionization gas-chromatography-mass spectrometry.

Free and conjugated IAA were determined by our method in olive and Wilking mandarine trees. Almost a three-fold increase in bound IAA was found in olive leaves and tissue following ethylene treatment. No dramatic changes in free IAA were noticed.

Seasonal fluctuations were found in IAA levels in olive and mandarine leaves. A gradual decrease in free IAA was seen during the period studied, and a large increase in bound IAA was found preceding flowering both in olive and mandarine trees which would give a low yield the coming year.

It is possible that the bound auxin serves as a regulatory pool of hormone in the plant and as a detoxification mechanism, and that the level of free IAA in the tree organs is controlled by variations of the ratio of the free and bound forms.

Studies of easy- and hard-to-root cuttings showed that IAA level decreased during rooting, but no correlation was found between the rooting process and the ratio of free (active) to conjugated (inactive) IAA in the cuttings.

GENERAL INTRODUCTION

The primary objective of our BARD proposal was to develop a microscale procedure for the isolation, purification and determination of the plant hormone indole-3-acetic acid (IAA) in tissue cultures and organs of fruit trees. With such a method on hand we planned to study factors which affect the concentration of IAA in tree crops such as citrus and olive.

Almost all current knowledge of in vivo control of hormone levels is based on studies of young seedlings of cereals. The availability of new chemical and physical methods for assays of IAA together with the ability to grow tissue cultures of fruit tree crops could permit the study of these economically important, but difficult-to assay plants.

Although bio-assays are available and are often used for IAA assay, such methods are limited since they measure the balance between growth promoters and inhibitors in the tissue. In order to measure the amount of endogenous IAA and its conjugates and products in the tissue we need methods which will yield pure IAA and IAA complexes that can be assayed by chemical means. Most of the work on auxin in trees was concentrated on the free IAA. Lately it was shown by Robert Bandurski and his group in Michigan State University that bound IAA is more abundant than free IAA in most plant tissues which were checked. The bound auxin probably serves as a regulatory pool of auxin in the plant and as a detoxification mechanism. It is very likely that the level of free auxin in the tree organs is controlled by variations of the ratio of free and bound auxin.

Beside regulating growth in plants auxins are known to participate in the regulation of physiological events. After we developed the new procedure for IAA determination, we decided to employ it for the study of some agriculturally-important phenomena in fruit trees. These were:

1. Alternate bearing in olive and Wilking mandarine;
2. Leaf abscission in olive following treatment with ethephon;
3. Rooting of easy-and-hard-to-root cuttings.

In addition the endogenous IAA content of olive callus tissues in relation to ethylene treatment was studied.

FREE AND CONJUGATED INDOLE-3-ACETIC ACID IN
ETHYLENE-TREATED LEAVES AND CALLUSES OF OLIVE

INTRODUCTION.

The effect of ethylene treatment on indole-3-acetic acid (IAA) levels in plant tissues has been studied using a variety of experimental systems. Unfortunately, no clear conclusion can be drawn from these studies. For example, reports of free IAA increasing following ethylene treatment (11) contradict studies showing decreased IAA levels (10,14,16,17). Similarly, studies showing increases in auxin conjugation (4,6) have been contradicted by reports showing no effect (8,9).

Some of these results can be explained by the difference in materials studied and the approaches taken. Of particular concern has been the extrapolations from IAA feeding experiments, the results of which probably have little to do with normal hormone metabolism. This is especially true in studies of auxin conjugation where feeding experiments show formation of amide and ester linked conjugates even in tissues normally devoid of one of these types of compounds (compare 1,2).

In this study changes in levels of free and conjugated indole-3-acetic acid resulting from ethylene treatment have been examined. An electron capture gas chromatographic procedure for IAA quantitation utilizing isotope dilution analysis was employed. The advantage of this method is that it has been validated on olive tissue by direct comparison to gas chromatography - selected ion monitoring - mass spectrometry (7).

These studies have examined changes in endogenous levels of free and bound IAA since these values should be indicative of changes induced by ethylene in the normal metabolic processes controlling auxin concentrations.

MATERIALS AND METHODS

Ethephon treatment of olive leaves - Olive branches were sprayed with 2000 ppm ethephon (adjusted to pH 7.0 with 0.1 M phosphate buffer) plus 0.03% Triton-x-100 and kept for 48 hrs in a humid atmosphere. Untreated olive branches were kept under identical conditions as control. After 48 hrs the treated leaves would fall at a slight touch. Forty gr of leaves from treated and untreated branches were used for extraction.

Ethylene treatment of olive calluses - Olive tissue cultures were prepared from bark on Double Inorganic Modified White medium (12) in 25x100 mm vials. After 6 weeks in 25⁰ (constant illumination) 15 vials were opened and inserted into a two-liter jar and hermetically sealed. Ethylene was injected into the jar through a rubber septum to make a final concentration of 100 ppm. Another 15 vials were kept under similar conditions with no ethylene as control. After 18 hours the ethylene concentration in both jars was measured by gas chromatography. The tissues were then removed from the vials, weighed and extracted.

Extraction - Leaves and calluses were ground in 70% acetone-water. Procedures for isotope dilution analysis, extraction,

solvent partitioning, high pressure liquid chromatography (HPLC), derivatization, a second HPLC step, and electron capture gas chromatography were exactly as described (7). The homogenate was divided into two equal fractions after the addition of ^{14}C -labeled IAA and one aliquot used for determination of free IAA. The second aliquot was reduced to water phase in vacuo hydrolyzed with 7N NaOH at 100 C for 3 hrs in a N_2 atmosphere as described (7). This procedure results in total hydrolysis of both ester and amide IAA conjugates.

RESULTS

Our studies show no differences in the levels of free IAA in leaves exposed to ethylene for 48 hr. (Table VI). It must be pointed out however, that the variation found in free IAA levels in this tissue was probably due to the fact that this material was field grown and was somewhat heterogenous with respect to leaf age. Nevertheless, it is clear that no consistent large change in free IAA level occurs in this tissue.

Olive tissues contain the bulk of their IAA as amide conjugates (Table VII) and have little or no ester IAA (unpublished results) as found by methods of selective hydrolysis previously described (2). Bound IAA levels show almost a 3-fold increase in 18 hrs, after ethylene treatment. It is important to note that these changes were seen in both intact leaves and in olive callus tissue.

Table VI. Effect of Ethephon treatment on content of free and bound IAA in olive leaves ($\mu\text{g/gr}$ fresh wt.) Sampling data June 1981.

Treatment	Free	Bound	Total
2000 ppm Ethephon	1.3 ± 0.45	5.1 ± 1.7	6.5 ± 1.3
0 ppm Ethephon	1.0 ± 0.27	1.8 ± 0.7	2.8 ± 0.7

Leaves (40 gr) were taken from fruitless branches, ground in aqueous acetone, IAA- 2^{14}C was added and amount of IAA was determined after conversion to the pentafluorobenzyl ester by gas chromatography using electron capture detector and isotope dilution analysis.

Table VII. Free and bound IAA levels in ethylene-treated and non-treated olive calluses ($\mu\text{g/gr}$ dry wt.)

Treatment	Free	Bound	Total
100 ppm ethylene	6.4 ± 3.0	46.7 ± 8.0	53.2 ± 8.2
0 ppm ethylene	7.6 ± 3.0	16.2 ± 4.5	22.8 ± 2.4

Calluses (20 gr) were exposed to 100 ppm ethylene or air for 18 hrs, macerated with aqueous acetone, IAA-2- ^{14}C added and IAA determined after conversion to the pentafluorobenzyl ester by gas chromatography using electron capture detector and isotope dilution analysis.

DISCUSSION

Our data show that free IAA levels following ethylene treatment do not change dramatically over the normal biological variation seen in this tissue. If alterations of IAA levels was responsible for any of the profound changes noted in the tissue after ethylene treatment then one might expect large changes in concentration.

This clearly is not the case. Changes of the magnitude noted by Lieberman and Knecht (13) for pea (about a 50% reduction) would probably be difficult to detect in olive which shows changes of this magnitude from branch to branch and day to day. Thus in olive, decreases in free IAA content of this magnitude can occur normally. These normal changes in IAA levels do not induce the profound physiological effects, such as leaf abscission, noted with ethylene treatment.

Studies of IAA content of intact plants and plant organs, however, ignore possible localized changes of IAA content which may be of profound importance. Recently Pengelly et al (15) showed that great differences in free and ester IAA content occurs between vascular and cortical tissue in the corn mesocotyl. It may be that IAA content of a target organ such as the abscission zone could also be differentially effected, yet these changes would be masked by the large amount of IAA extracted from the leaves.

Levels of bound IAA increase following ethylene treatment, and these results were demonstrated quite clearly and consistently. Previous reports only looked at conjugation of exogenous IAA in relation to ethylene treatment (4,6,8,9), and this may be quite different process from what occurs endogenously. Lieberman and Knecht (13) looked at changes in free plus ester IAA in Alaska pea, however both pea (2) and olive (7) contain little, if any, ester IAA. Thus, our data represents the first report of endogenous bound auxin changes accompanying ethylene treatment. We do not as yet know why bound IAA levels might increase. However, comparatively mild treatments which inhibit growth such as a light flash given to etiolated plants also results in increased conjugate formation (3). It may be that the accumulation of large amounts of a bound form of IAA which is resistant to enzymatic degradation (5) and may be compartmentalized differently from that of the free hormone has a unique role in the processes initiated by ethylene conjugate formation occurs as a result of the abscission process.

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FREE AND CONJUGATED INDOLE-3-ACETIC ACID LEVELS IN
LEAVES OF WILKING MANDARINE (CITRUS RETICULATA BLANCO)
AND OLIVE (OLEA EUROPA) IN RELATION TO FRUIT BEARING.

INTRODUCTION

Fruit trees in commercial groves usually do not bear equal crops every year. Most, including olive and the wilking mandarine, have a biennial cycle where a year of heavy fruit set ("on" year) is followed by a year of reduced bearing ("off" year). The causes for this biennial fruiting cycle, known commonly as alternate bearing, are largely unknown. Although enviromental conditions are known to effect the fruiting cycle, (5) endogenous factors appear to be of primary importance (5). Only a few preliminary investigations have been made previously concerning hormone changes in relation to the fruit set cycle (3,4). Therefore it would be of interest to determine changes in levels of free and bound indole-3-acetic acid (IAA) in leaves of tree crops over the biennial period.

MATERIALS AND METHODS

Second year leaves (40 g) were collected from freshly harvested branches of olive (Olea europaea L.cv Manzanillo) and citrus (Citrus reticulata) and ground immediately in 70% acetone-water.

Procedures for isotope dilution analysis, extraction, solvent partitioning, high pressure liquid chromatography (HPLC), derivatization, a second HPLC step and electron capture gas chromatography of IAA were as described (2). The homogenate was divided into two equal fractions after the addition of ^{14}C -labeled IAA and one aliquot used for the determination of free IAA. The second aliquot was reduced to the water phase in vacuo and hydrolyzed with 7 N NaOH at 100 C for 3 hr. in a N_2 atmosphere as described (2). This procedure results in total hydrolysis of both ester and amide linked IAA conjugates (1). The hydrolyzed fraction was acidified to pH 2.5 and the total (free plus bound) IAA determined as described (2).

These methods have been previously shown to be applicable to resinous materials, such as olive leaves (2).

RESULTS & DISCUSSION

IAA levels showed seasonal fluctuations in olive leaves (Fig 7). A gradual decrease in IAA content was seen over the period studied. During flowering (March) and fruit set no changes in leaf free IAA was noted. The bound IAA content, however, showed a more dynamic situation with a large increase in bound IAA preceding flowering in which would subsequently produce low fruit yields (Fig.7). Although large differences existed in bound IAA levels in these two phases of the biennial cycle, no outward physical differences were noted until May, when flowers drop off without setting fruit in the off years trees.

Levels of bound IAA was especially high in leaves which were taken from flowerless branches in April (Table VIII) or from branches with no fruitlets in May (Table IX).

Similar results were obtained with leaves of Wilking mandarine. However, the yellowed appearance of the leaves from June flush of the "on" trees suggested that younger leaves might be more appropriate material for the study of IAA levels for this variety. Samples taken just prior to flowering (31/3/81) showed that young leaves contained large amounts of bound IAA in their "off" year (Table X). Thus the "off" year was characterized by increases in bound IAA in both olive and citrus leaves. Because of the biennial nature of this phenomenon, a completed cycle has yet to be examined. Future studies will be conducted over several seasons and will examine more closely IAA changes in leaves of different ages.

Both free and bound IAA were found in floweres and fruits of olive and Wilking mandarine (Tables XI,XII,XIII).In the olive (Table XI) both the flowers and the fruitlets contained free and bound IAA, and as in the leaves, the amount of the bound exceeded the emount of the free. In the mandarine flowers, the level of free IAA was rather higher (6.9 $\mu\text{g}/\text{gr}.$) and exceeded the level of the bound IAA (5.5 $\mu\text{g}/\text{gr}$) (Table XII). The IAA in the fruit was found mainly in the rind (3.2 $\mu\text{g}/\text{gr}.$ dry wt. total IAA, Table XIII) and less in the pulp and seeds. There was much more bound IAA than free IAA.

מכון ויצמן למדע
המחלקה להנדסה חקלאית
תל-אביב

LEGEND

Fig. 7. Free and bound IAA content of olive leaves during the year.

- ▲—▲ Leaves taken from a tree which yielded 34kg. olive in 1980;
○—○ Leaves taken from a tree which yielded 5kg. olive in 1980.

Leaves (40g) were macerated in aqueous acetone. IAA-2-¹⁴C was added and free and bound IAA were determined by gas chromatography with electron capture detector after conversion to the pentafluorobenzyl ester^{and} by isotope dilution analysis.

Bound IAA was hydrolyzed with 7N KOH in 100 C for 3 hours.

Values for October, March and May are the average of 3 determinations (SE[±]0.3), other values are single determinations.

FREE

FLOWERING

↓ FRUIT SET

BOUND

OCT. NOV. DEC. JAN. FEB. MAR. APR. MAY. JUNE.
MONTH (1980-1981)

Table VIII: Free and bound IAA levels ($\mu\text{g/gr.fr.wt.}$) in leaves from branches with and without floweres from "off" and "on" olive trees. (sampling date Apr. 14th).

	Free		Bound	
	Off	On	Off	On
with flowers	0.6	3.0	8.1	9.2
without flowers	0.4	0.7	23.2	3.3

Table IX: Free and bound IAA levels ($\mu\text{g/gr.fr.wt.}$) in leaves from olive branches with and without fruit (Sampling date May 23th).

	Free	Bound
with fruit	1.0	6.3
without fruit	1.9	13.4
with dead fruit	1.2	13.3

Table X: Free and bound IAA levels ($\mu\text{g}/\text{gr. fresh wt.}$) in mature and young leaves of Wilking mandarine (Sampling date March 31st 1981).

	Mature leaves ¹		Young leaves ²	
	Free	Bound	Free	Bound
Off ³	3.2	7.6	0.4	6.0
On ⁴	3.6	9.0	0.2	18.0

1. Flush of June 1980
2. New flush
3. Tree with no fruit
4. Tree with 120 kg. fruit

Forty gr. of leaves from 'off' or 'on' trees were macerated in aqueous acetone, IAA-2-¹⁴C added and free and bound IAA were determined by gas chromatography with electron capture detector after conversion to the pentafluorobenzyl ester by isotope dilution analysis. bound IAA was hydrolyzed by 7N KOH in 100 C for 3 hours.

Table XI: Free and bound IAA levels ($\mu\text{g/gr.fr.wt.}$) in olives flowers and fruit from "off" and "on" trees. (Sampling date, flowers, May 2nd, fruit, May 27th).

	Flowers		Fruit	
	Free	Bound	Free	Bound
Of	0.7 ± 0.5	1.2 ± 0.2	-	-
On	0.4 ± 0.3	1.2 ± 0.7	1.7 ± 0.3	2.6 ± 0.5

Table XII: Free and bound IAA levels ($\mu\text{g/gr.fr.wt.}$) in flowers of "Wilking" mandarine (sampling date April 7th).

Free	Bound	Total
6.9 ± 1.1	5.5 ± 0.1	12.4 ± 1.0

Table XIII: Free and bound IAA levels ($\mu\text{g/gr. dry wt.}$) in fruit of "Wilking" mandarine (sampling date April 2nd).

	Free	Bound	Total
seeds	0.04	0.90	0.94
Rind	0.47	2.73	3.20
Pulp	0.04	1.16	1.20

% of dry weight: Seeds - 44.6%

Rind - 25.5%

Pulp - 16.0%

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LEVELS OF FREE AND BOUND IAA IN CUTTINGS
OF EASY-AND HARD TO ROOT VARIETIES OF GRAPEVINE
(VITIS VINIFERA) AND ROSE. (ROSA SP.)

INTRODUCTION

Since 1934, when Thimann and Went found that IAA could stimulate rooting in stem cuttings (5) there have been numerous papers on the subject. It was found that plants that normally root easily will usually respond readily to auxins, while poor rooters are much less responsive (4). Some other factors such as other growth regulators, juvenility, environment, light, nutrition, seasonal variations, and varied differences can affect the rooting of the cuttings (2). The endogenous content of IAA was determined in rooted and unrooted hardwood grapevine cuttings (2), but no comparison of free and bound IAA or a definite identifications of the auxin was made.

MATERIALS AND METHODS

Preparation of cuttings -

Two cultivars of grapevine, Vitis vinifera cv. Alphonse levalle (easy-to-root) and cv. SO₄ (hard-to-root), and two cultivars of Rose Rosa indica (easy-to-root) and R. canina (hard-to-root), were used. Single-node cuttings were dipped in 1% Benlett powder with or without 1% potassium salt of indolebutyric acid (KIBA) in talcum, according to treatment. Cutting surface was renewed before treatment.

The cuttings were kept for 12 days in 20 cm. deep Dutch peat and shredded polystyrene foam mixture at 1:2 ratio under mist spray in a glass house. Air temperature was between 20 to 25° .

Extraction - The bases of the cuttings (to the first node) were ground in 70% aqueous acetone containing isotope labeled IAA.

IAA was extracted and both free and bound IAA were extracted and determined by the method of Epstein and Cohen (1).

RESULTS AND DISCUSSION

It was reported (Odom & Carpenter, 4) that a higher percent of rooting is correlated with a higher auxin content in the cuttings. The higher the auxin content, the more likely it is that the cuttings will root. Nakano (3) showed that in hardwood cuttings of grapevine the activity of IAA-like substances in the cuttings was high at planting time in the well-rooted cuttings. In the unrooted cuttings the level of the auxin-like substances did not change. Similar results were obtained in our work: The easy-to-root grapevine cv. Alphonse levalle had a higher IAA content before rooting than the hard-to-root cv SO₄ (Table XIV). On the other hand, after IBA treatment IAA content decreased only in the cultivar which formed roots. The increase of IAA level in the treated cv SO₄ was probably due to the development of a callus at the base of the cuttings.

In the rose cuttings we did not include a treatment with KIBA. It is evident, however, (Table XV) that like in the grapevine, the level of IAA in the cutting which rooted (R. indica) is lower than in these which did not root (R. canina).

Although auxins play a very important part in the rooting, this process is probably not regulated by the ratio of free (active) to conjugated (inactive) IAA.

Table XIV: Free and bound IAA ($\mu\text{g/gr.fr.wt.}$) in Vitis vinifera cv Alphonse leleve (easy-to-root) and cv SO₄ (hard-to-root) with and without pretreatment with 1% potassium salt of indolebutyric acid (KIBA). Extraction was done 12 days after treatment.

Variety	No KIBA		1% KIBA	
	Free	Bound	Free	Bound
Alphonse ¹	5.1	6.5	6.5	2.4
SO ₄ ²	1.2	1.1	2.2	3.0

¹Without KIBA treatment there were 1-10 roots after 12 days, and with KIBA a big clump of roots.

²Without KIBA there were at the most 2 roots, with KIBA a callus was formed.

Table XV: Levels of free and bound IAA ($\mu\text{g/gr.fr.wt.}$) in cuttings of Rose indica (easy-to-root) and Rosa canina (hard-to-root). Extraction 12 days after planting.

	Free	Bound	Total
R. indica	0.8	0.8	1.1
R. canina	1.9	1.9	2.2

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EVALUATION OF THE RESEARCH ACHIEVEMENT WITH RESPECT TO THE
ORIGINAL RESEARCH PROPOSAL

The primary objective of our BARD proposal was to develop a microscale procedure for the isolation, purification and determination of the plant formone indol-3-acetic acid (IAA) in tissue cultures and organs of fruit trees. This goal was achieved and the procedure was developed and published and it is now being used in many plant hormone labs in the world. However, in order to do this work a high performance liquid chromatograph (HPLC) was essential, and we found it necessary to transfer money which was allotted for a lab technician to purchase this instrument. Unfortunately, our request for a lab technician for the second year of the grant was denied categorically. Therefore we had to make some changes in our original proposal, i.e. we concentrated more on work that could be done without a lab technician. For this reason the proposed work on the effect of external conditions on the ratio of free to conjugated IAA in tissue cultures was omitted and instead we checked the level of free and conjugated IAA in leaves and fruit of olive and Wilking mandarine in relation to alternate bearing or following ethylene treatment and in rooting of easy- and hard-to-root cuttings of grapevine and roses.

PUBLICATIONS

- Epstein, E. (1981). A new method for quantification of indoleacetic acid by HPLC, gas chromatography and isotops dulution. Isr.J.Bot. 30:49.
- Epstein, E. and J.D. Cohen (1981). Microscale preperation of pentaflouro-benzyl esters. Electron-capture gas chromatographic detection of indol-3-acetic acid from plants. J. Chromatogr. 209:413-420.
- Epstein, E. (1981). Concentration of free and bound indole-3-acetic acid (IAA) in leaves of fruit-bearing and barren olive and citrus. Plant physiol. 67(5):4.
- Epstein, E. Free and conjugated indole-3-acetic acid in ethylene-treated leaves and calluses of olive.(In preparation).
- Epstein, E. Free and conjugated indole-3-acetic acid leavls in leaves of Wilking mandarine (Citrus reticulata Blanco) and olive (Ulea europea) in relation to fruit bearing. (In preparation).
- Epstein, E. and J.D. Cohen (1980). Microscale preparation of indole-3-acetic pentafluorotoloene for electron capture detection - gas chromatography. Plant. Physiol. (Suppl.) 65:76.

DESCRIPTION AND RESULTS OF COOPERATION

Research support from BARD has allowed a close cooperative research effort to be conducted between our two laboratories. Dr. Jerry Cohen spent March 1980 at the Volcani Center working on development of a new electron capturing derivative for gas chromatographic analysis of Indole-3-Acetic Acid. He helped with the adaptation of this derivative for use with the instrumentation available at the Volcani Center and in confirming the results we obtained by using gas chromatography-mass spectrometry. Dr. Bandurski's group has continued to study the metabolism of corn seedling, especially the role of auxin conjugates in hormonal regulation. Our cooperative work on tree crops has shown that conjugates account for much of the Indole-3-Acetic Acid in mature tissues and that the levels of these compounds change during development and following ethylene treatment. These studies confirm and extend those obtained on young seedlings.

In October 1981 Dr. Epstein traveled to East Lansing to meet with Dr. R.S. Bandurski and Dr. J.D. Cohen for the purpose of consultation, writing of the final report and scientific papers and making plans for future work.