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# STUDIES ON THE EFFECT OF CALCIUM ION CONCENTRATION ON GROWTH, LIGNIFICATION AND PEROXIDASE ACTIVITY IN TOBACCO TISSUE

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STUDIES ON THE EFFECT OF CALCIUM ION CONCENTRATION ON GROWTH, LIGNIFICATION AND PEROXIDASE ACTIVITY IN TOBACCO TISSUE

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## ABSTRACT

Several cellular parameters were studied in W-38 tobacco callus tissues grown on media with five levels of calcium ion concentration. Tissues grown on 3 times the "normal" level of [$Ca^{++}$] showed no significant differences in growth or lignification when compared to the control level. Tissues grown at 1/100, 1/20 and 1/10 of the "normal" level of [$Ca^{++}$] showed fresh weight and dry weight yields lower than the 1 X control level. The non-extractable materials, however, contributed a progressively higher percentage to the dry weight. No correlation between scopolin and/or scopoletin concentrations in the tissues and the lignin content was possible due to the variable results obtained for the concentration of these substances in the tissues. Although reductions in the dry weight, as the [$Ca^{++}$] was reduced, gave an apparent progressive increase in lignin, as a result of expressing it as a % of dry weight, the three lower levels of $Ca^{++}$ concentration gave the same total yield of lignin. Thus $Ca^{++}$ concentration in the medium apparently has a direct effect on lignification rather than an indirect one related to reduced growth.

In another set of experiments using the above system, growth of tissues on higher or lower than the "normal" [$Ca^{++}$] in the medium resulted in increased soluble-peroxidase activity and in increased amounts of three cathodic isoperoxidases. Anodic electrophoretic analyses of

extracts of tissues grown on reduced [$Ca^{++}$] in the medium revealed changes in the relative amounts of the four observed anodic isoperoxidases. The amount of one rapidly migrating anodic isoperoxidase, $A_3$, was nearly the same for the tissues grown on low [$Ca^{++}$] as in tissues grown on the "normal" level of [$Ca^{++}$] in the medium. $A_1$ and $A_2$, two slowly migrating anodic isoperoxidases, were increased in tissues grown on reduced [$Ca^{++}$] in the medium.

The amount of peroxidase activity loosely associated with membranes is greater in tissues grown on lower than "normal" [$Ca^{++}$] in the medium and is probably associated with increased lignification. A preliminary electrophoretic analysis of the membrane-associated peroxidase for tissues grown on agar-containing medium with "normal" [$Ca^{++}$] indicates that there was some specificity as to the binding of isoperoxidases to the membrane. Little or no anodic isoperoxidase activity was membrane-associated. One cathodic isoperoxidase, $C_4$, which was not present in soluble extracts was found in [$Ca^{++}$] extracts of washed membranes.

Two anodic isoperoxidases were separated by isoelectric focusing. Preliminary characterization of these individual isoperoxidases indicates that they differ in physical, kinetic, and regulatory properties particularly in their regulation by scopoletin.

Based on these studies, possible physiological roles are suggested for some of the isoperoxidases.

# STUDIES ON THE EFFECT OF CALCIUM ION CONCENTRATION ON GROWTH, LIGNIFICATION AND PEROXIDASE ACTIVITY IN TOBACCO TISSUE

## CHAPTER I

## THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON GROWTH, LIGNIFICATION, AND CONCENTRATION OF SCOPOLIN AND SCOPOLETIN IN TOBACCO CALLUS TISSUE

### Introduction

Scopoletin and its 7-glucoside, scopolin, (Fig. I-1) are secondary plant metabolites. These compounds are not found in all higher plants but are frequently constituents of solanaceous plants (Harborne and Simmonds, 1964). Although other glycosides of scopoletin have been reported in plants (Sargent and Skoog, 1961; Yang, 1958), scopolin is apparently its most common glycoside in nature.

Scopolin has been shown to increase in concentration in tobacco plants which have been subjected to a wide variety of stress conditions. Sequeira (1967) reported that scopolin and scopoletin accumulated in the region of infection after plants were infected with the wilt-inducing bacterium *Pseudomonas solanacearum*. Increases in scopoletin generally paralleled those of scopolin during early stages of infection but scopoletin increased very rapidly to seventeen times normal as the disease progressed. Koeppe and coworkers at the University of Oklahoma have

shown that scopolin increased in concentration in tobacco plants which had been "stressed" by low temperature (1970a) and by excess amounts of ultraviolet (1969) and X-ray irradiation (1970b). Treatment of tobacco plants with the herbicides: "2,4-D" (Dieterman *et al.*, 1964), "Tordon" (Wender, 1970), and maleic hydrazide (Winkler *et al.*, 1969) have also been reported to result in increased scopolin concentration. Armstrong *et al.* (1970, 1971) have demonstrated the accumulation of scopolin in tobacco plants that were deficient in $Mg^{++}$, $K^{+}$, and $NO_3^{-}$. Substantial increases in the concentration of scopolin have been reported by Watanabe and coworkers in boron deficient tobacco plants (1961). Thus increases in scopolin have been reported in whole tobacco plants as a result of infection, mineral deficiency, various adverse environmental conditions, and treatment with herbicides.

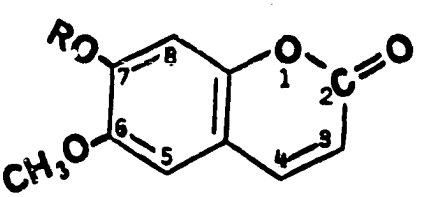



Fig. I-1 SCOPOLIN AND SCOPOLETIN

Scopoletin, R = H
Scopolin, R = Glucosyl

Tissue culture of tobacco callus has also been employed to study conditions under which the levels of scopolin and scopoletin are increased. Treatment of W-38 tobacco callus tissue in culture with varying amounts of rubber dust and carbon black has been shown to result in

increases in proline content as well as increases in the levels of scopolin relative to controls (Smith, Nau, and Wender, 1969). The nutritional requirements of W-38 and the effect of indoleacetic acid (IAA) and kinetin on scopolin and scopoletin levels have been among the numerous studies conducted on this cell line by Skoog and coworkers at the University of Wisconsin (1960).

Wender has enumerated a series of speculative possibilities which might explain the accumulation of scopolin in stressed tobacco tissues (1968). One of these was that if lignification were blocked during stress, then scopolin might accumulate. This speculative possibility was based on a biosynthetic scheme shown in Fig. I-2 which summarizes, in part, the work of Gamborg (1967); Hanson, Zucker, and Sondheimer (1967); Hanson (1966); and Steck (1967). A block on the way to lignin might result in a build up of glucosidoferulic acid and, thus, subsequently to an accumulation of scopolin. This speculative possibility was also suggested by the work of Dutta and McIlrath (1964) who had shown that the total lignin content of boron deficient sunflower tissue in culture decreased relative to those receiving adequate boron. Since boron deficiency in tobacco had been shown to cause an accumulation of scopolin in whole tobacco plants (Watanabe *et al.*, 1961), the suggestion of Wender seemed to be a reasonable possibility.

Work by Lipetz (1962) has shown histologically that high $Ca^{++}$ concentration in the medium resulted in a reduction of lignification in sunflower gall tissue in culture. Other tissue which he indicated showed similar results was tobacco gall, habituated tobacco, and freshly isolated carrot tissue. Since the reduction in lignification was

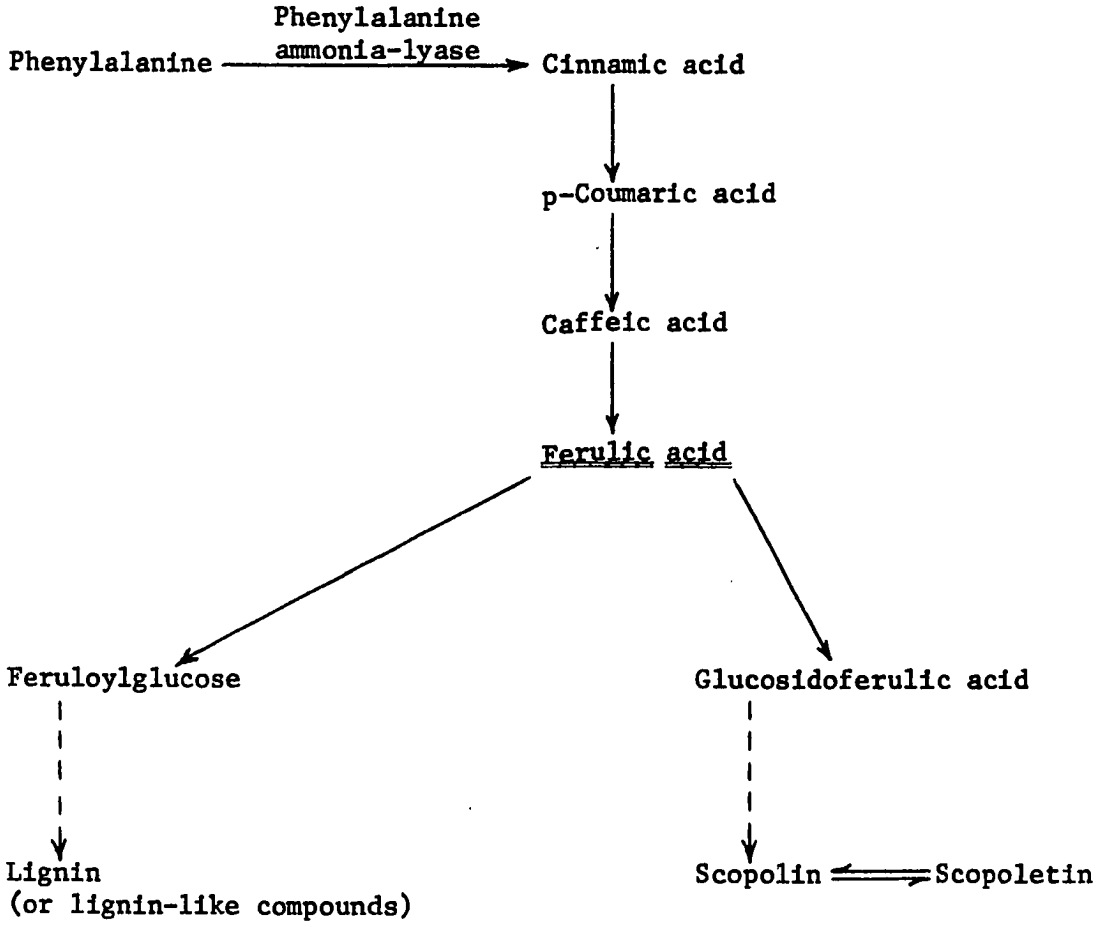



Fig. I-2 A proposed abbreviated biosynthetic route to scopolin and lignin from phenylalanine (from Gamborg, 1967; Hanson, Zucker, and Sondheimer, 1967; Hanson, 1966; and Steck, 1967).

observed when $CaCl_2$ replaced the $Ca(NO_3)_2$ used for the main study and did not occur when $KNO_3$ replaced the excess $Ca(NO_3)_2$, Lipetz concluded that the effect is due to $Ca^{++}$ and not to the anion. Work by Lipetz and Garro (1965) showed that this phenomenon may be the result of the release of peroxidase into the medium when tissues were grown on a medium containing high calcium ion. Parish and Miller (1969), in a study on the effect of calcium and phosphate on maturity and lignification in wheat, have shown that the degree of lignification in the internodes was greater in plants grown on low calcium. They also reported that the lignin content of internodes of plants receiving medium and high calcium treatments were not significantly different.

The work of Lipetz and of Parish prompted the author to initiate the present study which uses the variation of the concentration of calcium ion in the medium to study the relationship between scopolin, scopoletin, peroxidase (discussed in Chapter II), and lignification in W-38 tobacco tissue in culture. Results of a preliminary calcium study have been published elsewhere (Schafer and Wender, 1970) and are not included here.

## Materials and Methods

### Growth of Tissue

Cultures of W-38 tobacco (*Nicotiana tabacum* L., Wisconsin 38) callus tissue were employed for these studies. Cultures were originally given to Dr. S. H. Wender by Dr. Folke Skoog of the University of Wisconsin and have been maintained for four years in our laboratory by being transferred at 4-6 week intervals.

For growth in these experiments, tissues were supported by a piece of filter paper which was folded so as to adhere to the tube at the surface of 25 ml of liquid medium in 25 mm x 200 mm test tubes. This means of support of the tissue, as described by Heller (1965), avoids the use of agar which contains calcium. The revised medium (RM-1964) of Linsmaier and Skoog (1965) was used with 2 mg/l of indoleacetic acid (Matheson, Coleman, and Bell) and 200 µg/l of kinetin (Nutritional Biochemical Corporation), plus an increased amount of thiamine hydrochloride (1 mg/l). Eight tubes were inoculated for each of five levels of $CaCl_2 \cdot 2H_2O$: 4.4 mg/l; 22 mg/l; 44mg/l; 440 mg/l (which is the normal amount in the Linsmaier-Skoog medium); and 1320 mg/l. These will be referred to as 1/100 X Ca, 1/20 X Ca, 1/10 X Ca, 1 X Ca, and 3 X Ca, respectively.

Pieces of tissue approximately 8 mm in diameter and 2 mm thick were cut from five to six week old stock cultures which had been maintained on Linsmaier-Skoog medium with 2 mg/l indoleacetic acid and 200 µg/l kinetin with agar as support. All transfers were conducted in a laminar flow hood (Agnew-Higgins model M-142) using standard sterile technique. Eight pieces of tissue were placed in each of five sterile disposable Petri dishes and weighed. Weights were adjusted so that the amount of total inoculum in each level would be within ± 5% of each other. These pieces of tissue were then used to inoculate the tubes for the experiment.

Inoculation was conducted so that the weight of each piece of tissue could be determined. The five dishes were weighed on a type H6T Mettler balance. One piece of tissue from each dish was used to

inoculate one tube for each level, and the dishes were reweighed. This was repeated until the eight tubes for each level had been inoculated. The inoculated tubes were covered with flamed aluminum foil and allowed to stand vertically for 35 days at room temperature in a continuously lighted room where they received reflected light.

## Harvest and Extraction of Tissue

The fresh weight of each piece of tissue was determined. The eight pieces of tissue for each level were pooled and "fixed" by boiling in isopropyl alcohol-water azeotrope for five minutes. The "fixed" tissue was then ground in a blender at high speed and extracted, using the procedure of Wilson *et al.* (1968). Samples were then diluted to a final volume of 50 ml in a mixture of isopropyl alcohol : benzene : water (3:1:1) and were held at 0-4°C for subsequent analysis. Harvest weights were determined on a Mettler type H6T balance.

## Non-Extractable Residue Weight

For each sample, the residue remaining in the extraction thimble following the extraction was transferred to a preweighed sintered glass filter using isopropyl alcohol to effect the transfer. These were then subjected to repeated heating at 110°C, cooling in a desiccator, and weighing (Sartorius Analytical balance) until a constant weight was obtained. Due to the strong affinity of this material for water, it was necessary to correct for the absorption of water during weighing. This was accomplished by weighing the "n" filters in noted order and then reweighing the first three. In this way the average gain per "n + 1" weighings was determined. The average gain per weighing was calculated,

and the weight for each filter was corrected. The following is given as an example of the calculation: (corrected wt. #1) = (observed wt. #1) minus (1 X average wt. gain), (corrected wt. #2) = (observed wt. #2) minus (2 X average wt. gain), ... etc. The calculations were made on an Olivetti Underwood Programma 101. This correction procedure is based on the assumption that the amount of water absorbed is proportional to the length of time after the desiccator is first opened. This was apparently a reasonable assumption as only with the correction did weights become constant. The order in which the samples were weighed was varied from day to day to minimize any ordering effect.

## Extractable Residue Weight

The amount of extractable material in each sample was determined by transferring a 10 ml aliquot of each extract to a preweighed weighing bottle, removing the solvent in a vacuum oven, and repeated weighing after heating at 110°C until a constant weight was obtained. A Sartorius Analytical balance was used for all weighings.

## Dry Weight

Dry weight for each sample was calculated by adding the non-extractable residue weight and the extractable residue weight for the sample.

## Lignin Determination

Lignin was determined by a procedure based on that employed by Bergmann (1964). A sample of non-extractable residue (approximately 100 mg), weighed by difference after storage in a desiccator overnight, was hydrolyzed by shaking for 24 hrs in 15 ml of 72% sulfuric acid at room

temperature. The samples were then diluted to 3% sulfuric acid and subjected to further hydrolysis by autoclaving for 1 hr at 15 lbs pressure (121°C). The samples were then cooled, filtered through preweighed sintered glass filters, washed free of sulfate by repeated washing with distilled water (using 1 N $Ba^{++}$ to test for sulfate), and weighed to constant weight by repeated heating at 110°C.

## Scopolin Determination

The concentration of scopolin in the samples was determined by the paper chromatographic-fluorescence procedure used by Koeppe and coworkers at the University of Oklahoma (1969). Known amounts of pure scopolin were chromatographed with each set of samples, and the concentration of scopolin in each sample determined was calculated via a linear least squares program executed on an Olivetti Underwood Programma 101 Computer.

## Scopoletin Determination

The concentration of scopoletin in the samples was also determined by a paper chromatographic-fluorescence procedure. Aliquots of the sample were streaked on a 9¼ inch sheet of Whatman #1 paper which had been washed 24 hrs with 5% MeOH-$H_2O$ and dried approximately 2 hrs. These were then developed for 6 hrs in a descending chromatographic chamber in a mixture of isopropyl alcohol : formic acid : water (5:0.1:95). After the papers were dried, the positions of the zones corresponding to scopoletin were identified by fluorescence under ultraviolet radiation, circled with a pencil, cut out and eluted with 5% MeOH-$H_2O$. The eluates were diluted to volume in 5% MeOH-$H_2O$, and the quantity of

scopoletin was determined by measuring the fluorescence in a Turner Model 110 Fluorimeter. The quantity of scopoletin was determined by comparison with a curve established by running known quantities of pure scopoletin through the entire chromatographic and fluorescence procedures or by calculation via a least squares program executed on an Olivetti Underwood Programma 101 Computer using simultaneously run known amounts of pure scopoletin.

## Statistical Calculations

Means, standard deviations, and scopolin-scopoletin determinations were calculated utilizing an Olivetti Underwood Programma 101 Computer. The program for means and standard deviations was modified from a standard Olivetti program, and the standard deviations, s, obtained were based on the following formula:

$$s^2 = \frac{\Sigma x_i^2}{n - 1} - \frac{(\Sigma x_i)^2}{n(n - 1)}$$

This formula takes into account that a finite number of samples are used and, thus, has one degree of freedom removed. To test calculated means for significant difference, the confidence interval for the desired level of confidence was calculated using the method and "t" values given by Laitinen (1960). If the confidence intervals for two mean values were non-overlapping, they were considered statistically different.

The linear least squares program used for scopolin and scopoletin determinations was written using the equations and sample calculations of Laitinen (1960).

## Results

By varying the calcium concentration in the medium, various cellular parameters were affected. Visually there is a qualitative difference in the appearance of tissues grown at the various levels. After 35 days of growth, tissues show a notable increase in color (brown) as the amount of calcium ion in the medium is reduced. Tissues grown at 3 X Ca are very white, those at 1 X Ca generally white with some light brown areas, while those grown on 1/10 X Ca and 1/20 X Ca were progressively darker brown, and at 1/100 X Ca most of the tissue is generally dark brown in color. There is variation in color from piece to piece within a level and in individual pieces, but the general trend is quite striking. Although there was variation at the intermediate levels (1/10 X Ca and 1/20 X Ca), this general trend toward browner tissues at low $Ca^{++}$ levels was observed in all four experiments. Similarly, the non-extractable residues (to be discussed later) showed the same general darkening trend at lower calcium ion levels.

The yields (fresh weight) resulting from pooling eight pieces of tissue for each of the five levels are summarized in Table I-1. These data show that although there is considerable variation in yield for a given level from experiment to experiment, there is a significant reduction in fresh weight at lower calcium ion levels. This trend is evident in three of the experiments even for the 1/10 X Ca level although the mean value for this level in the four experiments is not statistically different from the 1 X Ca level (at a 95% confidence level). The mean values for the 1/20 X Ca and 1/100 X Ca levels show a significant reduction in fresh weight when compared to the normal level. The yield

of tissues from 3 X Ca is not significantly different from that at the normal calcium level.

Each piece of tissue used for inoculum was weighed. Although the mean values of the weight of the inoculum per tube for a level varied from 0.1565 g to 0.1315 g, the range within any given experiment was much smaller. Correction of the pooled fresh weight data (Table I-1) for the weight of inoculum neither reduces the variability nor affects the interpretation of the results. Thus, the variation in fresh weight per piece could not be due to differences in the weight of inoculum.

Table I-2 shows the mean values and standard deviation of the individual fresh weight per piece for each level of the four experiments. These values, of course, show the same trends as the pooled fresh weights in Table I-1, but also indicate that a large part of the variation in fresh weight is a result of variable growth from piece to piece at a given level within each experiment. This is particularly remarkable for tissues grown at 3 X Ca. Thus variation in fresh weight is due to variation in growth of the individual pieces of tissue at a given level (Table I-2) within a given experiment and to variation in growth of all levels from experiment to experiment (Table I-1), but is not the result of the weight of the inoculum used.

Calcium ion concentration also affects the amounts of "extractable" and "non-extractable" material in the tissues. These are summarized for the four experiments in Table I-3 and Table I-4, respectively, and represent the total weight from 8 pieces of tissue. The net amount of extractable material, extractable residue weight (ERW), is reduced as the amount of calcium ion in the medium decreases. The ERW of the

TABLE I-1

THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON FRESH WEIGHT

| EXPT. | CALCIUM CONCENTRATION X=440 mg/l $CaCl_2 \cdot 2H_2O$ | | | | |
|---|---|---|---|---|---|
| | 1/100 X | 1/20 X | 1/10 X | 1 X | 3 X |
| | Pooled weights in g | | | | |
| 1 | 5.7187 | 7.8587 | 10.0534 | 16.9298 | 13.6505 |
| 2 | 6.5450 | 7.5223 | 12.0754 | 11.6207 | 10.5213 |
| 3 | 6.9689 | 8.7537 | 10.2180 | 18.0138 | 20.7071 |
| 4 | 4.5396 | 5.2526 | 7.2333 | 13.0067 | 16.0365 |
| ave. | 5.9430 | 7.3468 | 9.8950 | 14.8927 | 15.2288 |
| ± s | 1.0699 | 1.4897 | 1.9973 | 3.0634 | 4.2940 |

s = standard deviation

TABLE I-2

THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON FRESH WEIGHT

| EXPT. | CALCIUM CONCENTRATION X=440 mg/l $CaCl_2 \cdot 2H_2O$ | | | | |
|---|---|---|---|---|---|
| | 1/100 X | 1/20 X | 1/10 X | 1 X | 3 X |
| | Average weight per piece ± s* in g | | | | |
| 1 | 0.7148 ±0.0734 | 0.9823 ±0.2840 | 1.2566 ±0.3138 | 1.8662 ±0.7304 | 1.7063 ±0.4102 |
| 2 | 0.8181 ±0.1081 | 0.9402 ±0.2088 | 1.5094 ±0.6443 | 1.4525 ±0.5228 | 1.3151 ±0.2621 |
| 3 | 0.8711 ±0.3336 | 1.0942 ±0.3385 | 1.2772 ±0.5015 | 2.2517 ±0.7332 | 2.5583 ±0.8815 |
| 4 | 0.5674 ±0.2200 | 0.6565 ±0.2109 | 0.9041 ±0.4208 | 1.6253 ±0.5701 | 2.0045 ±1.2494 |

*s = standard deviation

TABLE I-3

THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON THE EXTRACTABLE RESIDUE WEIGHT (ERW)

| EXPT. | CALCIUM CONCENTRATION X=440 mg/l $CaCl_2 \cdot 2H_2O$ | | | | |
|---|---|---|---|---|---|
| | 1/100 X | 1/20 X | 1/10 X | 1 X | 3 X |
| | Weight in mg | | | | |
| 1 | 54.4 (31.4)* | 79.5 (40.6)* | 95.0 (40.6)* | 207.5 (54.7)* | 192.5 (57.0)* |
| 2 | 75.5 (39.6) | 87.5 (42.8) | 169.0 (51.1) | 164.5 (54.1) | 160.5 (53.8) |
| 3 | 68.5 (32.7) | 85.0 (40.6) | 155.0 (50.0) | 169.0 (53.1) | 163.0 (55.0) |
| 4 | 65.0 (40.0) | 62.0 (35.0) | 79.5 (37.4) | 187.5 (55.4) | 257.5 (60.7) |
| ave. | 65.8 (35.9) | 78.5 (39.8) | 124.6 (44.8) | 182.1 (54.3) | 193.3 (56.6) |
| ± s | 10.0 ( 4.5) | 10.0 ( 3.3) | 43.5 ( 6.8) | 17.3 ( 0.9) | 44.7 ( 3.0) |

s = standard deviation

* Values in parentheses are the extractable residue weights expressed as a percentage of dry weight, i.e. of the sum of the non-extractable residue plus the extractable residue weight for each level.

TABLE I-4

THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON THE NON-EXTRACTABLE RESIDUE WEIGHT (NER)

| EXPT. | CALCIUM CONCENTRATION X=440 mg/1 $CaCl_2 \cdot 2H_2O$ | | | | |
|---|---|---|---|---|---|
| | 1/100 X | 1/20 X | 1/10 X | 1 X | 3 X |
| | Weight in mg | | | | |
| 1 | 119.2 (68.6)* | 116.4 (59.4)* | 138.8 (59.4)* | 171.8 (45.3)* | 145.5 (43.0)* |
| 2 | 115.4 (60.4) | 116.9 (57.2) | 162.0 (48.9) | 139.3 (45.9) | 137.8 (46.2) |
| 3 | 140.9 (67.3) | 124.2 (59.4) | 155.2 (50.0) | 191.1 (46.3) | 199.0 (45.0) |
| 4 | 97.3 (60.0) | 115.2 (65.0) | 133.4 (62.7) | 150.7 (44.6) | 166.5 (39.3) |
| ave. | 118.2 (64.1) | 118.1 (60.2) | 147.3 (55.2) | 163.7 (45.5) | 162.2 (43.4) |
| ± s | 17.3 ( 4.5) | 10.0 ( 3.3) | 10.0 ( 6.8) | 22.3 ( 0.7) | 26.4 ( 3.0) |

s = standard deviation

* Values in parentheses are the non-extractable residue weights expressed as a percentage of dry weight, i.e. of the sum of the non-extractable residue weight plus the extractable residue weight.

1/100 X Ca level is reduced to 1/3 the amount found in tissues grown on the normal (1 X Ca) level of calcium. The ERW for the 1/10 X Ca levels shows a remarkably wide variation in the four experiments with the extreme values being 2-fold different. Nevertheless, ERW expressed as a percentage of dry weight shows a consistent decrease as the amount of calcium ion in the medium is reduced.

There is an increase in the amount of non-extractable residue (NER) expressed as a percent of dry weight (Table I-4) in tissues grown on reduced amounts of calcium ion. The variation in the NER is not as great from experiment to experiment as the variation in the extractable material. Thus, although there is a decrease in the net amount of NER as the concentration of calcium ion is reduced (compared to 1 X level), reflecting reduction in overall growth, the amount of insoluble or non-extractable material (NER) in the cell increases relative to the amount of extractable material (ERW).

Table I-5 summarizes the effect on dry weight of calcium ion concentration in the medium. Values in this table were obtained by adding the NER and ERW for each level. The dry weight shows the same general trend and variation as the fresh weights (Table I-1, pg. 13). There is a significant reduction in the dry weight yield in the two lower $Ca^{++}$ levels when compared to the normal level (1 X Ca) of calcium ion in the medium. The general trend of decreased dry weight yield at lower $Ca^{++}$ is shown in all but experiment 2 where the 1/10 X Ca deviates. The general degree of correlation between fresh weight and dry weight is also reflected in Table I-6 which gives the percent water in the tissues for the various experiments. Although a slight decrease in percent $H_2O$ may

TABLE I-5

THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON DRY WEIGHT

| EXPT. | CALCIUM CONCENTRATION X=440 mg/l $CaCl_2 \cdot 2H_2O$ | | | | |
|---|---|---|---|---|---|
| | 1/100 X | 1/20 X | 1/10 X | 1 X | 3 X |
| | Weight in mg | | | | |
| 1 | 173.7 | 195.9 | 233.8 | 379.3 | 338.0 |
| 2 | 190.9 | 204.4 | 331.0 | 303.8 | 298.3 |
| 3 | 209.4 | 209.2 | 310.2 | 360.1 | 362.0 |
| 4 | 162.3 | 177.2 | 212.9 | 338.2 | 424.0 |
| ave. | 184.0 | 196.6 | 271.9 | 345.3 | 355.5 |
| ± s | 20.0 | 10.0 | 56.5 | 30.0 | 51.9 |

s = standard deviation

TABLE I-6

THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON THE WATER CONTENT OF TISSUE

| EXPT. | CALCIUM CONCENTRATION X=440 mg/l $CaCl_2 \cdot 2H_2O$ | | | | |
|---|---|---|---|---|---|
| | 1/100 X | 1/20 X | 1/10 X | 1 X | 3 X |
| | Percent water | | | | |
| 1 | 97.0 | 97.5 | 97.7 | 97.8 | 97.5 |
| 2 | 97.1 | 97.3 | 97.3 | 97.4 | 97.2 |
| 3 | 97.0 | 97.6 | 97.0 | 98.0 | 98.3 |
| 4 | 96.4 | 96.6 | 97.1 | 97.4 | 97.4 |
| ave. | 97.0 | 97.2 | 97.3 | 97.6 | 97.6 |
| ± s | 0.3 | 0.4 | 0.3 | 0.3 | 0.5 |

s = standard deviation

be suggested, there is no significant difference in percent $H_2O$ as the amount of calcium ion is varied. Thus, fresh weight and dry weight show similar trends in this system and there is no significant difference in the water content of the tissues grown at the various levels of calcium ion in the medium.

The data in Table I-7 indicate that the effect of calcium ion concentration in the medium on the concentration of scopolin and scopoletin, and on the scopolin-scopoletin ratio is quite variable from experiment to experiment. The variation is so great that no meaningful conclusions on the effect of $Ca^{++}$ on these parameters can be based on these data. Although samples were held at 0-4°C for variable lengths of time before analysis, the reassay of some samples (necessary occasionally when the duplicate analyses had varied from each other by more than 10%) indicated that no significant changes in the content of scopolin or scopoletin occurred on the storage of samples up to three months. The methods of extraction and of analysis used in this study were the same as those used by Koeppe (1969) who reported no significant change in scopolin or scopoletin content after storage of tobacco samples at 0-4°C for up to a year. Thus, the observed variation is not likely to be a result of the analytical procedures employed.

The effect of calcium ion concentration in the medium on lignin content of the tissues is shown in Table I-8. The amount of lignin per 100 mg of NER is increased as the calcium ion concentration is reduced. There is an approximately 150% increase when the 1/10 X Ca level is compared with the 1 X level. This trend toward increased lignin per 100 mg of NER continues as the amount of $[Ca^{++}]$ is reduced further, reaching a

TABLE I-7

THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON THE CONCENTRATION OF SCOPOLIN, SCOPOLETIN, AND THE SCOPOLETIN/SCOPOLIN RATIO IN THE TISSUES

| EXPT. | CALCIUM CONCENTRATION X=440 mg/l $CaCl_2 \cdot 2H_2O$ | | | | |
|---|---|---|---|---|---|
| | 1/100 X | 1/20 X | 1/10 X | 1 X | 3 X |
| | | | SCOPOLIN (µg/g fresh wt.) | | |
| C-1 | 27.5 | 64.3 | 69.6 | 43.8 | 53.7 |
| C-2 | 95.1 | 74.4 | 82.6 | 121.8 | 106.4 |
| C-3 | 38.7 | 21.5 | 23.8 | 82.6 | 52.7 |
| C-4 | 23.9 | 27.3 | 45.6 | 70.7 | 79.4 |
| ave. | 46.3 | 46.9 | 53.8 | 79.7 | 73.0 |
| ± s | 33.1 | 26.4 | 23.9 | 32.4 | 25.4 |
| | | | SCOPOLETIN (µg/g fresh wt.) | | |
| C-1 | 2.32 | 2.66 | 2.82 | 3.88 | 3.17 |
| C-2 | 2.60 | 3.62 | 3.59 | 2.02 | 2.96 |
| C-3 | 2.28 | 1.90 | 1.96 | 3.61 | 2.96 |
| C-4 | 2.69 | 1.72 | 2.18 | 3.44 | 2.97 |
| ave. | 2.47 | 2.48 | 2.64 | 3.24 | 3.02 |
| ± s | 0.20 | 0.86 | 0.73 | 0.83 | 1.00 |
| | | | SCOPOLETIN/SCOPOLIN RATIO | | |
| C-1 | 11.8 | 24.2 | 24.7 | 11.3 | 16.9 |
| C-2 | 36.6 | 20.6 | 21.2 | 60.3 | 35.9 |
| C-3 | 17.0 | 11.3 | 12.1 | 22.9 | 17.8 |
| C-4 | 8.8 | 15.9 | 20.9 | 20.6 | 26.7 |
| ave. | 18.6 | 18.0 | 19.7 | 28.8 | 24.4 |
| ± s | 12.5 | 5.6 | 5.4 | 21.6 | 8.9 |

s = standard deviation

TABLE I-8

THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON LIGNIN CONTENT OF TISSUES

| EXPT. | CALCIUM CONCENTRATION X=440 mg/1 $CaCl_2 \cdot 2H_2O$ | | | | |
|---|---|---|---|---|---|
| | 1/100 X | 1/20 X | 1/10 X | 1 X | 3 X |
| | | | mg Lignin/100 mg NER | | |
| C-1 | 10.6 | 8.7 | 6.2 | 2.8 | 2.2 |
| C-2 | 10.3 | 8.8 | 7.7 | 4.0 | 4.0 |
| C-3 | 9.9 | 9.0 | 7.7 | 2.5 | 1.6 |
| C-4 | 9.4 | 9.6 | 8.5 | 3.3 | 2.1 |
| ave. | 10.05 | 9.02 | 7.52 | 3.15 | 2.48 |
| ± s | 0.52 | 0.40 | 0.96 | 0.66 | 1.05 |
| | | | Total Lignin in mg (per eight pieces) | | |
| C-1 | 12.6 | 10.1 | 8.6 | 4.8 | 3.2 |
| C-2 | 11.9 | 10.3 | 12.5 | 5.5 | 5.5 |
| C-3 | 13.9 | 11.2 | 11.9 | 4.8 | 3.2 |
| C-4 | 9.1 | 11.1 | 11.3 | 5.0 | 3.5 |
| ave. | 11.87 | 10.67 | 11.07 | 5.02 | 3.85 |
| ± s | 2.02 | 0.55 | 1.72 | 0.33 | 1.10 |
| | | | Lignin as % Dry Weight | | |
| C-1 | 7.2 | 5.1 | 3.6 | 1.2 | 0.9 |
| C-2 | 6.2 | 5.0 | 3.7 | 1.8 | 1.8 |
| C-3 | 6.6 | 5.3 | 3.8 | 1.3 | 0.8 |
| C-4 | 5.6 | 6.2 | 5.8 | 1.4 | 0.8 |
| ave. | 6.40 | 5.40 | 4.10 | 1.42 | 1.07 |
| ± s | 0.67 | 0.54 | 0.80 | 0.26 | 0.47 |

s = standard deviation

NER = non-extractable residue

3-fold higher value at 1/100 X Ca compared to the 1 X Ca level.

Tissues grown on the three lower levels of $Ca^{++}$ have statistically the same total amount of lignin. This value (11 mg) is over twice as high as the total (5 mg) amount found in the 1 X Ca level. There is no significant difference in the total amounts of lignin between the 1 X Ca and 3 X Ca levels.

The quantity of lignin in tissues expressed as a percentage of dry weight shows an even greater degree of increase than the mg of lignin/100 mg of NER. The 1/100 X Ca level shows twice the amount of lignin compared to the 1 X Ca level. There is no significant difference in the amount of lignin (as percent of dry weight) in the 3 X level compared to the 1 X level by any of these three means of expressing lignin content. In summary, the data in Table I-8 indicate that lowering the calcium ion concentration in the medium results in a significant and highly reproducible increase in lignin content as reflected by all three of these means of expressing the amount of lignin in the tissues.

## Discussion

The interpretation of results obtained using a nutritional system, such as the one employed in the present study, is quite complex. Various cellular parameters are affected. Growth of the tissues is generally affected and some of the observed changes may simply result from differences in rate of growth.

There is an apparent correlation between the amount of browning in the tissue and the amount of lignification in the tissues. One of the

most striking results of growth of the W-38 at various levels of $Ca^{++}$ is the reproducible trend of tissues grown on lower levels of calcium ion concentration toward being darker in color at the end of the 5 weeks of growth. Upon extraction of the tissues, the same trend toward increased brown coloration is also apparent in the non-extractable residues from tissues grown at progressively lower concentrations of $Ca^{++}$ in the medium. Thus, the coloration is associated with some alcohol-water insoluble material. Since lignin is one component of the non-extractable residue, and since the amount of lignin (as % dry) weight increases (Table I-8, pg. 20) as the concentration of calcium ion in the medium is reduced, the increased browning is probably associated with the degree of lignification. Lipetz (1962) has reported that sunflower gall tissue grown on White's medium was reddish-brown in color, while tissue grown on 10 X White's medium was white to cream colored. He went on to demonstrate, histologically, that the reddish-brown pigment was deposited on the cell wall and appeared to be lignin. The present data, in which quantitative increases in lignin parallel the progressive increase in brown coloration in some component of the non-extractable material, support the suggestion of Lipetz (1962) that reddish-brown pigmentation of tissue grown in culture on low calcium is associated with increased lignin content.

The amount of calcium ion in the medium also affects the growth and degree of lignification in tobacco callus tissues. As a convenience in discussion, Table I-9 summarizes the effect of $[Ca^{++}]$ on the growth of W-38 tobacco tissues in culture (data from Tables I-1 through I-6).

TABLE I-9

THE EFFECT OF CALCIUM CONCENTRATION IN THE MEDIUM
ON THE GROWTH OF W-38 TOBACCO CALLUS TISSUE

| Amount of $CaCl_2 \cdot 2H_2O$ X = 440 mg/l of medium | fresh wt. g | extractable residue mg | non-extractable residue mg | dry wt. mg | % $H_2O$ |
|---|---|---|---|---|---|
| 1/100 X | 5.94 (40)** ±1.07 | 65.8 (36)** ±10.0 | 118.2 (72)** ±17.3 | 184.0 (53)** ±20.0 | 97.0 ±0.3 |
| 1/20 X | 7.35 (49) ±1.49 | 78.5 (43) ±10.0 | 118.1 (72) ±10.0 | 196.6 (57) ±10.0 | 97.2 ±0.4 |
| 1/10 X | 9.90 (66) ±2.00 | 124.6 (68) ±43.5 | 147.3 (90) ±10.0 | 271.9 (79) ±56.5 | 97.3 ±0.3 |
| 1 X | 14.89 (100) ±3.06 | 182.1 (100) ±17.3 | 163.7 (100) ±22.3 | 345.3 (100) ±30.0 | 97.6 ±0.3 |
| 3 X | 15.22 (102) ±4.29 | 193.3 (106) ±44.7 | 162.2 (99.0) ±26.4 | 355.5 (103) ±51.9 | 97.6 ±0.5 |
| Data from table | I-1* | I-3 | I-4 | I-5 | I-6 |

* Values from Table I-1 rounded off to 2 decimal places.
** Values in parentheses are values based on 1 X Ca ≡ 100.

Values represent the net weight from eight pieces of tissues pooled for each of the five levels of $[Ca^{++}]$. Values in parentheses are the relative values of the parameter compared to the 1 X Ca with the control level being assigned a value of 100, e.g., the relative value of fresh weight for a given level equals 100 times the value of fresh weight for the given level divided by the fresh weight for the 1 X Ca level.

As was anticipated, reduced growth (as reflected by both fresh weight and dry weight yields) occurred at reduced calcium ion concentrations in the medium. There was no significant difference in the yield at 1 X Ca and 3 X Ca. These findings in which W-38 tobacco callus was grown at the surface of liquid medium are in general agreement with the finding of Murashige and Skoog (1962) that 440 mg $CaCl_2 \cdot 2H_2O$/l gave optimum growth.

There appears to be a slight increase in the relative amount of dry weight in tissues grown on lower calcium ion concentration. Although the fresh weight and dry weight showed the same general trend of reduction at low $[Ca^{++}]$, a close comparison of the relative values of fresh weight to the relative value of dry weight for each level (Table I-9) reveals that there is, however, a slight increase in the relative amount of dry weight as $[Ca^{++}]$ in the medium is lowered. That this increase is slight is reflected by the finding that there is no significant change in the percent $H_2O$ in the tissues grown at the various levels. Dutta and McIlrath (1964) also reported no significant change in the percent water in sunflower tissues grown on medium with and without boron, although increased water content has been reported in boron deficiency (Gauch and Dugger, 1954). Therefore, the present findings

indicate that growth of W-38 tobacco callus on medium with reduced [$Ca^{++}$] results in decreased net yield and possibly in a slight increase in the amount of dry weight yield relative to fresh weight yield but no significant change in the amount of water in the tissues.

The amount of non-extractable residue (as % dry weight, see Table I-4, pg. 15) is significantly increased as the [$Ca^{++}$] in the medium is reduced. Since this material should be composed, in large part, of cell wall material, it would appear likely that the growth of tissue on medium with low [$Ca^{++}$] results in cell wall material comprising a higher percentage of the dry weight. That such an increase in cell wall material is reasonable has been suggested by previous reports. Sorokin and Sommer (1949) found that calcium deficient pea roots showed premature development of tracheal elements. Lyon and Garcia (1944) also reported increased thickening of cell walls in stems of calcium deficient tomatoes. The results of the present study indicate that the increase in the relative amount of dry weight compared to the relative weight for each level is due to an increase in the relative amount of non-extractable material. This is further supported by the close parallel between the relative value for fresh weight and the relative value for extractable material of the various levels of [$Ca^{++}$]. If the mg of extractable material per gram fresh weight is calculated for each level, the value is nearly a constant for the five levels. Thus, reduced calcium ion concentration in the medium affects fresh weight yield and the net amount of soluble material to a much greater extent than the amount of insoluble material produced by tobacco callus tissues.

The results of the analysis of the effect of [$Ca^{++}$] on the concentration of scopolin and scopoletin, and on the scopolin-scopoletin ratio in the extracts of the tissues are too variable to allow any definitive conclusion as to the relationship of these compounds to lignification. The lack of reproducibility of the concentration of these substances in the tissues is not readily apparent. The constancy in the concentration of scopolin and scopoletin in samples held in the cold makes it appear unlikely that the variability observed is due to the extraction, storage, or analytical procedures employed. The weight of the inoculum for the different levels and from experiment to experiment was quite similar and could not explain the observed variability. One possibility, however, is that the variation in the concentration of scopolin and scopoletin observed in the tissues was due to variable content or potential for the synthesis of these compounds in the inoculum which was unaffected by the level of the $Ca^{++}$ in the medium on which they were subsequently grown. An alternative possible, less likely, explanation for the variability could be that differences arose as a result of the variation in the amount of surface area of the piece of tissue contacting the filter paper and thus there was a widely variable amount of loss of scopoletin into the medium. Thus, although one goal of this study was to test a speculative possible hypothesis that increased scopolin concentration in the tissues might occur when lignification was decreased, the variability of the present results makes it difficult to draw any conclusion concerning this hypothesis.

The lignin content of W-38 tobacco callus tissues increases as the calcium ion concentration is reduced. The range of lignin content (as %

dry weight) increases, progressively, from 1.4% (1 X Ca level) to 6.7% at the lowest level of $Ca^{++}$. This is somewhat lower than some other reports. Bergmann has reported lignin content of 5.6% for tobacco tissue grown in culture with 2,4D as the auxin, and 15.8% when the medium was supplemented with $10^{-7}$ mg/l kinetin (percentages as % dry weight). The values reported by Schafer and Wender (1970) in a preliminary calcium study in tobacco callus culture were also higher than those in the present report. The inhibition of lignification by indoleacetic acid in whole plants has been reported (Petinov and Urmantsev, 1964; Stafford, 1965). Thus, the different levels of lignification by tobacco tissues in culture may be a result of the purity of the auxin or which auxin was employed.

Parish and Miller (1969) have reported changes in lignin content in the top three internodes of wheat plants grown on three levels (low, normal, high) of calcium and phosphate ion in various combinations. The mean value of the percent lignin (as % dry weight) for the top three internodes was 12.9% for tissues grown on low levels of calcium and low phosphate compared to 3.6% for tissues grown on normal calcium and low phosphate. They found, however, that if phosphate was present at the normal level, no significant change in lignin content was observed with low $Ca^{++}$. Increased amounts of lignin have been demonstrated (Lipetz, 1962), histologically, in tissue cultures grown on medium containing lower levels of calcium ion.

While lignin as percent dry weight increased, total lignin content of the W-38 tobacco callus tissues grown on the three reduced levels of

calcium ion concentrations were all the same (within experimental error) and were over twice the amount found in the 1 X Ca control level. Thus, the observed progressive increase in percent lignin (as % dry weight) as $[Ca^{++}]$ was reduced, resulted from decreased dry weight yield, not from progressive increases in lignin synthesis. This is in agreement with the contention of Lipetz (1962) that the effect of $[Ca^{++}]$ on lignification is not a result of reduced growth. His study demonstrated that although addition of kinetin to sunflower tissue grown in culture on medium containing low calcium grew as well as controls, increased lignification still occurred. Lipetz and Garro (1965) later demonstrated the additional release of peroxidase from cell membranes, which had been washed free of peroxidase activity, when treated with $5 \times 10^{-2}$ M $Ca^{++}$. On the basis of their findings, they suggested that the high $[Ca^{++}]$ releases membrane-bound peroxidase from the cellular membrane and thus reduces lignification in tissue. The present work in no way conflicts with this hypothesis. This hypothesis will be discussed further in Chapter II of this report. The present findings indicate that reduction of the $[Ca^{++}]$ below some undetermined level, between 3 mM and 0.3 mM for W-38 tobacco callus tissue, results in a significant (100%) increase in the synthesis of lignin and that apparent progressive increase in lignin (as % dry weight) result from decreased growth.

## Summary

Several cellular parameters were studied in W-38 tobacco callus tissues grown on medium with five levels of calcium ion concentration. Tissues grown on 3 times the normal level of $[Ca^{++}]$ showed no significant

differences in growth or lignification when compared to the control level. Tissues grown at 1/100, 1/20, and 1/10 of the normal level of $[Ca^{++}]$ showed reduced fresh weight and dry weight yields compared to the 1 X control level. The non-extractable materials contributed a progressively higher percentage to the dry weight (dry weight = extractable residue + non-extractable residue) as the $[Ca^{++}]$ was reduced. The amount of extractable material showed a reduction which closely paralleled the reduction in fresh weight as the $Ca^{++}$ concentration was reduced. No correlation between scopolin and/or scopoletin concentrations in the tissues and the lignin content was possible due to the variable results for the concentration of these substances in the tissues. Although reductions in the weight gave an apparent progressive increase in lignin, as a result of expressing it as a % of dry weight, as the $[Ca^{++}]$ was reduced, the three lower levels of $Ca^{++}$ concentration gave the same total yield of lignin. This value, approximately 11 mg, was 100% higher than the 5 mg value for the control level. Thus $Ca^{++}$ concentration in the medium apparently has a direct effect on lignification rather than an indirect one related to reduced growth.

# CHAPTER II

## THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON THE PEROXIDASE CONTENT AND THE ISOPEROXIDASE PATTERN

### Introduction

Brown (1969) in a recent review on the biochemistry of lignin formation discusses evidence indicating the probable involvement of peroxidase in lignification. In 1962, Lipetz demonstrated, histochemically, that the degree of lignification in several different tissues in culture was inhibited by excess calcium ion concentration in the medium. Later, Lipetz and Garro (1965) demonstrated that cellular membrane material, which had been washed until the washes contained no significant peroxidase activity, released additional peroxidase activity when treated with a $5 \times 10^{-2}$ M or greater concentration of $Ca(NO_3)_2$. They also tested other ions and found the following order of effectiveness in the ability to release peroxidase from washed membranes: $Ca^{++} \approx Sr^{++} > Ba^{++} > Mg^{++} > NH_4^+$. Thus, Lipetz and Garro concluded that tissues grown on low calcium contain more lignin because more peroxidase (required for lignification) is bound to the cellular membrane.

Parish and Miller (1969) have reported that the top three internodes of wheat plants grown on low calcium solution contain increased amounts of lignin provided the amount of phosphate in the nutrient

solution was also low. The specific activity of total peroxidase was lower in tissues grown on nutrient solution containing lower and higher calcium ion than normal or on lower and higher phosphate ion than normal in the nutrient solutions. They also found that tissue grown on low calcium had an increased percentage of bound peroxidase which could be released by treatment of washed cellular membranes with $10^{-2}$ M $Ca^{++}$. Increased phosphate did not affect the amount of bound peroxidase but did lower lignin content. Thus, although low $Ca^{++}$ may cause an increase in lignification by increasing the amount of bound peroxidase as suggested by Lipetz and Garro (1965), other factors may dominate this effect, as Parish and Miller (1969) have shown for phosphate ion.

Although the existence of multiple forms of peroxidase is well documented for various tissues (recent review on isoenzymes in plants: Shannon, 1968), there have been no studies on possible changes in isoperoxidase patterns in relation to lignification. A change in the amount of one or more isoperoxidase(s) which also possessed indoleacetic acid (IAA) oxidase activity could influence the degree of lignification in the tissue in the following way: the increased IAA oxidase activity could result in a reduction in the level of IAA, and since it has been reported that IAA inhibits lignification at a concentration of $10^{-5}$ in *Phleum pratense* (Stafford, 1965), this could reduce IAA to a level which would allow increased production of lignin by tissues.

Thus the present work was initiated to study the effect of calcium ion concentration in the medium on the total peroxidase activity, the membrane-bound peroxidase activity, and the isoperoxidase patterns of

the tissues. Tobacco callus tissues (W-38) were once again grown in culture at five levels of [$Ca^{++}$], since it had been shown that [$Ca^{++}$] reproducibly and predictably alters the degree of lignification in the tissues (Chapter I; Schafer and Wender, 1970). There has been a report (Dvorak, Cernohorsha and Ledinska, 1969) of changes in the isoperoxidase patterns in pumpkin as a function of calcium nutrition, but the degree of lignification was not studied. The present work also includes the results of a preliminary study on the possible specificity of binding of isoperoxidase to the membrane in tobacco callus tissues.

## Materials and Methods

### Growth of Tissue

For studies on the effect of $Ca^{++}$ on isoperoxidases, from four to eight tubes were inoculated for each of five $Ca^{++}$ levels, as described in Chapter I, except that the inocula were not weighed in these studies. The five concentrations of $CaCl_2 \cdot 2H_2O$ used were: 4.4 mg/l; 22 mg/l; 44 mg/l; 440 mg/l (which is the normal amount in Linsmaier-Skoog medium); and 1320 mg/l. These will be referred to as 1/100 X Ca, 1/20 X Ca, 1/10 X Ca, 1 X Ca, and 3 X Ca, respectively. The tubes were inoculated with tissue from 5 week old stock cultures and allowed to grow for 35 days as before. Three complete experiments were performed.

In order to obtain sufficient $Ca^{++}$ releasable membrane-bound peroxidase for electrophoresis, five week old W-38 tobacco callus stock-culture tissue grown on agar was employed. Stock cultures of W-38 were maintained on Linsmaier-Skoog (1965) medium containing 200 µg/l of

kinetin, 2 mg/l of indoleacetic acid plus an elevated level of thiamine HCl (1 mg/l).

Extraction of Supernatant and Membrane-Bound Enzyme

To study the effect of [$Ca^{++}$] on isoperoxidases, a sufficient number of pieces of tissue required to obtain from 5-7 g of tissue were pooled and weighed. The tissues were then transferred to a cold mortar and pestle and ground to reduce the chunks of tissue into small pieces. All subsequent operations of the extraction procedure were conducted at 0-5°C unless stated otherwise. This material was then transferred to a blender cup (Sorvall Omnimixer) using 2 ml of 0.05 M tris-maleate (pH 7.0) buffer per gram fresh weight of the sample and was blended for 6 min at 8,000 rpm. The resulting homogenate was filtered through a layer of cheese cloth and centrifuged 20 min at 27,000 x g in a refrigerated centrifuge (Sorvall RC2-B) at 0-5°C. The supernatants were removed and the volume recorded. These extracts were subsequently used for assay and electrophoretic analysis. No loss of enzyme activity nor change in the isoperoxidase pattern was observed in samples stored at room temperature up to one month, although some unidentified material sometimes precipitated on standing.

In order to determine the amount of $Ca^{++}$ releasable membrane-bound peroxidase present, the pellet for each sample was suspended in 10 ml of cold 0.05 M tris-maleate buffer (pH 7.0). All subsequent operations were conducted at 0-4°C. The suspension was allowed to stand 5 min in an ice bath, and then recentrifuged at 27,000 x g for 10 min. The supernatant, Wash 1, was decanted and held for assay. The pellet was

again suspended in 10 ml of buffer, and 5 ml (i.e. one-half) of the resulting suspension of membrane material was transferred to another tube. Both tubes were allowed to stand 5 min, and then recentrifuged as before. The supernatants from the two tubes for each level were combined. The combined solution was designated, Wash 2. Five ml of 50 mM $Ca(NO_3)_2$ in 0.05 M tris-maleate buffer (pH 7.0) was added to one of the two tubes for each level. To the second tube for each level (control) was added 5 ml of the same buffer without $Ca^{++}$. Once again the membrane material in both tubes for each level was suspended, and the suspensions were allowed to stand in an ice bath. After 5 min, the tubes for both treatments were centrifuged at 27,000 x g for 10 min. The supernatant was removed from each tube and held for assay. These supernatants were designated, Treatment 1Ca, and Treatment-1b, respectively. The $Ca^{++}$ treatment was repeated on the once extracted residue, and the supernatant was designated, Treatment-2c. All solutions were maintained at 0-4°C until assayed.

To obtain a large amount of $Ca^{++}$ releasable membrane-bound peroxidase,47.5 g of W-38 (agar grown) was broken in a chilled mortar and pestle, and then ground for 6 min at 8,000 rpm in a blender (Sorvall omnimixer) using 100 ml of 0.05 M tris-maleate buffer (pH 7.0) to effect the transfer. The homogenate was then filtered through cheese cloth and the resulting filtrate centrifuged at 27,000 x g for 10 min in a Sorvall RC2-B at 4°C. The pellet was washed twice by suspension in 100 ml of cold buffer, being allowed to stand 5-10 min in an ice bath and recentrifuged. The pellet was then suspended in 20 ml of 100 mM $Ca(NO_3)_2$ in 0.05 M tris-maleate buffer (pH 7.0), and stirred continuously

overnight in the refrigerator. The suspension was centrifuged and the resulting extract subjected to electrophoretic analyses.

## Peroxidase Assay

Enzyme activities were determined by a modified procedure of Lance (1955). The final reaction mixture was 3 ml and contained 13 mM guaiacol, 5 mM $H_2O_2$ and 0.04 M tris-maleate buffer (pH 7.0). The reaction was initiated by the addition of the enzyme preparation and the absorbance at 470 mμ was measured at 1-min intervals on a Hitachi-Perkin Elmer model 139 spectrophotometer. It was sometimes necessary to filter samples from the washing experiments through glass wool to remove trace amounts of suspended material.

## Protein Determination

Protein was estimated by the method of Itzhaki and Gill (1964), using bovine serum albumin as a standard.

## Electrophoresis

A Buchler Polyanalyst Disk Electrophoresis apparatus was used. The method of Orstein and Davis (1962) was used for anodic electrophoresis with a running pH of 9.3. Cathodic electrophoresis, at a running pH of 4.2, was performed as described in the Buchler instruction manual. Temperature was maintained at approximately 20°C by passing tap water through the jacket of the apparatus. Peroxidase bands were visualized by placing the gels in a mixture of 2 parts 1% guaiacol in pH 7.0 buffer, 2 parts 0.05 M tris-maleate buffer (pH 7.0), and 1 part $H_2O_2$ for varying lengths of time. Standard time for anodic gels was for 2 hrs

while cathodic gels were stained for 30 min.

## Results

The same general pattern of growth and appearance of tissues on the various levels of calcium ion concentration in the medium was observed in the present experiments as had been observed in the experiments in Chapter I. After 35 days growth, the tendency toward increased browning in tissues grown on reduced [$Ca^{++}$] in the medium was again evident.

Growth of callus tissue on medium containing higher or lower than the normal amount of calcium ion concentration results in increased peroxidase activity in the soluble crude extracts of the tissues. Table II-1 summarizes the results of three experiments on the effect of [$Ca^{++}$] on the soluble-peroxidase activity in tobacco callus tissues grown in

TABLE II-1

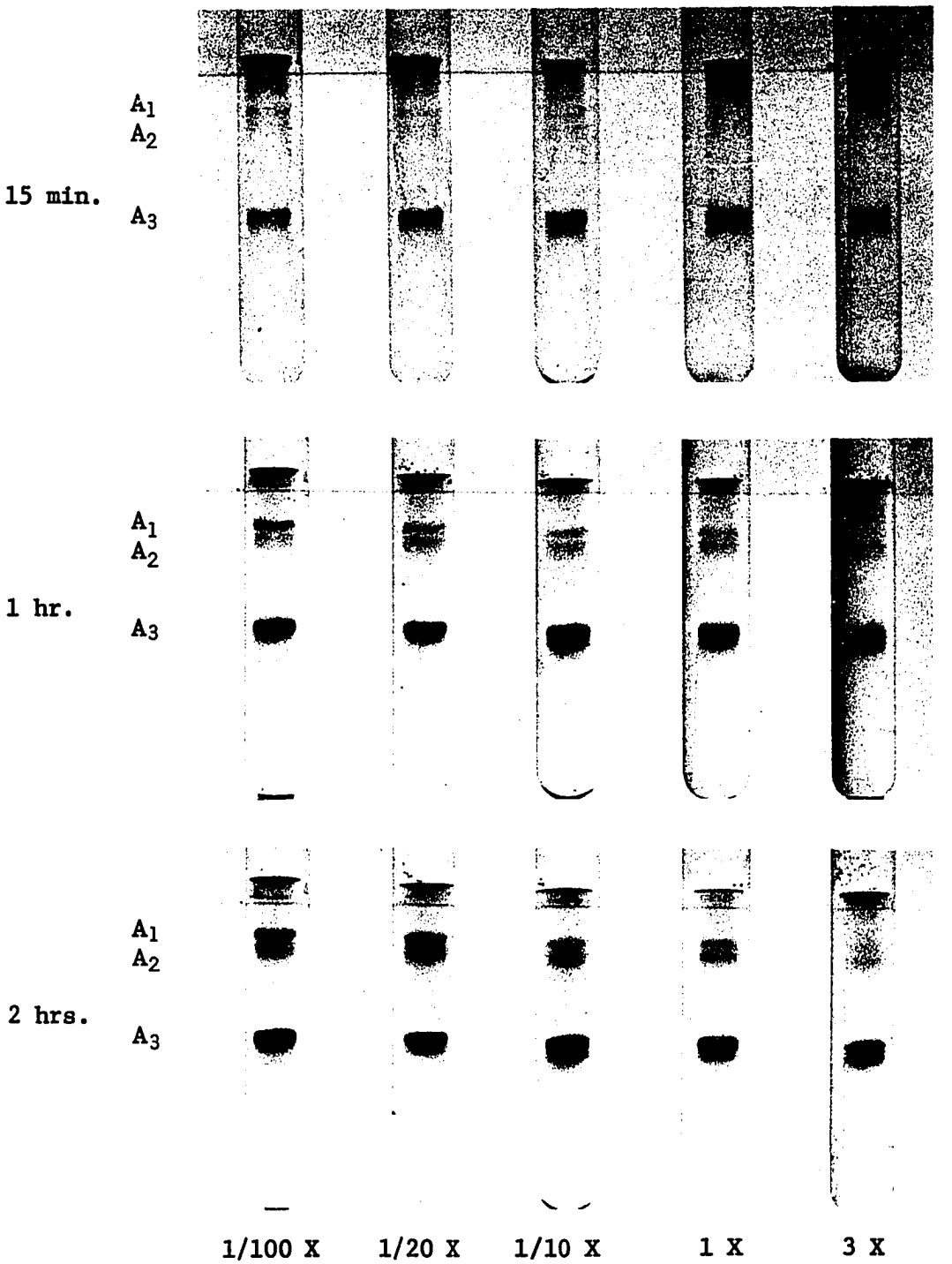
THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON SOLUBLE-PEROXIDASE ACTIVITY OF TOBACCO CALLUS TISSUE

| EXPT. | CALCIUM CONCENTRATION X=440 mg/l $CaCl_2 \cdot 2H_2O$ | | | | |
|---|---|---|---|---|---|
| | 1/100 X | 1/20 X | 1/10 X | 1 X | 3 X |
| | peroxidase activity expressed as ΔO.D./min/g fresh wt. | | | | |
| 1 | 133 | 135 | 78 | 66 | 85 |
| 2 | 136 | 146 | 109 | 79 | 84 |
| 3 | 144 | 134 | 103 | 65 | 80 |
| ave. | 138 | 138 | 97 | 70 | 83 |
| ± s | ±5 | ±6 | ±16 | ±7 | ±0 |

s = Standard Deviation

culture on five levels of [$Ca^{++}$]. A progressive increase in soluble-peroxidase activity was observed as the [$Ca^{++}$] was reduced to the 1/20 X Ca level. The average peroxidase activity for tissues grown at 1/20 X Ca and 1/100 X Ca were both almost 100% higher than the 70 ΔO.D./min/g fresh weight observed for the 1 X Ca, normal level. The 3 X Ca level showed an apparent slight increase in soluble-peroxidase activity compared to the 1 X level. Variation of calcium ion concentration in the medium on which tobacco callus tissues are grown results in a reproducible and significant change in the soluble-peroxidase activity in crude extracts of the tissues.

In an effort to determine the effect of [$Ca^{++}$] on the isoperoxidase pattern, aliquots of the soluble extracts of the tissues grown at the five levels of [$Ca^{++}$] were applied to polyacrylamide gels and subjected to disk gel electrophoresis at a running pH of 9.3. The gels were then routinely stained for 2 hrs. Fig. II-1 shows that the anodic isoperoxidase pattern obtained was, however, a function of staining time. A rapidly migrating major anodic band, $A_3$, was visualized very rapidly upon placing gels in the staining mixture. At 15 min $A_3$, as well as rapidly migrating minor band, $A_4$, which is unmarked in the figure but is just below $A_3$, was already quite apparent. Two slowly migrating bands, $A_1$ and $A_2$, were hardly visible at 15 min. At 1 hr, $A_1$ and $A_2$ had become quite evident. Although a further staining of $A_1$ and $A_2$ had occurred at 2 hrs, $A_3$ and $A_4$ appeared nearly the same at 2 hrs as they had at 1 hr. Since the only significant change observed if gels were stained over 2 hrs was decreased resolution caused by diffusion, 2 hrs was adopted as the standard staining time for anodic gels.

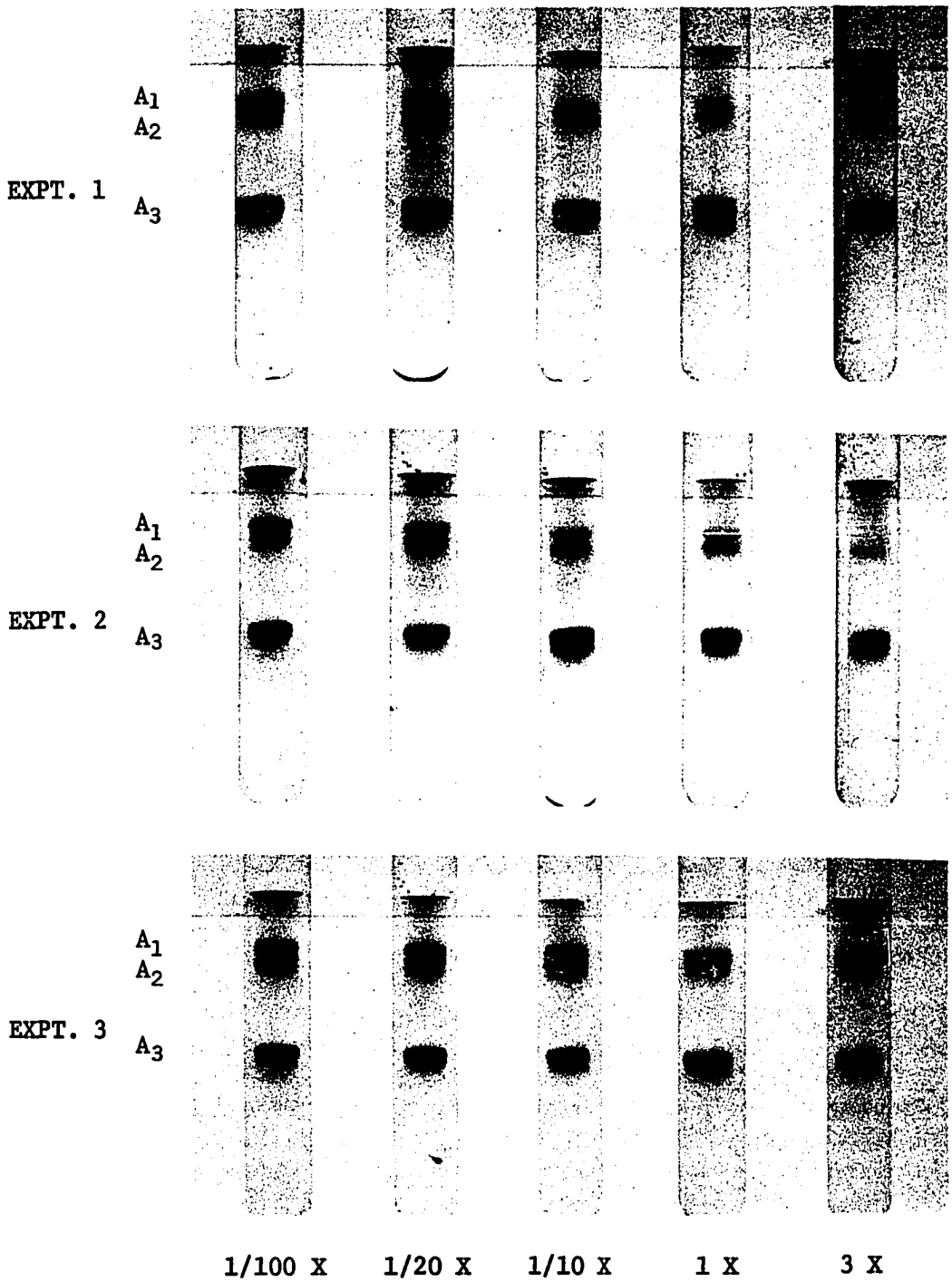



$Ca^{++}$ concentration in the medium where X = 440 mg/l $CaCl_2 \cdot 2H_2O$

Fig. II-1 COMPARISON OF ANODIC ISOPEROXIDASE PATTERN AS A FUNCTION OF STAINING TIME. - Polyacrylamide gels were run at pH 9.3. 25 µl of supernatant extract of EXPT. 2 was applied to each of the five gels. The gels were stained for the times indicated.

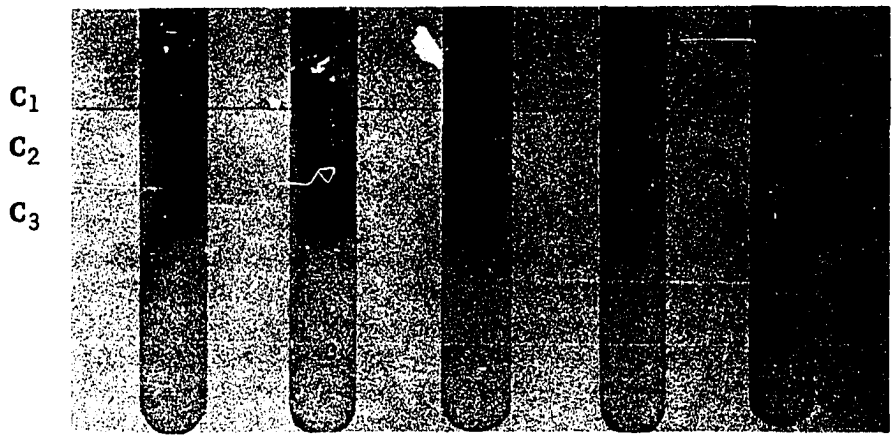
Comparison of the anodic isoperoxidase pattern for each of the levels of the three experiments (Fig. II-2) reveals that the changes in the $[Ca^{++}]$ in the medium affects the relative amounts of the four anodic isoperoxidase bands. While $A_3$ seems to be affected little by a reduction of the $[Ca^{++}]$ in the medium the amounts of $A_1$ and $A_2$ present are increased as the $[Ca^{++}]$ in the medium is reduced to 1/20 X Ca. Although $A_2$ is increased in soluble extracts from tissues grown on lower $[Ca^{++}]$, it would appear it is present in greatest amount at the 1/20 X Ca level and is present in slightly smaller amounts at 1/100 X Ca. Band $A_1$, however, appears to be present at approximately the same level in extracts from tissues grown at 1/20 X Ca and 1/100 X Ca. Analysis of extracts of tissues grown at 3 X the normal level of $[Ca^{++}]$ in the medium indicates that the amount of $A_3$ is reduced compared to the 1 X Ca level, while the amount of the rapidly migrating minor anodic isoperoxidase, $A_4$, increases in amount relative to the 1 X Ca level. The 3 X Ca level tissues also apparently had equivalent to slightly reduced amounts of $A_1$ and $A_2$. Band $A_4$, although only a minor band in 1 X Ca normal tissues, was present at a very low level or nearly absent in extracts of tissues grown at 1/100 X Ca and 1/20 X Ca. The relative absence of $A_4$ at $[Ca^{++}]$ reduced below the 1/10 X Ca level is best seen in the Fig. II-1 at 15 min staining. These experiments show that changes in the $[Ca^{++}]$ in the medium have different effects on the various anodic isoperoxidases present in the tissues.

Although problems with attaining really satisfactory results for the cathodic isoperoxidase patterns prevented the running of the cathodic isoperoxidase patterns for extracts from the above experiments,




$Ca^{++}$ concentration in the medium where X = 440 mg/l $CaCl_2 \cdot 2H_2O$

Fig. II-2 COMPARISON OF THE ANODIC ISOPEROXIDASE PATTERN FOR THREE EXPERIMENTS. - Polyacrylamide gels were run at pH 9.3. 25 µl of supernatant extract was applied to each gel. The gels were stained for two hours.




$Ca^{++}$ concentration in the medium where X = 440 mg/l $CaCl_2 \cdot 2H_2O$

Fig. II-3 CATHODIC ISOPEROXIDASE PATTERN AS A FUNCTION OF CALCIUM ION CONCENTRATION IN THE MEDIUM. - Polyacrylamide gels were run at pH 4.2. 25 µl of supernatant extract was applied to each gel. Staining time was 30 min.

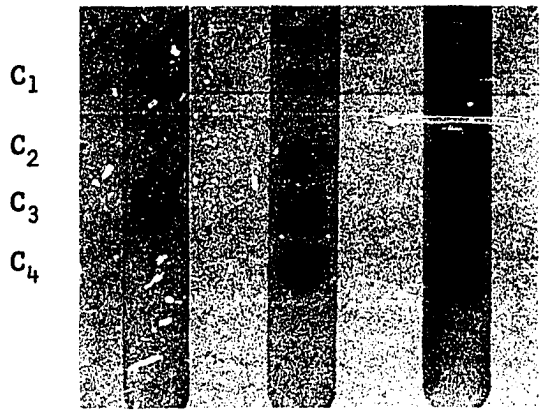



Fig. II-4 COMPARISON OF THE CATHODIC ISOPEROXIDASE PATTERNS FOR THE SUPERNATANT EXTRACT AND THE CALCIUM ION EXTRACT OF WASHED CELLULAR MEMBRANE FROM AGAR GROWN W-38 TOBACCO CALLUS. - Polyacrylamide gels were stained for 30 min. The samples applied to the gels were: (1) 25 µl of supernatant extract; (2) 50 µl of 100 mM $Ca^{++}$-buffer extract; (3) 150 µl of 100 mM $Ca^{++}$-buffer extract.

Fig. II-3 shows the cathodic isoperoxidase patterns as a function of $[Ca^{++}]$ in the medium for another experiment. Equivalent amounts of extracts from a previous $[Ca^{++}]$ experiment which had been frozen were run at pH 4.2 in a cathodic system and stained for 30 min. Freezing had little affect on total peroxidase activity of crude extracts of W-38, and extracts which had been frozen showed similar anodic isoperoxidase patterns to those obtained with unfrozen extracts. Although three bands are designated $C_1$, $C_2$, and $C_3$, subsequent studies in our lab have indicated that the diffuse wide bands, $C_1$ and $C_3$, in Fig. II-3 are probably composed of more than one band (Pickering, 1971). Interestingly, both $C_1$ and $C_3$ showed increased staining in extracts of tissues grown on medium with higher or lower than normal levels of $[Ca^{++}]$. $C_1$ and $C_3$ seem to be present in approximately the same amounts for the three lower levels of $[Ca^{++}]$ in the medium. Band $C_2$ which is almost absent in the pattern for the 1 X Ca level is quite pronounced in extracts of tissues grown at 1/100 X Ca and 1/20 X Ca. This limited information on the effect of $[Ca^{++}]$ in the medium on the cathodic isoperoxidase pattern seems to suggest a general increase in the cathodic isoperoxidases in tissues grown on medium with greater or lesser $[Ca^{++}]$ than normal.

Membrane-associated peroxidase activity is increased in tissues grown at lower $[Ca^{++}]$ in the medium. Table II-2, summarizes the data for the washing of the cellular membranes and the treatment of cellular membranes with $Ca^{++}$. Although there is great variability in the data, there is, without doubt, not only more peroxidase activity released on $Ca^{++}$ treatment of washed membranes from tissues grown on lower $[Ca^{++}]$ in the medium, but also higher peroxidase activity in all the preliminary

TABLE II-2

THE EFFECT OF CALCIUM ION CONCENTRATION IN THE MEDIUM ON MEMBRANE ASSOCIATED PEROXIDASE

| EXPT. | CALCIUM CONCENTRATION X=440 mg/l $CaCl_2 \cdot 2H_2O$ | | | | |
|---|---|---|---|---|---|
| | 1/100 X | 1/20 X | 1/10 X | 1 X | 3 X |
| | peroxidase activity expressed as ΔO.D./min/g fresh wt. | | | | |
| | | | Wash 1 | | |
| E-1 | 1.925 | 2.290 | 3.470 | 0.954 | 0.465 |
| E-2 | 4.320 | 4.415 | 3.573 | 1.483 | 1.115 |
| E-3 | 6.052 | 3.583 | 4.976 | 1.885 | 0.784 |
| ave. | 4.099 | 3.429 | 4.006 | 1.441 | 0.788 |
| ± s | 2.073 | 1.071 | 0.841 | 0.467 | 0.325 |
| | | | Wash 2 | | |
| E-1 | 1.185 | 0.818 | 0.757 | 0.394 | 0.138 |
| E-2 | 1.392 | 1.449 | 1.447 | 0.339 | 0.219 |
| E-3 | 1.949 | 0.693 | 1.072 | 0.566 | 0.291 |
| ave. | 1.508 | 0.987 | 1.102 | 0.433 | 0.216 |
| ± s | 0.395 | 0.405 | 0.485 | 0.188 | 0.077 |
| | | | Treat 1B | | |
| E-1 | 0.533 | 0.567 | 0.584 | 0.280 | 0.051 |
| E-2 | 0.848 | 0.607 | 1.126 | 0.148 | 0.182 |
| E-3 | 0.817 | 0.352 | 0.485 | 0.188 | 0.047 |
| ave. | 0.733 | 0.509 | 0.732 | 0.205 | 0.093 |
| ± s | 0.174 | 0.137 | 0.345 | 0.068 | 0.077 |
| | | | Treat 1Ca | | |
| E-1 | 1.481 | 1.636 | 3.943 | 0.700 | 0.251 |
| E-2 | 2.560 | 3.035 | 7.147 | 0.678 | 0.910 |
| E-3 | 1.179 | 0.932 | 1.212 | 0.408 | 0.259 |
| ave. | 1.740 | 1.868 | 4.101 | 0.595 | 0.473 |
| ± s | 0.726 | 1.070 | 2.971 | 0.163 | 0.378 |

s = Standard Deviation

washes. Thus, although the data seem to indicate that there is probably relatively little tightly bound $Ca^{++}$ releasable peroxidase activity in tissues of this system, the data do suggest that there is an increased amount of loosely associated membrane peroxidase in tissues grown on low calcium ion concentration.

A comparison of the isoperoxidase pattern of membrane associated peroxidase with the isoperoxidase pattern for soluble peroxidase is shown in Fig. II-4 (pg. 41). For this study, cellular membranes from W-38 callus tissues grown on agar medium containing normal [$Ca^{++}$] were used. Although as shown above, these tissues contained less membrane-associated peroxidase, tissues grown on normal [$Ca^{++}$] do contain lignin and have some membrane-bound peroxidase. Thus since a system for the analysis of cathodic isoperoxidases was developed after completion of the three above experiments, tissue grown on agar with medium containing normal [$Ca^{++}$] was used to study possible specificity in the binding of isoperoxidases to the membrane. The tissues were washed twice and then treated overnight with 100 mM [$Ca^{++}$]. The supernatant and $Ca^{++}$ extracts of the washed membranes were subjected to electrophoresis. The $Ca^{++}$ extract contained extremely small amounts of anodic isoperoxidase activity. Comparison of the cathodic patterns, however, reveal that there is indeed specificity in the cathodic isoperoxidases which are membrane bound. While $C_1$ and $C_3$ are the major bands in soluble extracts, little $C_1$ is membrane bound relative to $C_3$. $C_2$ is also present in the $Ca^{++}$ membrane extracts as is demonstrated when a 150 µl of $Ca^{++}$ extract is applied to the gels. One very rapidly migrating cathodic band, $C_4$, is

present in relatively large amount in $Ca^{++}$ extracts of membranes but apparently it is not present in our soluble extracts.

Thus, the present work indicates that tissues grown on low [$Ca^{++}$] in the medium contain increased amounts of two slowly migrating anodic isoperoxidases and all cathodic isoperoxidases. They also exhibit increased amounts of loosely associated membrane peroxidase activity as well as total soluble peroxidase activity. Of the two rapidly migrating anodic bands, the major band, $A_3$, appears unchanged by growth on reduced [$Ca^{++}$] in the medium, while the rapidly migrating anodic band, $A_4$, is decreased or absent in these tissues. Growth of tissue on higher than normal level of [$Ca^{++}$] results in higher total soluble peroxidase activity, in reduced amounts of all anodic bands, except $A_4$, and in increased amounts of all the cathodic isoperoxidases.

## Discussion

Previous work (Chapter I of the present study; Schafer and Wender, 1970) has demonstrated that W-38 tobacco callus tissues grown on reduced [$Ca^{++}$] exhibit reduced growth, but show an increase in lignin content. Thus, since both growth and lignification are related to peroxidase (IAA oxidase) activity, the changes in peroxidase activity and in the isoperoxidase pattern in extracts of tobacco tissues grown on varied [$Ca^{++}$] may be related to either or both parameters. While the observed increase in membrane-associated peroxidase activity in tissues grown on reduced [$Ca^{++}$] is probably related in some manner to lignification, the observed increase in soluble-peroxidase is probably not. Tissues grown on both higher or lower than the normal [$Ca^{++}$] in the medium exhibited

increased soluble-peroxidase activity although tissues grown on higher calcium show little change in lignin content. Reduced soluble-peroxidase activity has been reported (Parish and Miller, 1969) in the internodes of wheat plants grown with low calcium ion and low phosphate ion concentration, although lignin content and membrane-bound peroxidase were increased. The findings of the present study, as well as those of Parish and Miller (1969), support the suggestion (Lipetz, 1962; Lipetz and Garro, 1965) that high [$Ca^{++}$] may reduce the degree of lignification in plant tissues by releasing from the membrane a peroxidase which is associated with lignification.

Although Lipetz and Garro (1965) first demonstrated the release of additional peroxidase activity upon [$Ca^{++}$] treatment of cellular membranes which had been washed free of peroxidase activity, there have been no studies analyzing the electrophoretic characteristics of released isoperoxidases and their relation to lignification. There has been one report (Whitmore, 1971) on the electrophoretic analysis of [$Ca^{++}$] releasable membrane-bound peroxidases in wheat coleoptiles, but lignin content was not determined and no correlation with lignification was discussed. Thus, the present study included an electrophoretic analysis of the membrane-bound peroxidase activity.

Specificity in the binding of the various isoperoxidases was observed in tobacco callus tissues grown on agar-medium containing a normal level of [$Ca^{++}$]. Little anodic peroxidase activity was membrane-bound. Cathodic electrophoretic analysis of membrane-bound peroxidase compared with soluble peroxidase indicates that the various cathodic bands are present in different relative proportions. $C_2$ and $C_4$, which

were present among the membrane-bound isoperoxidases but were undetected in soluble extracts, should be considered as isoperoxidases which are possibly associated with lignification. Of the two, $C_4$ appears the most suspect since whenever it was detected, it was bound to the membrane. Difficulties in the development of an electrophoretic analysis of cathodic isoperoxidases precluded analysis of the [$Ca^{++}$] extracts of membranes for the above experiments. Although it is possible that the observed increase in membrane-bound peroxidase from tissues grown on low [$Ca^{++}$] might result from the binding of one or more other isoperoxidases, this seems unlikely, since tissues grown on normal [$Ca^{++}$] contain lignin. Any more conclusive association of particular isoperoxidases with lignification will require further experimental studies.

The observed increase in soluble-peroxidase activity in tissue grown on lower than normal [$Ca^{++}$] may be related to reduced growth. Peroxidase activity has been associated with or considered a component of "IAA oxidase" (Goldacre, 1951; Galston *et al.*, 1953). Galston *et al.* (1968) reported that while growth potential decreased progressively, peroxidase activity increased progressively in sections of tobacco tissues from the stem when sections from the upper to the lower portion of the stem were analyzed. Peroxidase activity thus showed an inverse correlation with growth potential. Increased peroxidase activity related to reduced growth, correlates well with the generally accepted view that destruction of IAA by IAA oxidase results in reduced growth. This hypothesis is based on the observation of Thimann (1934) that the products of the enzymatic oxidation of IAA yielded products which no longer stimulated growth. On the other hand, Meudt (1970) and Moyed and

Tuli (1968) have presented evidence indicating an alternate possible role for IAA oxidase suggesting that IAA oxidase may convert IAA to an active substance which is responsible for growth. Although increased soluble-peroxidase activity may be related to growth, a consideration of changes in the pattern of the various isoperoxidase components may be more meaningful since the functions of various isoperoxidases may be different.

Isoperoxidase patterns as well as growth and lignification change significantly as a function of $[Ca^{++}]$ in the medium. $A_1$ and $A_2$ are present in increased amount in tissues grown on low $[Ca^{++}]$. The amount of $A_3$, however, appears to be nearly the same at all levels of $[Ca^{++}]$. Lee in a recent series of papers (1971a, 1971b, 1971c) has reported the effect of various growth hormones on growth and the pattern of certain anodic IAA oxidases in tobacco. These IAA oxidases also showed peroxidase activity. In these studies, increased amounts of one rapidly migrating band, which he designates $A_5$, have been associated with increased growth. Comparison of the patterns of Lee (1971c) with those of the present study indicates that Lee's IAA oxidase $A_5$ is probably the same enzyme as the peroxidase $A_3$ in the present study. On the basis of the present work and that of Lee, it seems reasonable to suggest that this rapidly moving peroxidase (IAA oxidase) may convert IAA, as has been suggested by Moyed and Tuli (1968) and Meudt (1970), into an active growth promoting substance. The level of this isoperoxidase (IAA oxidase) has been shown to be affected by concentration of IAA (Lee, 1971c), kinetin (Lee, 1971a), and gibberillic acid (Lee, 1971b) in the growth medium.

The results of the present study do not conflict with the findings of Lee (1971a, 1971b, 1971c) since the level of growth hormones is constant and $A_3$ remains unchanged in tissues grown on normal and low [$Ca^{++}$] in the medium. They do suggest, however, that other isoperoxidases are also probably related to growth. The growth of the tissues could be controlled in the present system by one or more additional peroxidases which function as enzymes which convert the active product derived from IAA (by the action of $A_3$) into an inactive substance. Thus, a reduced level of the growth promoting substance would result and a reduced level of growth occur in agreement with the present results. $A_1$ and $A_2$ show changes in amount with [$Ca^{++}$] which could be consistent with this role. Thus the changes in the pattern of anodic isoperoxidases may relate to the pattern of growth.

The changes observed in the cathodic isoperoxidase patterns may be related to growth. The same general pattern of increase was observed in the amount of the cathodic isoperoxidases and in the soluble-peroxidase activity when tissues were grown at the various levels of [$Ca^{++}$] in the medium. The increased soluble-peroxidase activity in tissues grown on higher than normal [$Ca^{++}$], however, is not associated with a significant change in fresh weight yield relative to the normal level for this system (see Table I-9, pg. 23). If one postulated that the cathodics function as IAA oxidases in the more generally accepted sense, as destroyers of a growth stimulating substance, reduced growth at high [$Ca^{++}$] would be predicted. One could, however, speculate that the increase in the anodic band, $A_4$, could offset the effect of the increased amounts of the cathodics, if $A_4$ functioned as an even more

efficient enzyme catalyzing the conversion of IAA to an active hormone than $A_3$. $A_4$ was present in increased amounts in tissues grown on 3 X Ca. Any conclusions or serious suggestions as to the meaning of the changes in cathodic isoperoxidase patterns as a function of either growth or of $[Ca^{++}]$ in the medium will have to await more complete experiments.

## Summary

Tobacco callus cultures were grown on five levels of $[Ca^{++}]$ in the medium. Growth of tissues on higher or lower than the normal $[Ca^{++}]$ in the medium resulted in increased soluble-peroxidase activity and in increased amounts of the three cathodic isoperoxidases. Anodic electrophoretic analyses of extracts of tissues grown on reduced $[Ca^{++}]$ in the medium revealed changes in the relative amounts of the four observed anodic isoperoxidases. The amount of one rapidly migrating anodic isoperoxidase, $A_3$, was nearly the same for the tissues grown on low $[Ca^{++}]$ as in tissues grown on the normal level of $[Ca^{++}]$ in the medium. $A_1$ and $A_2$, two slowly migrating anodic isoperoxidases, were increased in tissues grown on reduced $[Ca^{++}]$ in the medium.

The amount of peroxidase activity loosely associated with membranes is greater in tissues grown on lower than normal $[Ca^{++}]$ in the medium and is probably associated with increased lignification demonstrated in tissues grown on low $[Ca^{++}]$ (Chapter I). A preliminary electrophoretic analysis of the membrane-associated peroxidase for tissues grown on agar-containing medium with normal $[Ca^{++}]$ indicates that there was some specificity as to the binding of isoperoxidases. Little or no anodic

isoperoxidase activity was membrane-associated. One cathodic isoperoxidase, $C_4$, was found in [$Ca^{++}$] extracts of washed membranes but not in soluble extracts. $C_2$, one of the major soluble cathodic isoperoxidases was present in relatively small amount in [$Ca^{++}$] extracts of washed membranes.

Possible physiological roles are suggested for some of the individual isoperoxidases based on changes in the relative amounts observed in these studies correlated with the experiments of Chapter I, and on the work of others.

# CHAPTER III

## THE SEPARATION AND PRELIMINARY CHARACTERIZATION OF TWO ANODIC ISOPEROXIDASES FROM TOBACCO CALLUS TISSUES

### Introduction

The existence of multiple forms of peroxidase in plants is now well documented (Shannon, 1968). Although various, excellent physiological studies such as those by Galston and coworkers (1968) and by Lee (1971a, 1971b, 1971c), have been made on the isoperoxidases of tobacco, the detailed enzymic properties (i.e. substrate specificity, effector studies, kinetic, and physical properties) of the individual isoperoxidases have not been reported for this plant. A few such studies, however, have been conducted in other species (Macnicol, 1966; Evans, 1970; and McCune, 1961). The work reported here was a preliminary study on the separation and characterization of two anodic isoperoxidases from tobacco.

The oxidation of indole-3-acetic acid (IAA) is one of the several catalytic actions exhibited by peroxidase preparations from plants (Galston *et al.*, 1953; Siegel and Galston, 1967). Further, this IAA oxidase activity has been reported to be both inhibited and stimulated by scopoletin (Andreae, 1952; Schaeffer *et al.*, 1967). More recently Imbert and Wilson (1970) found that scopoletin inhibited IAA oxidase

activity at high concentrations (12.5-250 n mole/ml), but stimulated activity at low concentrations (0.25-10 n mole/ml). The above studies, however, utilized extracts which were mixtures of various isoperoxidases. Thus the characterization in the present study includes a comparison of the effect of scopoletin on the peroxidase activity of each of the two isolated isoperoxidases. Part of the results reported here was included in a recent communication (Schafer, Smith and Wender, 1971).

## Methods and Materials

Two extracts of tissues prepared in the third experiment described in Chapter II were utilized in this study. Five ml of the 1 X Ca level extract was used for the first isoelectric focusing experiment (pH 3-10). For the second (pH 3-6) narrow gradient experiment, 5 ml of the 1/10 X Ca extract was subjected to isoelectric focusing.

### Isoelectric Focusing

Using the method of Vesterburg and Svensson (1966), isoelectric focusing was accomplished on a LKB 440 ml isoelectric focusing column. The sucrose gradients were prepared manually with 5 ml of extract replacing the 4.6 ml of less dense solution normally used for solution number 24. Runs were made at 4°C for 48 hrs (pH 3-10 gradient) and 64 hrs (pH 3-6 gradient) at a potential of 450-600 volts. After isoelectric focusing, the contents of the column were fractionated into 4 ml volumes, and the pH of all fractions having significant peroxidase activity was determined so that the isoelectric point of each peroxidase peak could be ascertained.

## Peroxidase Activity

Enzyme assays were run by a procedure based on that of Lance (1955). The final reaction mixture was 3 ml and contained 13 mM guaiacol, 5 mM $H_2O_2$ and 0.04 M tris-maleate buffer (pH 7.0). The reaction was initiated by the addition of enzyme preparation and the absorbance at 470 mμ was measured at 1 min intervals in a Hitachi-Perkin Elmer model 139 spectrophotometer. Scopoletin in tris-maleate buffer was added at various concentrations in studies on the effect of scopoletin on peroxidase activity. For the preincubation experiment, mixtures minus the component described in Table III-2 (pg. 59) were incubated at room temperature in normal light for 10 min and the reaction was initiated by the addition of the indicated component. Reaction rates were then followed normally. pH profiles were determined by measuring activity in range of 0.04 M tris-maleate buffers. Michaelis constants ($K_m$) were determined by the method of Lineweaver-Burk (1934).

## Electrophoresis

The fractions with peroxidase activity were subjected to polyacrylamide gel electrophoresis analysis. The method of Orstein and Davis (1962) was used for polyacrylamide-gel electrophoresis using a Buchler Polyanalyst disk electrophoresis apparatus. Peroxidase bands were visualized by placing the gel for 2 hrs in a mixture of 2 parts 1% guaiacol in pH 7.0 buffer, 2 parts 0.05 M tris-maleate buffer (pH 7.0) and 1 part 0.5% $H_2O_2$.

## Results

As was demonstrated in Chapter II, when the cells of W-38 tobacco callus tissue culture are broken and then subjected to anodic

polyacrylamide gel electrophoresis at pH 9.3, four bands of activity appear after application of guaiacol and $H_2O_2$ to the gel. Since these four bands appear to coincide in relative electrophoretic mobility to the four anodic isoperoxidases recently reported by Stafford and Galston (1970), their nomenclature is used to distinguish the anodic isoperoxidases that were separated. A schematic representation of a typical electrophoresis run in our laboratory is shown in Fig. III-1A.

In attempts to separate the anodic isoperoxidases, extracts containing the isoperoxidases were subjected to isoelectric focusing with gradients from pH 3 to 10 and pH 3 to 6. Isoelectric focusing, using either gradient, yields two separated peaks of approximately the same peroxidase activity (guaiacol assay) with one peak at pH 3.96 and another at pH 4.79. Polyacrylamide gel electrophoretic analysis demonstrated that the pH 4.79 band of peroxidase activity contains only $A_1$ (Fig. III-1B). Furthermore, analysis of the fractions in the peroxidase activity (guaiacol assay) band centered at pH 3.96 indicates that fractions between 4.00 and 4.05 contain $A_3$ as the only detected peroxidase active enzyme, while those between 3.93 and 4.00 contain $A_3$ incompletely separated from demonstrable amounts of $A_2$ (Fig. III-1B). Although Fig. III-1B represents the results of analysis of the pH 3-6 run, similar results were observed upon analysis of fractions 94, 115, and 118 for the pH 3-10 run. Isoperoxidase $A_4$ was not detected in the fractions. Thus the initial separation of two individual isoperoxidases allowed preliminary studies on the properties of these two isoperoxidases.

Table III-1 summarizes a comparison of the properties of the two isoperoxidases. The two isoperoxidases differ in all parameters tested.

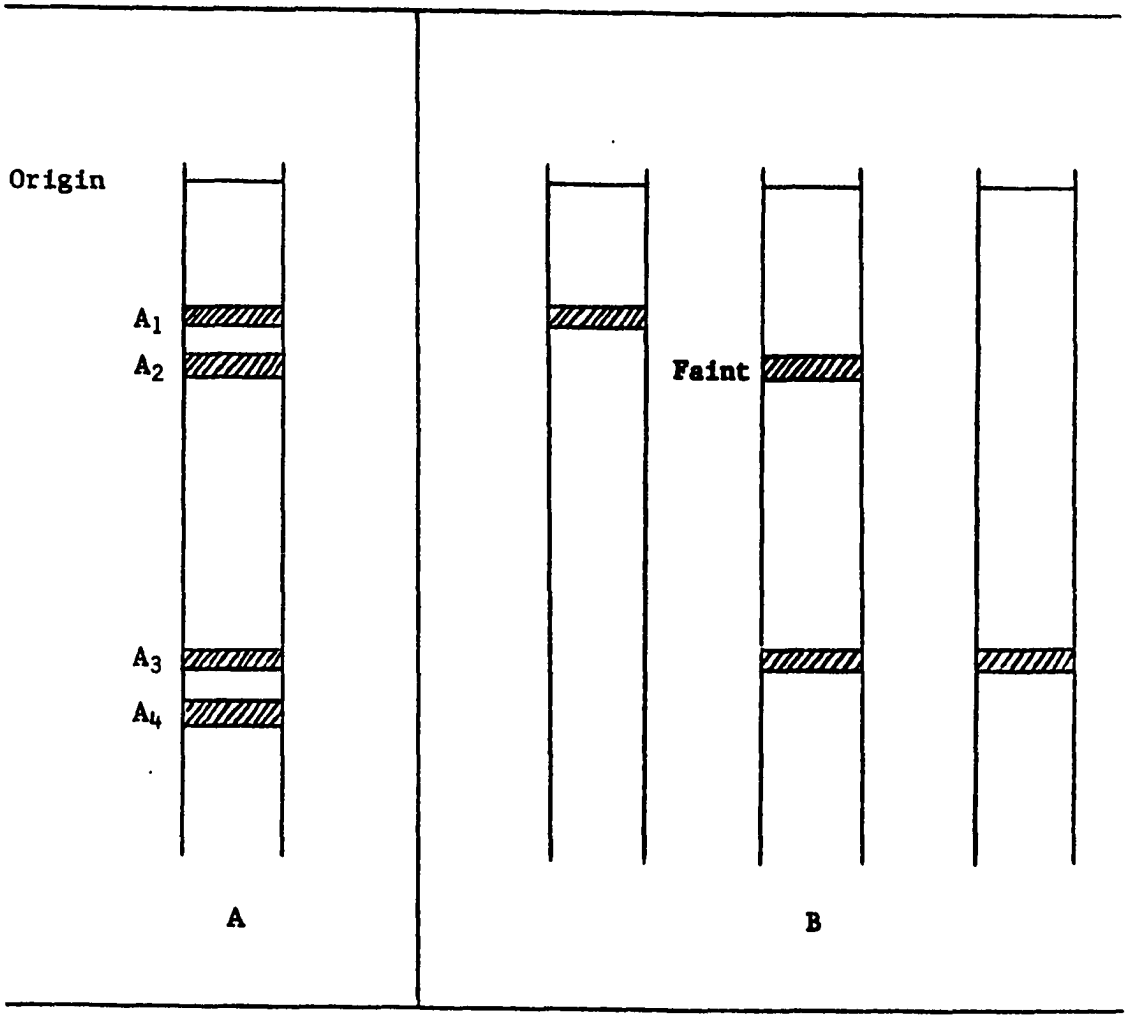



Fig. III-1 ANODIC PEROXIDASES FROM TOBACCO CALLUS TISSUE CULTURE W-38. Visualization was accomplished by reaction with guaiacol and $H_2O_2$ after polyacrylamide gel electrophoresis at pH 9.3. A. Extracts of the tobacco tissue. B. Fractions 56, 84, and 90 collected from isoelectric focusing with a gradient from pH 3 to 6.

The pH optima for the two bands differ by 0.6 pH unit with an estimated error of ± 0.3 pH. $A_3$ has a higher affinity for guaiacol than $A_1$ as is reflected by the $K_m$. As discussed in Chapter II, $A_3$ attains maximum or steady state staining much more rapidly than does $A_1$.

The finding that guaiacol-$H_2O_2$ activity of $A_3$ is stimulated by scopoletin while $A_1$ is unaffected is especially interesting. Fig. III-2 shows the effect of various concentrations of scopoletin on the guaiacol-$H_2O_2$ activity. Scopoletin at concentrations up to approximately 0.5 mM was increasingly stimulatory although the observed increase in stimulation between 0.33 mM and 0.5 mM scopoletin was small. Preincubation of scopoletin in the presence of $A_3$ and $H_2O_2$ for 10 min at room temperature,

TABLE III-1

COMPARISON OF PROPERTIES OF THE TWO ISOPEROXIDASES SEPARATED BY ISOELECTRIC FOCUSING

| | BAND $A_1$ | BAND $A_3$ |
|---|---|---|
| Isoelectric point | | |
| pH 3-6 run | 4.79 ± 0.1 | 3.96 ± 0.03 |
| pH 3-10 run | (4.8 - 5.1) | (3.8 - 4.1) |
| pH Optimum | 6.0 ± 0.3 | 6.6 ± 0.2 |
| $K_m$ for guaiacol* | 9.1 mM | 4.5 mM |
| Maximum staining on polyacrylamide gel | 2 hrs | < 1 hr |
| Stimulation of peroxidase activity by 0.33 mM scopoletin | none | 65%-75% |

* Determined at pH 7.0

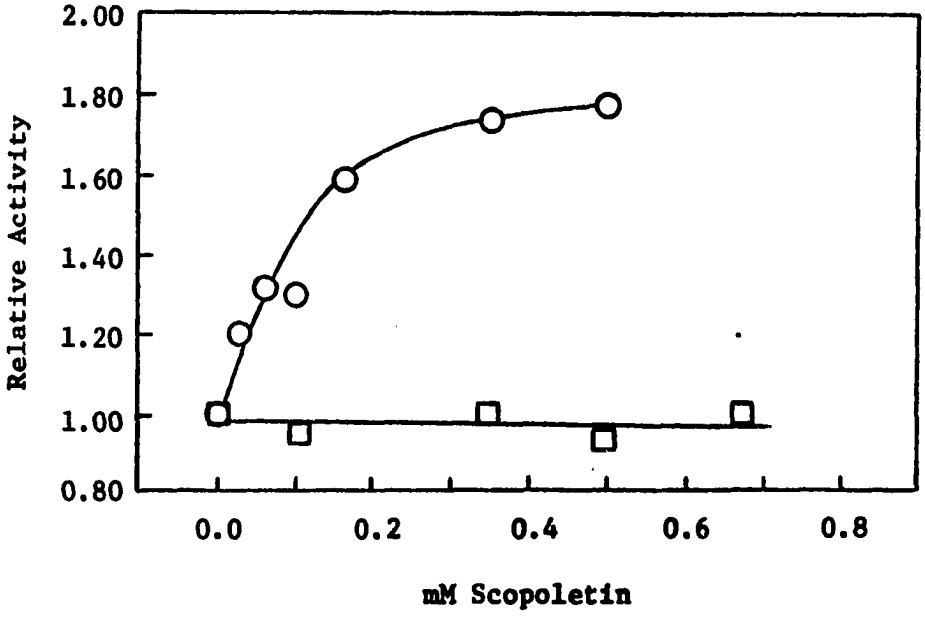



Fig. III-2 THE EFFECT OF SCOPOLETIN ON ISOPEROXIDASES $A_1$ and $A_3$ FROM TOBACCO CALLUS TISSUE CULTURE W-38.

□ = Band $A_1$

○ = Band $A_3$

however, results in loss of ability to stimulate the enzyme catalyzed guaiacol-$H_2O_2$ reaction. Table III-2 shows the results of such a preincubation experiment. Upon the addition of $A_3$ enzyme solution to a mixture containing scopoletin and $H_2O_2$ which had been preincubated for 10 min, the peroxidase (guaiacol assay) activity was stimulated. This indicates that the loss of the ability of scopoletin to stimulate peroxidase activity was not the result of a non-enzymatic reaction of scopoletin with $H_2O_2$. Since the addition of $H_2O_2$ to a preincubated mixture containing enzyme plus scopoletin still showed stimulated activity, $H_2O_2$

TABLE III-2

PREINCUBATION EXPERIMENT

| | ASSAY MIXTURES | | | |
|---|---|---|---|---|
| | minus Guaiacol | minus $H_2O_2$ | minus Enzyme | Control |
| | ml added | | | |
| 1 mM Scopoletin in 50 mM pH 7.0 tris-maleate buffer | 1.00 | 1.00 | 1.00 | — |
| 50 mM pH 7.0 Tris-maleate buffer | 1.15 | 1.15 | 1.15 | 2.15 |
| 1 g Guaiacol/100 ml (in water) | 0.50* | 0.50 | 0.50 | 0.50 |
| 0.2% $H_2O_2$ in water | 0.25 | 0.25* | 0.25 | 0.25 |
| Diluted $A_3$ enzyme solution | 0.10 | 0.10 | 0.10* | 0.10* |
| ΔO.D./min/0.1 ml | 0.027 | 0.038 | 0.039 | 0.024 |

* Added after 10 min preincubation to a mixture of the other components

must participate in the conversion of scopoletin to a non-stimulatory substance. Furthermore, the appearance of a blue coloration when a mixture of scopoletin, $H_2O_2$, and $A_3$ are preincubated 10 min also suggests a reaction occurs. The blue coloration was progressively replaced by a yellow coloration upon standing for increasingly longer periods. $A_3$, therefore, catalyzes a reaction of scopoletin and $H_2O_2$ yielding a product which does not stimulate peroxidase (guaiacol-$H_2O_2$) activity.

## Discussion

It has been shown that two anodic isoperoxidases present in extracts of W-38 tobacco callus tissues are separable by isoelectric focusing. On the basis of electrophoretic mobility at pH 9.3, $A_1$ and $A_2$ appear to be similar. Both of these isoperoxidases migrate relatively slowly at pH 9.3, while $A_3$ shows rapid migration at this pH. However, when the two separated peaks of peroxidase activity were analyzed, the slowly migrating $A_2$ was found still mixed with the rapidly migrating, $A_3$, in some fractions from the peak of peroxidase activity centered at pH 3.96. Thus $A_2$ and $A_3$ have very similar isoelectric points. These observations suggest that there are significant qualitative as well as quantitative differences in the amino acid sequences and/or tertiary structure among the three major anodic isoperoxidases of tobacco callus tissue.

Perhaps the most significant difference in the two separated isoperoxidases was the effect of scopoletin on their peroxidase (guaiacol-$H_2O_2$) activity. While the peroxidase activity of $A_3$ was stimulated 65-75% by 0.33 mM scopoletin, the activity of $A_1$ was unaffected by the

presence of scopoletin in the reaction mixture over the range (up to 0.67 mM) tested. Imbert and Wilson (1970) have reported that scopoletin inhibited IAA oxidase activity (preparations also showed peroxidase activity) from 12.5-250 n mole/ml, but stimulated activity from 0.25-10 n mole/ml. The amounts of scopoletin used for our highest stimulatory effect would be inhibitory in the system of Imbert and Wilson (1970). Their results were obtained with extracts which probably contained a mixture of isoperoxidases since no separation was conducted. The present results suggest that the results of Imbert and Wilson may reflect the combined effects on one or more different isoperoxidases having IAA oxidase activity.

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