THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

PART I. A STUDY OF SCOPOLIN AND SCOPOLETIN METABOLISM

PART II. PHENYLALANINE AMMONIA-LYASE IN TOBACCO TISSUE CULTURE: INHIBITION STUDIES

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PART I. A STUDY OF SCOPOLIN AND SCOPOLETIN METABOLISM

PART II. PHENYLALANINE AMMONIA-LYASE IN TOBACCO TISSUE CULTURE: INHIBITION STUDIES

APPROVED BY

Simon H. Wender

Edie Smith

Francis Johnson

Stanley C. Nash

John A. Fitch

DISSERTATION COMMITTEE

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TABLE OF CONTENTS

LIST OF TABLES

PART I. A STUDY OF SCOPOLIN AND SCOPOLETIN METABOLISM

Table | Page

LIST OF ILLUSTRATIONS

PART I. A STUDY OF SCOPOLIN AND SCOPOLETIN METABOLISM

I. A STUDY OF SCOPOLIN AND SCOPOLETIN METABOLISM

CHAPTER I

INTRODUCTION

Scopoletin, 6-methoxy-7-hydroxy coumarin, has been reported to occur in a wide variety of plants (Winkler, 1967). It may, indeed, be the most common coumarin in the plant kingdom (Robinson, 1963). Scopolin, the 7-glucoside of scopoletin, is the most widespread glycoside of scopoletin and both frequently occur together in plants (Wender, 1970). Scopolin and scopoletin occur in tobacco, and scopoletin has been identified and quantitatively determined in cigarette smoke (Yang, 1958).

Scopolin and scopoletin increase in concentration under a wide variety of abnormal or stress conditions (Wender, 1970). Most early investigations reported only the accumulation of scopoletin in plants subjected to stress conditions; however, the work from this laboratory has shown that increases in the concentration of scopolin generally accompanied the increases in scopoletin. Lack of analysis for scopolin or hydrolysis of scopolin to scopoletin may explain why increases in scopoletin alone were reported by early workers.

Scopoletin and scopolin accumulate in diseased plants. Winkler (1967) listed 9 diseases or afflictions that have been reported to cause an increase in scopoletin and/or scopolin. The report that black rot

resistant sweet potatoes synthesize more scopoletin than the fungus-susceptible variety suggests that scopoletin might play a role in disease resistance (Minamikawa *et al.*, 1963), but further research is needed before one can draw a conclusion therefrom.

Mineral deficiencies also cause accumulations of scopolin and scopoletin in plants. The concentration of scopolin increased twenty-fold in leaves of boron-deficient tobacco plants (Watanabe *et al.*, 1961). Armstrong *et al.* (1970) also observed an accumulation of scopolin in nitrogen-deficient tobacco plants.

Synthetic growth regulators, such as "Tordon" (4-amino-3,5,6-trichloropicolinic acid) (Wender, 1970), 2,4-D (2,4-dichlorophenoxyacetic acid) (Dieterman *et al.*, 1964), and maleic hydrazide (Winkler, 1969), when sprayed on tobacco plants, caused an increase in scopolin and scopoletin.

Various environmental stress conditions, such as low daytime temperatures and high intensity UV and X-ray irradiation, have also produced scopolin accumulation in tobacco (Koeppe *et al.*, 1969, 1970a, 1970b).

Although the evidence for a functional role for scopolin and scopoletin is inconclusive, most hypotheses center around possible roles in growth regulation. Tryon (1956) reported a correlation between scopoletin content and differentiating ability of tobacco tissue culture. Scopoletin inhibits indole-3-acetic acid oxidase and peroxidase (Sequeira, 1964; Andreae, 1952). Shaeffer *et al.* (1967) have shown that both scopolin and scopoletin inhibit the rate of indole-3-acetic acid destruction, although scopoletin is more than 10 times as effective

as scopolin. In tobacco tissue culture, scopoletin in lower concentrations apparently stimulated growth which is an effect that Skoog and Montaldi (1961) attributed to scopoletin's sparing effect on IAA.

Einhellig *et al.* (1970) determined the effects of scopoletin on the growth of tobacco seedlings. A threshold level of inhibition was found to be between 1×10^{-4} and 1×10^{-3} M with 1×10^{-4} M scopoletin showing no inhibition, 1×10^{-3} M great inhibition and 5×10^{-4} M having an intermediate effect on growth. The higher levels of scopoletin inhibited photosynthesis but did not affect respiration.

Despite the widespread occurrence of scopoletin and scopolin and their possible role in growth regulation, the metabolism of these compounds has received relatively little attention. The biosynthetic path for scopolin and scopoletin is much better established than the conversion of these coumarins to other compounds. Reid (1958) first showed that L-phenylalanine was a moderately good precursor of scopoletin in tobacco. Runeckles (1963a) found radioactive scopolin in tobacco leaf disks after feeding C^{14}-ferulic acid. Steck (1967a,b) showed that in tobacco leaves scopolin can be formed from ferulic acid via glucosidoferulic acid. Although the biosynthetic pathway from ferulic acid to scopolin does not include scopoletin, Fritig *et al.* (1967) and Steck (1967b) found that scopoletin is readily converted to scopolin. (See Fig. 1.).

From a metabolic point of view, phenolic compounds such as scopolin have usually been regarded as end products (G.H.N. Towers, 1964). There are few examples of investigations of the metabolism of C^{14}-labeled polyphenols or phenolic glycosides. At the beginning of my investigation, no work had been done on the metabolism of scopolin. It

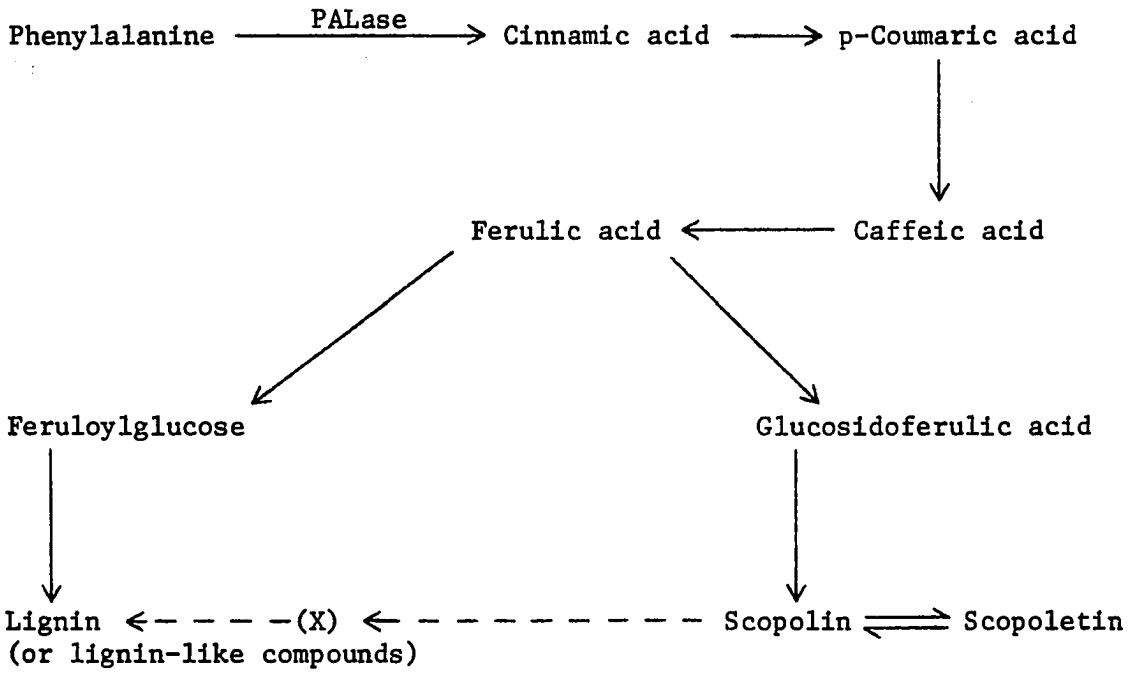


FIGURE 1. A Proposed Abbreviated Biosynthetic Route to Scopolin and Lignin from Phenylalanine. (from Gamborg, 1967; Hanson, Zucker, and Sondheimer, 1967; Steck, 1967a and 1967b; Wender, 1970; Schafer and Wender, 1970; Sargent and Skoog, 1960)

PALase = Phenylalanine ammonia-lyase

– – – – → Postulated

——— → Shown by Tracer Studies

was not known if scopolin was a metabolic end product or if it was rapidly metabolized.

Sargent and Skoog (1960) reported that in tobacco tissue culture, indoleacetic acid and kinetin influence the level of scopolin and scopoletin. The change in levels of these coumarins in response to different concentrations of growth regulators suggested to them that scopolin could be converted to some non-fluorescent product called "X" which presumably is utilized in lignin formation. Runeckles (1963b) found a complex polyphenol from flue-cured tobacco leaves which contained scopolin. Although this complex may only be formed during the curing process and not occur naturally in the plant, its existence and the postulated existence of "X" suggested that scopolin might not be an end product. Therefore, experiments were designed to determine the metabolic fate of scopolin and scopoletin. Radioactive isotopes were used for the investigation as well as a search for an enzyme that catalyzed one of the steps. Dieterman *et al.* (1969) found that C^{14}-quercetin was incorporated into an isopropyl alcohol-insoluble fraction of tobacco. Taylor (1968) reported that radioactivity from labeled chlorogenic acid was found in the insoluble residues. Therefore, the alcohol insoluble fraction as well as the alcohol soluble fraction of the plant extracts were examined for radioactivity.

Little is known about the translocation of phenolic compounds. Bate-Smith (1962) was unable to find any evidence for phloem transport of phenolic compounds. Koeppe (1968) and Einhellig (1969) suggested that scopoletin but not scopolin is transported throughout the plant. In the present study, two different types of experiments were designed

to possibly determine which compound is translocatable, if indeed either was.

Several hypotheses have been presented to explain the accumulation of scopolin and scopoletin in plants subjected to stress conditions (Wender, 1970). The hypotheses may be divided into two main groups. The first possibility consists of increased biosynthesis of scopolin under stress conditions. Wender (1970) presented a number of speculative possibilities about which enzyme or pathway might be increased in order to achieve an accumulation of scopolin. The second hypothesis suggests that scopolin is converted to "X" in healthy rapidly growing tobacco tissue. Furthermore, Sargent and Skoog (1960) assumed the healthy tobacco tissues exhibit a rapid turnover of scopolin to "X". Injury or viral infection would block the pathway from scopolin to "X" and scopolin would accumulate.

Since the first group of hypotheses requires a slow turnover of scopolin in healthy plants and the second group of hypotheses necessitates a rapid turnover of scopolin, the two hypotheses could be tested by measuring the turnover rate of scopolin. The experiments to determine the metabolic fate of scopolin and scopoletin were designed in such a way that the biological half-life of scopolin could be determined.

CHAPTER II

METHODS AND MATERIALS

Growth of Tobacco Seedling

Tobacco seeds (*Nicotiana tabacum* L. var. One Sucker) were germinated in a crock of pure washed quartz sand. The crock of sand had been autoclaved overnight at 121°C. After the seeds were planted, the crock was covered with a glass lid, left in the laboratory for twenty-four hours, and then transferred to a Percival growth chamber. The light intensity of the growth chamber was 1200 ft-c; the photoperiods were 16 hours light and 8 hours dark. The light/dark temperatures were 85/70° F, respectively. The seedlings were watered daily with either Fe-EDTA double strength Hoagland's nutrient solution (Hoagland and Arnon, 1950) or distilled water.

When the tobacco seedlings were about 1-2 cm tall, they were transplanted to 40 ml opaque plastic vials. The vials contained a nutrient solution consisting of one part Fe-EDTA Hoagland's solution and two parts distilled water. The transplanted tobacco seedlings were left about 24 hours in the laboratory and then were moved to a growth chamber. After the seedlings had grown for about two weeks in the vials, they were selected for experimentation on the basis of uniform size.

Uptake of Radioactive Compound

In all experiments reported here, the radioactive solution was made up of four parts of distilled water containing the radioactive compound to one part double strength Fe-EDTA Hoagland's nutrient solution.

In one set of experiments the tobacco seedlings were transferred to vials containing known amounts of radioactive solution. Each sample group was allowed to take up and metabolize the radioactive compound for various lengths of time. The plants in each group were then washed, fixed and extracted.

In another series of experiments, all the plants were left in the vials containing the radioactive solution for the same length of time. The roots on all the plants were washed with distilled water, and the plants were transferred to vials containing the usual non-radioactive nutrient solution (Fe-EDTA Hoagland's solution : distilled water, 1:2 v/v). The seedlings were allowed to metabolize the radioactive compound they had previously taken up for different time periods. At the end of each defined time period a sample group was washed, fixed and extracted. Each group or sample consisted of either three or four plants.

The washings from the roots plus the radioactive solution left in the vials were quantitatively diluted. Aliquots from these solutions were measured for radioactivity in order to determine the amount of radioactivity remaining unabsorbed at the end of the experiment. Since both the amount of radioactivity at the beginning of the experiment and the radioactivity left at the end of the experiment were known, it was possible to calculate how much radioactivity was taken up by the seedlings (cpm taken up = cpm original minus cpm remaining at the end of the experiment).

Fixation and Extraction

After the roots of the plants were washed with distilled water, the seedlings were separated into roots and shoots. The roots and shoots were weighed (fresh weight) and were fixed by boiling them in isopropyl alcohol azeotrope (88% isopropanol, 12% water) for 5 minutes.

The fixed plant material was thoroughly ground in a blender and transferred into an extraction thimble (either paper or glass). Samples which had a fresh weight of 10 g or less were extracted with 40 ml of hot isopropanol : water (1:1, v/v) followed by 50 ml of hot isopropanol : benzene : methanol : water (2:1:1:1, v/v/v/v) (IBMW) and finally by 40 ml of isopropanol azeotrope. When the fresh weight of the sample exceeded 10 grams the plant material was extracted with a quantity of isopropanol : water (1:1, v/v) equal to four times the fresh weight, followed by IBMW (5x fresh weight) and finally with hot isopropanol azeotrope (4x fresh weight). The thimble containing the washed plant material was placed in a Soxhlet extractor and extracted for 24 hours with isopropanol azeotrope. The solvent was then changed to isopropanol, and the plant material was extracted another 24 hours. Solvents from all the extraction steps were combined and the extract was evaporated to dryness under reduced pressure. The residue was brought to a known volume with IBMW.

The plant material had thus been divided into two main fractions. The combined extracts represented an IBMW-soluble fraction, and the residue left in the thimble represented an IBMW-insoluble fraction.

Scopoletin, Scopolin, and Fabiatrin Determinations

Descending, one dimensional paper chromatography was utilized in the quantification of scopolin and fabiatrin (Koeppe, 1968). Sixteen

sheets of Whatman #1 paper (23 x 57 cm) were washed in a chromatocab for 20-24 hours with 5% methanol. The washed papers were dried just prior to use. An aliquot of the IBMW soluble extract was streaked along a 15 cm origin and the papers were developed with a ternary solvent system of methylisobutyl ketone : formic acid : water (14:3:2, v/v/v) (KFW). In some cases, papers streaked with standards were developed along with the papers streaked with crude extract. After development for twenty hours in KFW, the papers were dried and the fluorescent bands of fabiatrin and scopolin could be observed under a UV light.

The same procedure, but employing a different solvent system, was used to isolate scopoletin. The papers were washed and streaked as usual, then developed in a solvent system containing isopropanol : formic acid : water (5:0.1:95, v/v/v) (IFW). After development for six hours, scopoletin had separated from all other fluorescent bands.

The fluorescent bands corresponding to either scopolin, scopoletin or fabiatrin were circled with a pencil, cut out, and eluted with 5% methanol for 20 hours. The eluates were brought to a known volumn with 5% methanol and the fluorescence of each was measured on a model 110 Turner fluorometer. The range selection on the fluorometer was set at 1X, and the meter sensitivity was set at 6. The primary filter was #7-60 and the secondary filters were #2A plus #48 (Kodak Wratten filter). The instrument was zeroed using a blank which had been run through procedures identical with those of the particular set of samples for which fluorescence was being measured.

Standard reference curves were prepared for scopolin and scopoletin by running known quantities of the authentic compounds through the entire analytical procedure (e.g. Einhellig, 1969; Koeppe, 1968; Armstrong,

1968). The quantity of scopolin, scopoletin and fabiatrin was determined either by using the standard reference curve or from standards developed with the unknown samples.

Either the whole sample or an aliquot from the sample used in the fluorescence measurement was transferred to a scintillation vial. The sample was evaporated to dryness in a vacuum oven and counted for radioactivity. Since both the radioactivity and the quantity of the substance were determined, the specific activity could be calculated.

Measurement of Radioactivity in the IBMW Insoluble Material

The radioactive IBMW-insoluble material was oxidized to $C^{14}O_2$, which was trapped in a phenethylamine solution. An aliquot of this solution was counted for radioactivity (Jeffay and Alvarey, 1961; Duncombe and Rising, 1969).

The residue remaining in the Soxhlet thimble after the extensive extractions was dried in a vacuum oven under reduced pressure at $60^{o}C$. When paper extraction thimbles were used, the IBMW-insoluble material was scraped out and weighed. Glass thimbles were weighed before and after the extraction procedure in order to determine the weight of the IBMW-insoluble material.

One to nine milligrams of IBMW-insoluble material were weighed on a microbalance (Cahn G-2 Electrobalance) and placed in a small, three-necked round-bottomed flask. A separatory funnel (50 ml) containing the oxidation mixture was connected to the middle neck, a gas inlet tube from a nitrogen tank was connected to a second neck, and a gas outlet tube was fitted to the third neck. The gas outlet tube was further connected by

glass and rubber tubing to a series of four 20 ml glass scintillation vials. The first vial contained about 10 ml of 1% HCl, the second and third vials contained 5 ml of a solution of dioxane and phenethylamine (1:1, v/v) (Packard), and the fourth vial had 10 ml of saturated barium hydroxide. Each inlet tube was placed beneath the solution near the bottom of the vial, and each outlet was at the top of the vial (see Figure 2).

With the nitrogen gas turned off, the oxidation mixture (10-20 ml) was added to the round bottom flask. A small amount of the oxidation mixture was kept in the separatory funnel to prevent entrance of CO_2 into the funnel. After the oxidation mixture was added, nitrogen gas was passed through the system for 2 minutes. The flask was then slowly heated with a small direct flame until the mixture boiled for 30 to 60 seconds. The elapsed time for heating was 3 minutes. The flask was allowed to cool for 5 minutes with nitrogen passing through the system. The nitrogen stream was discontinued and the middle two vials containing the dioxane-phenethylamine solution were removed. The round bottom flask was removed and the middle two inlet tubes were washed with 1N NaOH followed by 1% HCl, leaving the apparatus ready for another sample. Three to four samples per hour were combusted by using this procedure.

Two ml aliquots of the dioxane-phenethylamine were transferred to scintillation vials and counted for radioactivity.

The oxidation mixture (van Slyke and Folch, 1940) consisted of 25 g of CrO_3, 167 ml of syrupy phosphoric acid, and 333 ml of fuming sulfuric acid (20% free SO_3). Just prior to use, 5 grams of KIO_3 were added to 100 ml of the acid mixture and the resulting solution was heated to

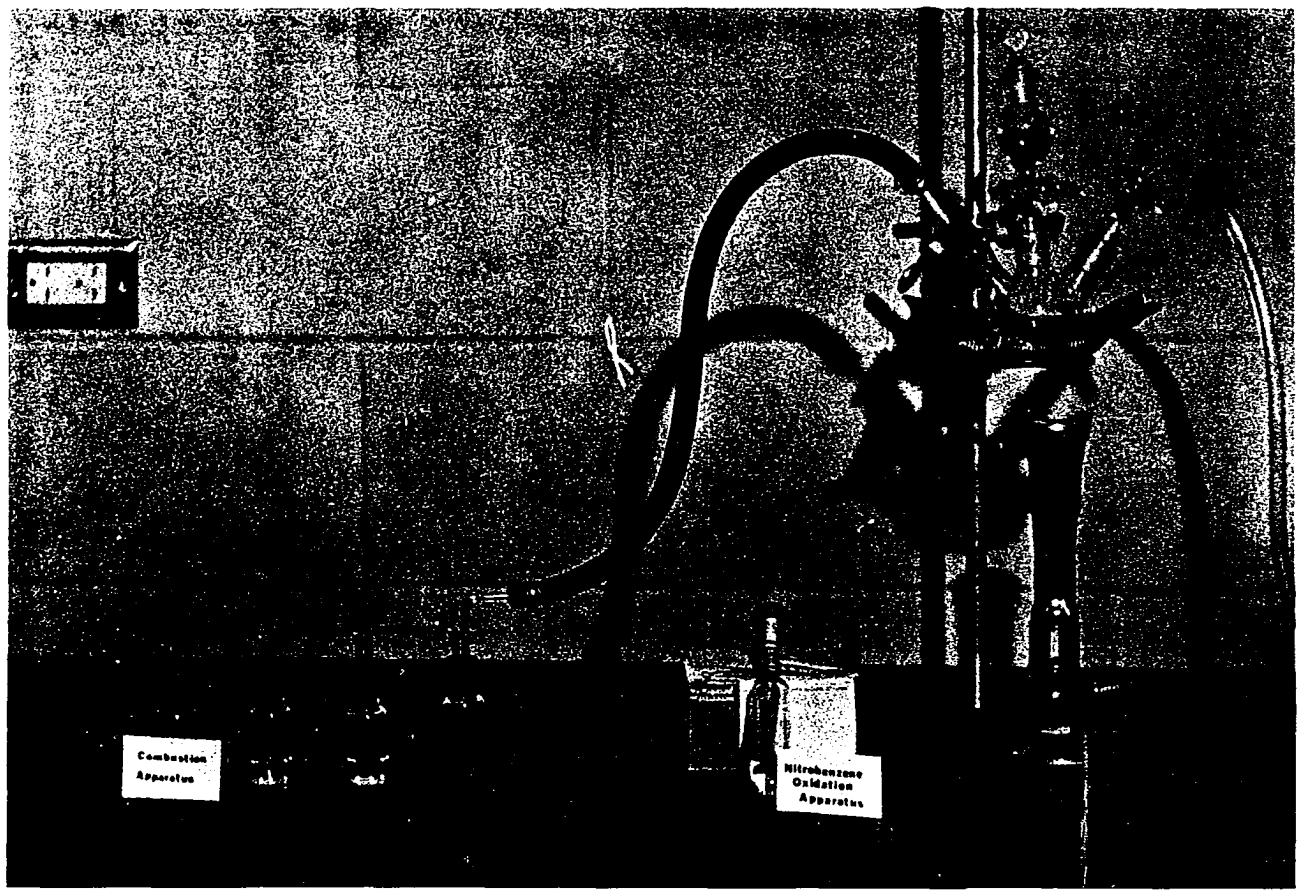


FIGURE 2. Nitrobenzene Apparatus and Combustion Apparatus.

160°C. As soon as the solution cooled, it was used in the combustion procedure. Recently, another method for the determination of $C^{14}O_2$ in plant insoluble material has been developed (Watson and Williams, 1970). It is very similar to the above method except more elaborate equipment and a closed system are required.

Determination of Radioactivity

The radioactivity was measured by a Beckman DPM-100 liquid scintillation counter. The per cent error at the 96% confidence level was not greater than 5%, usually 2%. Ten ml of the scintillation solution, Beckman's cocktail-D were used for each vial. Cocktail-D consists of 5 g of PPO (2,5 diphenyloxazone) (Beckman), 100 g of naphthalene (Beckman) and enough p-dioxane (Matheson Coleman and Bell; Scintillation Quality) to make one liter.

The extent of the quenching was determined by using an external standard reference, and every sample was counted on the external standard ratio channel. To find the relationship between the external standard ratio number and the counting efficiency, the following experiment was performed. The external standard ratio number and the cpm were obtained for an unquenched sample. Several drops of a quenching solution were added and a lower external standard ratio and lower cpm were obtained. The process was repeated several times. The cpm of the most unquenched sample were designated as relative 100% efficiency. The cpm at relative 100% efficiency correspond to the maximum efficiency of the instrument for C^{14} or about 90% of the disintegrations per minute; therefore, cpm at relative 100% efficiency = 90% dpm. The cpm of the quenched samples were also changed to some relative per cent efficiency value ((cpm of

quenched sample)/(cpm of relative 100% efficiency sample) x 100 = relative percent efficiency of quenched sample).

Empirically it was found that the plot of the inverse of the percent efficiency vs. the inverse of the external standard ratio was a straight line (Fig. 3 and 4). Mr. Phil Schafer found the slope and intercept of plots such as Figures 3 and 4 using a least square program for the Olivetti Underwood Programma 101. By using the slope and intercept in another program devised by him it was possible to convert the cpm of quenched samples to cpm at the relative 100% efficiency level.

Determination of Radiochemical Purity of Scopoletin, Scopolin and Fabiatrin

Scopoletin-4-C^{14} was synthesized in this laboratory by Alexis Zane (1963) and extensively purified by column chromatography and crystallization.

To determine if any decomposition had occurred since 1963, the radiochemical purity of the scopoletin-4-C^{14} was examined by chromatography. A portion of the scopoletin-4-C^{14} was dissolved in methanol and spotted on thin-layer plates coated with a microcrystalline cellulose called Avicel-SF (FMC Corporation, American Viscose Division). The plates were developed in several different solvent systems and in each case only one fluorescent spot was observed. A more sensitive method for determining impurities was column chromatography. Relatively large amounts of samples could be used making it possible to detect very small amounts of impurities. A 1.1 cm diameter column was packed to a depth of 12 cm with Polyclar AT in water. A 0.5 ml aliquot of scopoletin-4-C^{14} dissolved in distilled water was added to the column and the

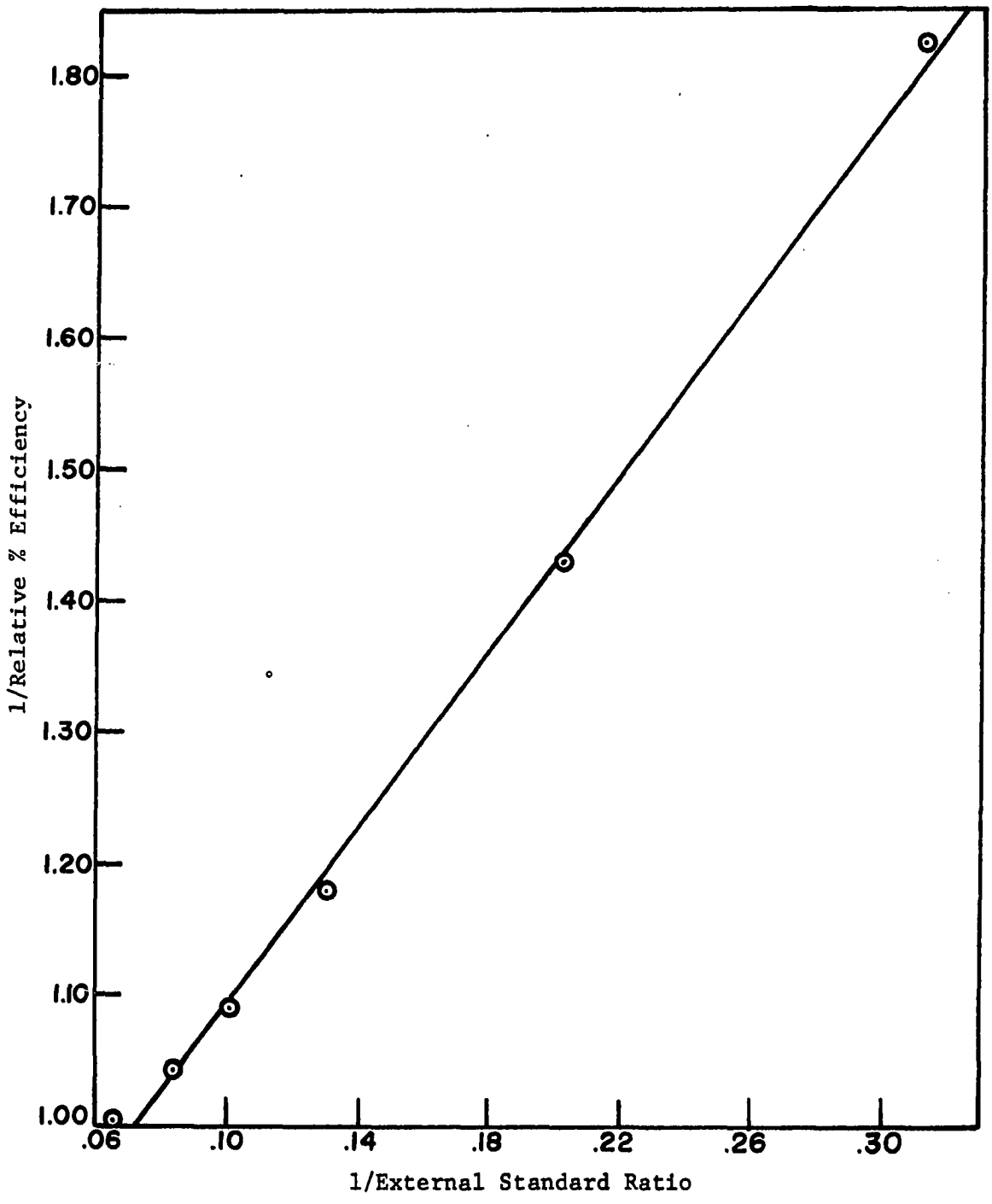


FIGURE 3. A Double Reciprocal Plot of Relative Percent Efficiency vs. External Standard Ratio. The Plot was Used to Correct for the Quenching Caused by Pigments in the Tobacco Shoots. (See text for details.)

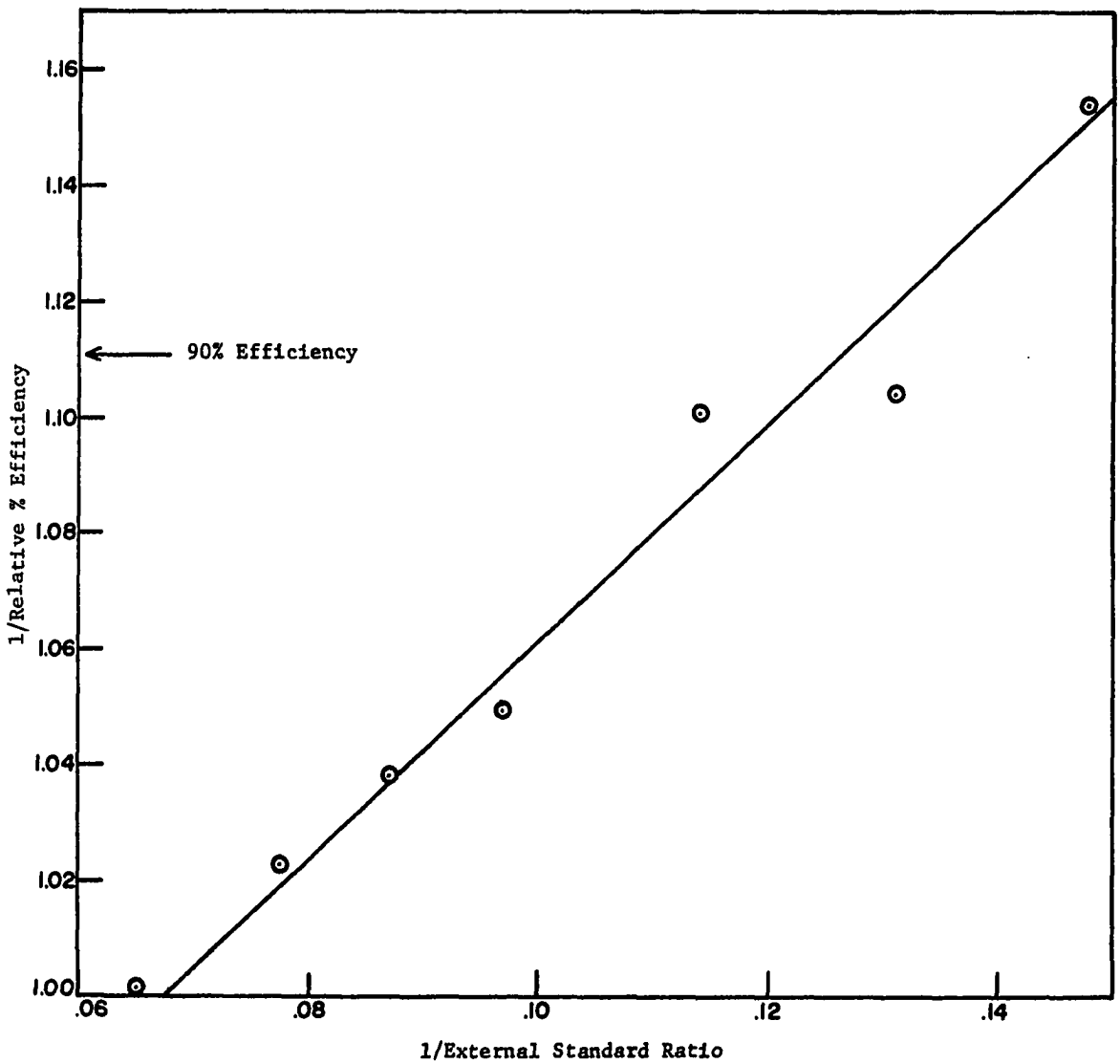


FIGURE 4. A Double Reciprocal Plot of Relative Percent Efficiency vs. External Standard Ratio. The Plot Was Used to Correct for Quenching Caused by Pigments in the Tobacco Roots. (See text for details.)

scopoletin was eluted with water. Fractions of 3.5 ml were collected, and the fluorescence and radioactivity of each fraction were determined as usual. The fluorescence and radioactivity of each fraction were plotted as seen in Figure 5. Columns packed with Polyclar AT in benzene: methanol (97:3, v/v) and with Sephadex G-15 were also used in the same type of procedure. The results indicated the radiochemical purity of the scopoletin-4-C^{14} was at least 99%.

The radiochemical purity of the scopolin and fabiatrin obtained from the paper chromatograms developed in KFW (see page 10) was also determined by column chromatography. IBMW soluble crude extracts (roots and shoots) were streaked on paper and developed in KFW for 20 hours. The scopolin and fabiatrin zones were cut out and eluted in 5% methanol. The eluates from the paper strips were chromatographed on a 0.9 cm diameter column packed to a depth of 75 cm with Sephadex G-25 (medium). The rest of the procedure was the same as used for scopoletin-4-C^{14}. Figure 6 shows no major radioactive impurity and a fluorescent impurity amounting to about 3% of the total fluorescence. Figure 7 shows that the fabiatrin obtained from the paper chromatographs had no major radioactive impurities. Two fluorescent impurities, however, were present.

Biosynthesis of Double Labeled and Glucose-1-C^{14} Scopolin

Five 125 ml flasks of eight day old tobacco culture cells (WR-132) were filtered on a Buchner filter and washed on the same filter with 400 ml of a defined salt medium (Linsmaier and Skoog, 1965) minus the inositol and sucrose. One half of the washed cells was used for the biosynthesis of the double labeled scopolin and half was used for the biosynthesis of glucose-1-C^{14} scopolin. Two 125 ml Erlenmeyer flasks

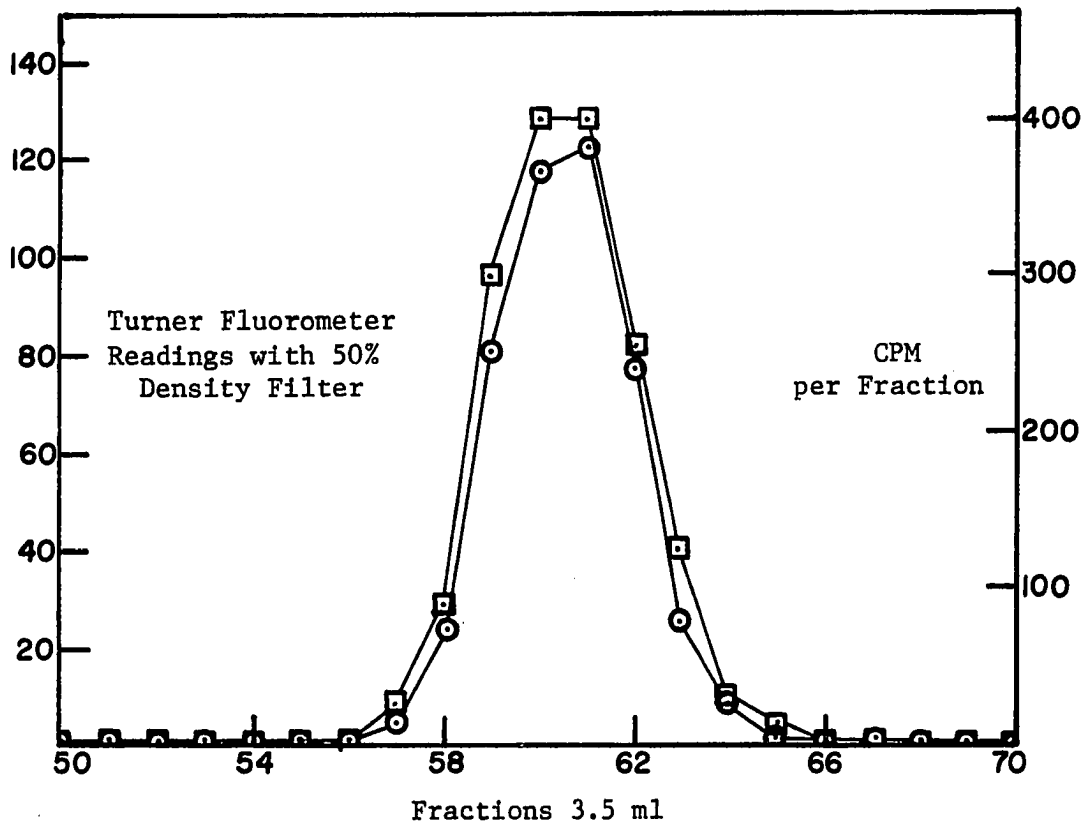


FIGURE 5. Chromatography of Scopoletin-4-C^{14} on a 1.1 x 12 cm Column of Polyclar AT Using Distilled Water as Eluting Solvent.

□ = CPM per fraction

○ = Fluorescence as measured by a Turner Fluorometer

Fluorometer readings above 100 were made with a 10% density filter.

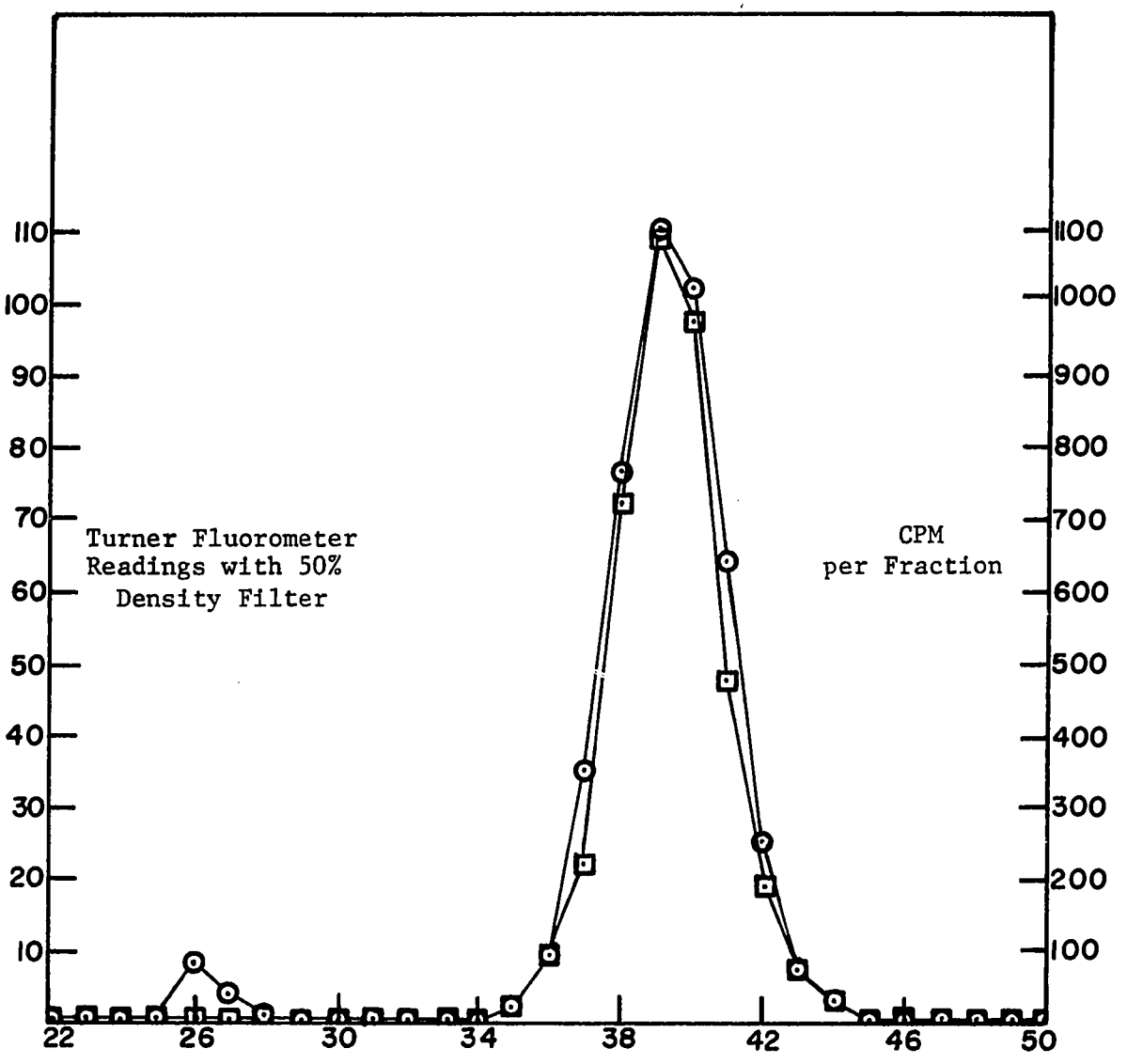


FIGURE 6. Chromatography of Scopolin-4-C^{14}, Obtained from Paper Chromatograms of Crude Extracts, on a 0.9 x 75 cm Column of Sephadex G-25 Using Distilled Water as Eluting Solvent.

□ = CPM per fraction

○ = Fluorescence as measured by a Turner Fluorometer

Fluorometer readings above 100 were made with a 10% density filter.

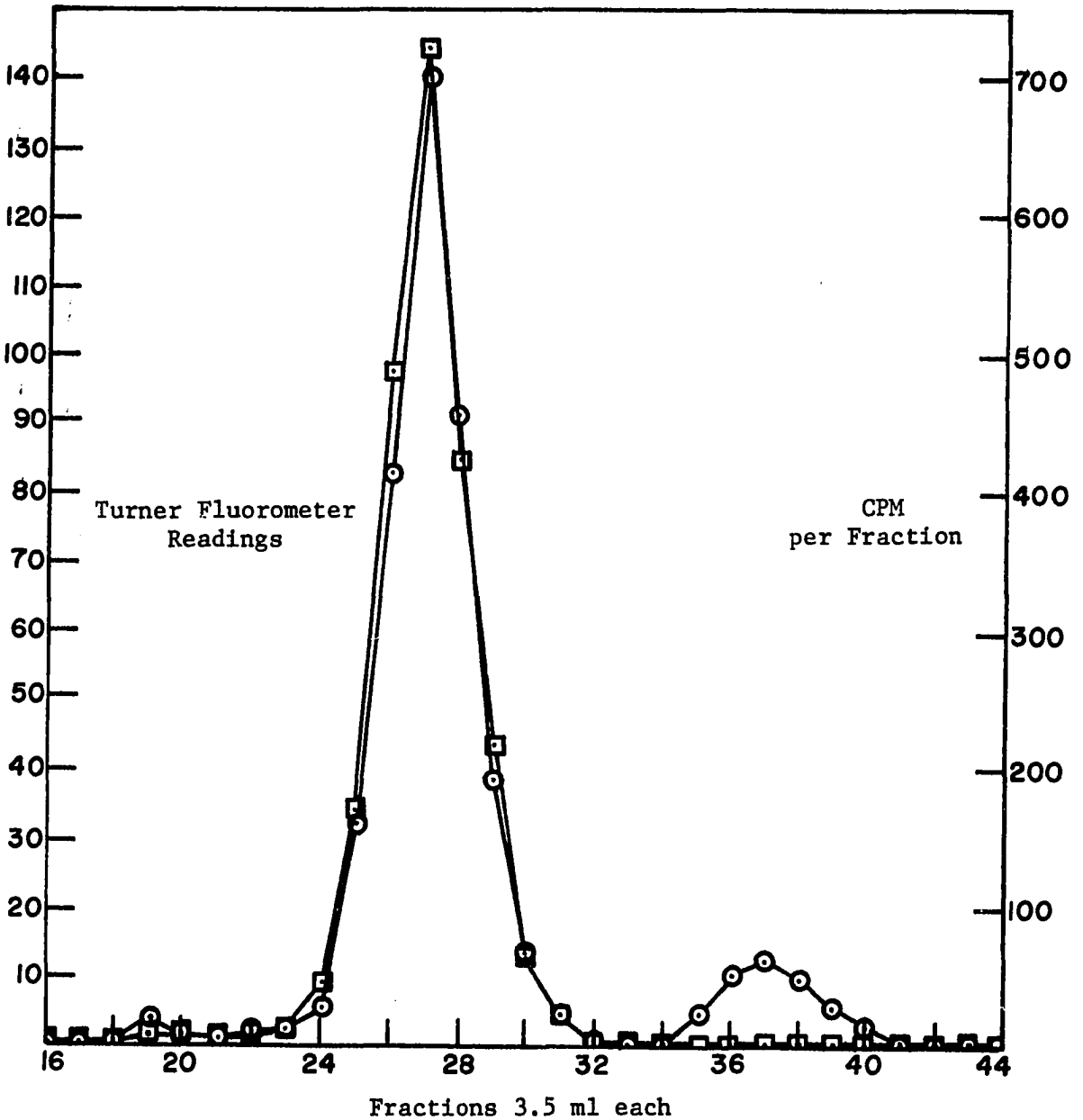


FIGURE 7. Chromatography of Fabiatrin-4-C^{14}, Obtained from Paper Chromatography of Crude Extracts, on a 0.9 x 75 cm Column of Sephadex G-25 Using Distilled Water as Eluting Solvent.

□ = CPM per fraction

○ = Fluorescence as measured by a Turner Fluorometer

Fluorometer readings above 100 were made with a 50% density filter.

were used in each procedure. For the biosynthesis of the double labeled scopolin, each flask contained the tobacco cells, 2 mg of glucose-1-C^{14} (10 μ Ci), and 3 mg of tritium labeled scopoletin in 50 ml of tissue culture medium (minus the inositol and sucrose). The flasks used for the biosynthesis of glucose-1-C^{14} scopolin were identical to the above set except non-radioactive scopoletin was substituted for tritium labeled scopoletin. The complete mixture was incubated with shaking for 2.5 hours. The cells were collected by filtration and the spent media were saved. The cells while still on the filter were washed with 300 ml of deionized water. The plant tissue was fixed and extracted as previously described (page 9). An aliquot of the double labeled scopolin had a H^3 to C^{14} ratio of 1.24/1 after the second purification step and a ratio of 1.25/1 after the last purification step.

Purification of Radioactive Scopolin

The plant extracts were evaporated to a small volume and applied as 31 cm streaks to 46 x 57 cm sheets of Whatman No. 3 MM chromatography paper. The papers had been washed for 24 hours in a wash solution of methanol : water : acetic acid : n-butanol (10:6:1:2, v/v/v/v). The papers were next washed in 5% methanol for 24 to 48 hours.

After the papers were streaked with plant extract, they were developed in KFW for 20 hours. The scopolin band was cut out and eluted in 5% methanol overnight. The eluates were evaporated to a small volume and again applied to washed Whatman No. 3 MM chromatography paper. The papers were developed in (BAW) n-butanol : acetic acid : water (4:1:2, v/v/v). The scopolin zones were cut out and eluted in 5% methanol overnight.

The eluates were reduced to a small volume (1-5 ml) and applied to a 2.5 cm diameter column packed to a depth of 40 cm with Sephadex G-25 (fine). The fractions which fluoresced blue (scopolin) under UV light were brought to a known volume and frozen.

Sometimes a column packed with Polyclar AT was used as a preliminary purification step before the paper chromatography. The Polyclar AT used for column chromatography and enzyme purification was washed using a modification of a method previously reported (Mizelle, 1965). Two hundred fifty grams of Polyclar AT (General Aniline and Film Corporation) were hydrated in approximately one liter of water for 2-24 hours. The water was removed by filtration and the Polyclar AT was soaked and filtered consecutively in 385 ml dimethylformamide, 770 ml acetic acid, 2310 ml water, 1540 ml methanol and, finally, at least 4000 ml water. The Polyclar AT was dried in an oven at 60°C for several days. Deionized or distilled water was always added to the dry Polyclar AT at least 12 hours before use.

Procedures Used in the Examination of the IBMW Insoluble Material

The dry IBMW insoluble material was weighed and placed in a 30 ml filtering crucible. The crucible was placed in a 250 ml beaker and enough 1% HCl was added to cover a part of the crucible (usually about 150 ml of 1% HCl per gram of material). Ice was placed on a watch glass which covered the beaker, and the mixture was slowly refluxed, with occasional stirring, for four hours. In an alternate procedure, the protein was removed from the IBMW insoluble material by enzymic hydrolysis. The IBMW insoluble material was incubated for 12 hours at 37°C with a 1%

solution of protease (Sigma Type IV) (0.5 g of IBMW insoluble material per 100 ml of enzyme solution) in 0.05 M phosphate buffer, pH 7.6. In both procedures the mixtures were filtered and washed with distilled water. The filtrate was diluted to a known volume and saved. The residue was dried in a vacuum oven at 60°C and weighed.

This residue was oxidized by an alkaline nitrobenzene procedure. A mixture of the residue, redistilled nitrobenzene (0.15 ml per 0.1 g of plant residue), and 2 N sodium hydroxide (2.5 ml per 0.1 g of plant residue) was transferred to a small glass bomb (Fig. 2). The bomb was placed in a beaker of cooking oil on a hot plate. The complete apparatus was constantly agitated on a reciprocal shaker. The temperature was raised to 160°C and the oxidation was allowed to proceed for three hours. After the bomb cooled, the oxidation mixture was transferred to a liquid-liquid extractor (Ciereszko, 1966) and extracted with ether for twenty-four hours. This ether extract containing the nitrobenzene was counted for radioactivity. The pH of the extraction mixture in the liquid-liquid extractor was adjusted to about seven, using phosphoric acid. The mixture was again extracted for twenty-four hours with ether. The ether was removed from the extractor, evaporated to dryness and the resulting residue was taken to a known volume with methanol and saved. The oxidation mixture was lowered to a pH of about one, and the mixture was again extracted with ether for twenty-four hours. The ether phase was removed; the ether evaporated; and the resulting residue was taken to a known volume with methanol and saved. The aqueous mixture left in the liquid-liquid extractor was filtered and the residue was washed with distilled water. Both the filtrate and residue were saved (Dieterman, 1961; Browning, 1967).

The second procedure for defining lignin was a 72% sulfuric acid procedure. The protein was removed from 1 g samples of IBMW insoluble material by hydrolysis in 1% HCl for four hours. The resulting residue was placed in a small beaker and 72% sulfuric acid (15 ml) was slowly added with stirring. The mixture was allowed to stand for 2 hours in a water bath at 18-20°C, with frequent stirring. The material was washed into a one liter beaker, and water (560 ml) was added for dilution to a sulfuric acid concentration of 3%. Ice was placed on a watch glass which covered the beaker, and the mixture was slowly refluxed with occasional stirring for four hours. The insoluble lignin was allowed to settle, and the mixture was filtered into a preweighed filtering crucible. The residue was washed free of acid with distilled water, dried, and weighed (Browning, 1967). The residue is called Klason lignin or 72% sulfuric acid lignin.

CHAPTER III

RESULTS

Continuous-feeding of Scopoletin-4-C^{14}

The first experiment was a continuous feeding one, i.e., the tobacco seedlings were left in a scopoletin-4-C^{14} nutrient solution for various lengths of time. As mentioned earlier, Einhellig *et al.* (1970) had examined the effects of different concentrations of scopoletin on growth of tobacco seedlings and determined the concentrations of scopoletin that inhibit growth; therefore, a concentration of scopoletin (1.94×10^{-4} M) was used in this experiment that would give maximum radioactivity yet would not inhibit the growth of the seedlings. The specific activity of the scopoletin-4-C^{14} was 3.5 μ curies/μ mole. Six groups of four plants each were used in the experiment. A plant group was harvested after 1, 4, 11, 28, 50 and 74 hours. The plants were extracted as described in the experimental section.

Isolation and Identification of Fabiatrin

The radioactive root extracts were streaked on Whatman #1 chromatography paper and developed for 20 hours in KFW. After the papers were dry, they were cut into sections as shown in Figure 8. The different sections of the paper were eluted with 5% MeOH and counted for radioactivity.

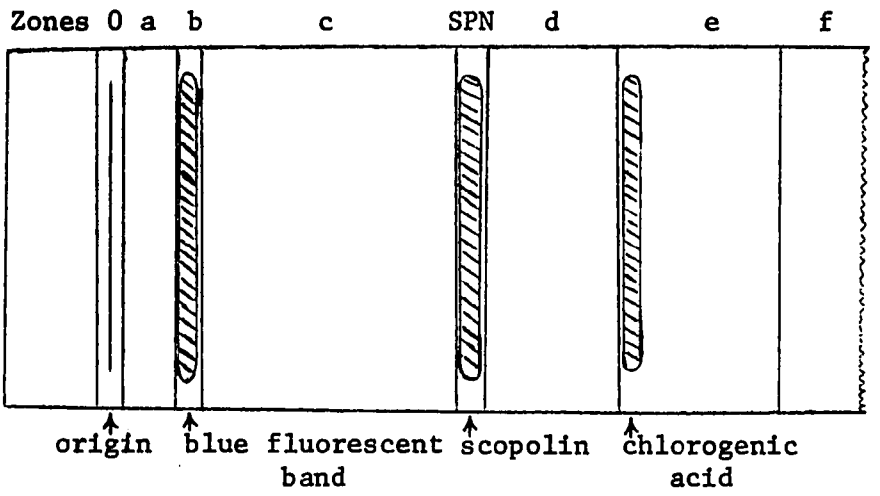


FIGURE 8. A Paper Chromatogram of a Radioactive Root Extract Developed in Methylisobutyl Ketone : Formic Acid : Water (14:3:2, v/v/v) (KFW).

The section containing the blue fluorescent band (zone b, Fig. 8) was found to be radioactive. When the paper chromatographs were developed in IFW instead of KFW, the blue fluorescent band ran ahead of scopolin indicating that the substance was more polar than scopolin.

In order to obtain greater quantities of the blue fluorescent compound, 100 ml of non-radioactive IBMW root solution (23.5 g of roots) was evaporated to dryness and redissolved in 25 ml of water. The aqueous solution was extracted three times with ethyl acetate, and the ethyl acetate was discarded. Seventy-five ml of ethyl acetate: pyridine (2:1, v/v) were added. A small aqueous layer at the bottom of the separatory funnel containing "tars" was discarded. The remaining solution was evaporated to dryness and the residue dissolved in 25 ml of water. The aqueous solution was chromatographed on a 6 cm diameter column packed to a depth of 30 cm with Polyclar AT in water. The

column was eluted with water, and about 50 ml fractions were collected. The first fractions contained sugars while the next few fractions contained amino acids. Next came the unknown blue fluorescent zone followed by a larger blue fluorescent zone, scopolin. The compounds with free phenolic groups were adsorbed to the column (Loomis and Battaile, 1965). The fractions, which contained the unknown blue fluorescent zone, were evaporated to a small volume and applied as a 31 cm streak to a number of 46 x 57 cm Whatman No. 3 MM chromatography papers which were then developed in BAW. Several blue fluorescent bands were seen. Each band was cut out and eluted from the paper with 5% methanol. The eluates were evaporated to about 0.5 ml and spotted on Avicel-SF thin layer plates along with the radioactive blue fluorescent compound. The plates were developed in BAW and EPW (ethyl acetate : pyridine : water (2:1:2, v/v/v)). The eluate that contained the blue fluorescent compound whose R_f corresponded to the unknown radioactive compound was further purified by paper chromatography. The papers were developed in IFW and BPW (butanol : pyridine : water (14:3:3, v/v/v)). A column packed with Polyclar AT in benzene : methanol (95:5, v/v) was used as the last purification step.

A portion of the purified material was dissolved in water and equal amounts were pipetted into 15 ml pear-shaped flasks and evaporated under reduced pressure to dryness. To each flask either 2 ml of 1 N HCl, 0.1 N HCl or H_2O were added. The three flasks were heated for one hour on a steam bath. The acid was removed by a flash evaporator, and a small amount of isopropanol azeotrope was added to each flask. The unknown samples were spotted on Avicel SF thin-layer plates along with scopolin, scopoletin, glucose, xylose, and a number of other monosaccharides.

The plates were developed in EPW. Both the 1 N HCl sample and the 0.1 N HCl sample had three fluorescent spots while the H_2O sample had only one. The fluorescent spots were circled and the plate was sprayed with aniline-oxalate reagent (Partridge, 1950). Two, sometimes three spots, could be seen when the sprayed plates were heated in the oven at 105°C for 10 minutes. The R_f values of the different spots are shown in Table 1.

Enzymatic hydrolysis of the suspected fabiatrin was accomplished by spraying the purified fabiatrin band, which had been streaked on 3 MM Whatman chromatography paper, with 0.1% emulsin (0.1 M acetate buffer, pH 5.3). The paper was kept warm and moist for two hours and developed in BAW for 13 hours. The results are shown in Table 2.

These results are consistent with the identification of the unknown blue-fluorescent band as fabiatrin, the β-primeveroside 6(β-D-xylosido)-β-D-glucopyranosido derivative of scopoletin (Chaudhury *et al.*, 1948; Sargent and Skoog, 1961; and Birkofer *et al.*, 1967). The UV spectrum was very similar to a published spectrum of fabiatrin (Sargent and Skoog, 1961) as was the melting point of 226°C compared with 226°-228°C (Chaudbury *et al.*, 1948) and 230°C (Birkofer *et al.*, 1967).

Continuous-feeding of Scopoletin-4-C^{14} (continued)

After fabiatrin had been identified, the radioactivity of fabiatrin, scopolin and scopoletin, the specific activity of scopolin and fabiatrin, and the activity of various fractions from the scopoletin-4-C^{14} uptake experiment were determined. The results can be seen in Tables 3, 4 and 5 and Figure 9.

As shown in Figure 9, Table 3, and Table 5, most of the scopoletin

TABLE 1

R_f VALUES OF SUSPECTED FABIATRIN AND SOME OF ITS HYDROLYSIS PRODUCTS *I*

Standards	R_f	Unknown	R_f
Scopolin	0.605	Suspected Scopolin	0.607
Scopoletin	0.94	Suspected Scopoletin	0.94
Xylose (red spot)	0.36; 0.54*	Suspected Xylose (red spot)	0.37; 0.54*
Glucose	0.26; 0.38*	Suspected Glucose	0.26; 0.38*
		Suspected Fabiatrin	0.45
		Suspected Primeverose	0.18*

*Developed twice

Fabiatrin was hydrolyzed in HCl, spotted on Avicel-SF thin-layer plates, and developed in ethyl acetate : pyridine : water (2:1:2, v/v/v).

TABLE 2

R_f VALUES OF SUSPECTED FABIATRIN AND SOME OF ITS HYDROLYSIS PRODUCTS *II*

Standards	R_f	Unknown	R_f
Scopolin	0.62	Suspected Scopolin	0.61
Scopoletin	0.88	Suspected Scopoletin	0.87
Xylose	0.40	Suspected Xylose	0.38
Glucose	0.32	Suspected Glucose	0.32
		Suspected Fabiatrin*	0.46

Fabiatrin was hydrolysed by 0.1% emulsin, streaked on Whatman No. 3 MM paper and developed in butanol : acetic acid : water (4:1:2, v/v/v) (BAW).

*Sargent and Skoog (1961) obtained an R_f of 0.47 in BAW for fabiatrin.

TABLE 3

ACTIVITIES OF FRACTIONS AND COMPOUNDS AT VARIOUS TIMES DURING THE CONTINUAL FEEDING OF SCOPOLETIN-4-C^{14} TO TOBACCO SEEDLINGS

Hours of uptake	Total cpm taken up	Total cpm recovered	IBMW Insol.	IBMW Soluble		Scopolin		Fabiatrin
			Roots	Roots	Shoots	Roots	Shoots	Roots
1	15.7	12.5	7.07	4.43	0.990	4.13	0.671	0.175
4	38.2	29.0	15.8	11.3	1.93	10.2	1.18	0.575
11	88.1	61.5	33.2	25.9	2.42	22.4	1.36	0.990
28	176	126	60.9	54.7	10.2	40.8	5.88	2.48
50	264	179	94.2	73.3	11.7	49.1	7.82	3.64
74	351	249	141	88.5	19.1	61.2	10.4	6.64

The activities are measured in cpm x 10^4.
Scopoletin-4-C^{14} was at a concentration of 1.94 x 10^{-4} M

Sol. = soluble
Insol. = insoluble
IBMW = isopropanol : benzene : methanol : water, (2:1:1:1, v/v/v/v)

TABLE 4

ACTIVITIES OF FRACTIONS AND COMPOUNDS EXPRESSED AS PERCENT AT VARIOUS TIMES

DURING THE CONTINUAL FEEDING OF SCOPOLETIN-4-C^{14} TO TOBACCO SEEDLINGS

Hours of uptake	% cpm recovered	% cpm in IBMW Sol.[1]	% cpm in IBMW Insol. (roots)[1]	% cpm in IBMW Sol. (roots)[1]	% cpm in IBMW Sol. (shoots)[1]	$\frac{fab}{spn} \times 100$	% cpm (spn + fab)[1]	% cpm (spn + fab)[2]	% cpm in IBMW Insol. (roots)[3]
1	79.5	34.5	45.0	28.2	6.3	4.2	31.6	91.6	56.6
4	75.7	34.6	41.1	29.5	5.0	5.6	31.3	90.3	54.2
11	69.7	32.1	37.6	29.4	2.7	4.4	35.5	87.4	53.9
28	71.6	36.9	34.6	31.0	5.8	6.1	27.9	75.7	48.5
50	67.8	32.2	35.6	27.7	4.4	7.4	22.9	71.2	51.7
74	70.9	30.6	40.3	25.2	5.4	10.8	22.3	72.7	56.8

[1] of total cpm absorbed
[2] of total cpm in IBMW soluble
[3] of total cpm recovered

fab = fabiatrin
spn = scopolin
IBMW = isopropanol : benzene : methanol : water, (2:1:1:1, v/v/v/v).
sol. = soluble
insol. = insoluble

TABLE 5

ACTIVITIES AND SPECIFIC ACTIVITIES OF SCOPOLIN, SCOPOLETIN AND FABIATRIN AT VARIOUS TIMES DURING THE CONTINUAL FEEDING OF SCOPOLETIN-4-C^{14} TO TOBACCO SEEDLINGS

Hours of uptake	Roots					Shoots	
	Scopolin		Fabiatrin		Scopoletin	Scopolin	
	Activity	Sp. Act.	Activity	Sp. Act.	Activity	Activity	Sp. Act.
1	4.13	1.2	0.175	1.6	0.365	0.671	0.55
4	10.2	2.6	0.575	2.5	0.305	1.18	1.2
11	22.4	2.6	0.990	2.7	0.503	1.36	1.4
28	40.8	3.0	2.48	2.8	0.575	5.88	2.3
50	49.1	3.1	3.64	3.0	0.885	7.82	2.4
74	61.2	3.0	6.64	2.9	1.235	10.4	2.2

Activity is measured in cpm x 10^4.
Specific activity is measured in μ curies/μ mole.
The specific activity of the original scopoletin-4-C^{14} solution was 3.5 μ curies/μ mole.

4-C^{14} in the soluble portion was found in the form of its 7-glucoside, scopolin. The scopoletin-4-C^{14} was at a virtual steady state while the scopolin-4-C^{14} increased at a rate that could be described by a rectangular hyperbola (Fig. 9). Since the specific activity of the scopolin in the roots at the different time periods was about the same, the concentration of scopolin increased at about the same rate as the radioactivity. The plot of the reciprocal of radioactivity in scopolin (roots) vs. the reciprocal of hours of uptake of radioactive scopoletin is shown in Figure 10. From this plot, the maximum cpm of radioactive scopolin that could accumulate under these conditions in the roots was calculated to 77.5 x 10^4 cpm.

The average amount of radioactivity recovered was 72.5% of the total radioactive scopoletin taken up. Part of the loss of radioactivity was due to the small (about 2% of the total radioactivity), but significant amount of radioactivity in the IBMW insoluble portion of the leaves.

An average of 33.4 percent of the total radioactivity taken up was located in the IBMW insoluble portion of the roots. Of the total radioactivity recovered, an average of 53.6 percent was located in the IBMW insoluble portion of the roots and 46.4 percent was located in the IBMW soluble portion (roots and shoots). The IBMW soluble portion of the roots had almost 6 times as much radioactivity as the average amount of radioactivity found in the IBMW soluble portion of the shoots. The ratio of radioactivity in the insoluble fraction to that in the soluble fraction was about the same for all time periods. This means that part of the radioactive scopoletin was used by the plant to form insoluble material at the time it was taken up or very shortly afterwards. Likewise, the proportion of radioactivity in the shoots as compared to the

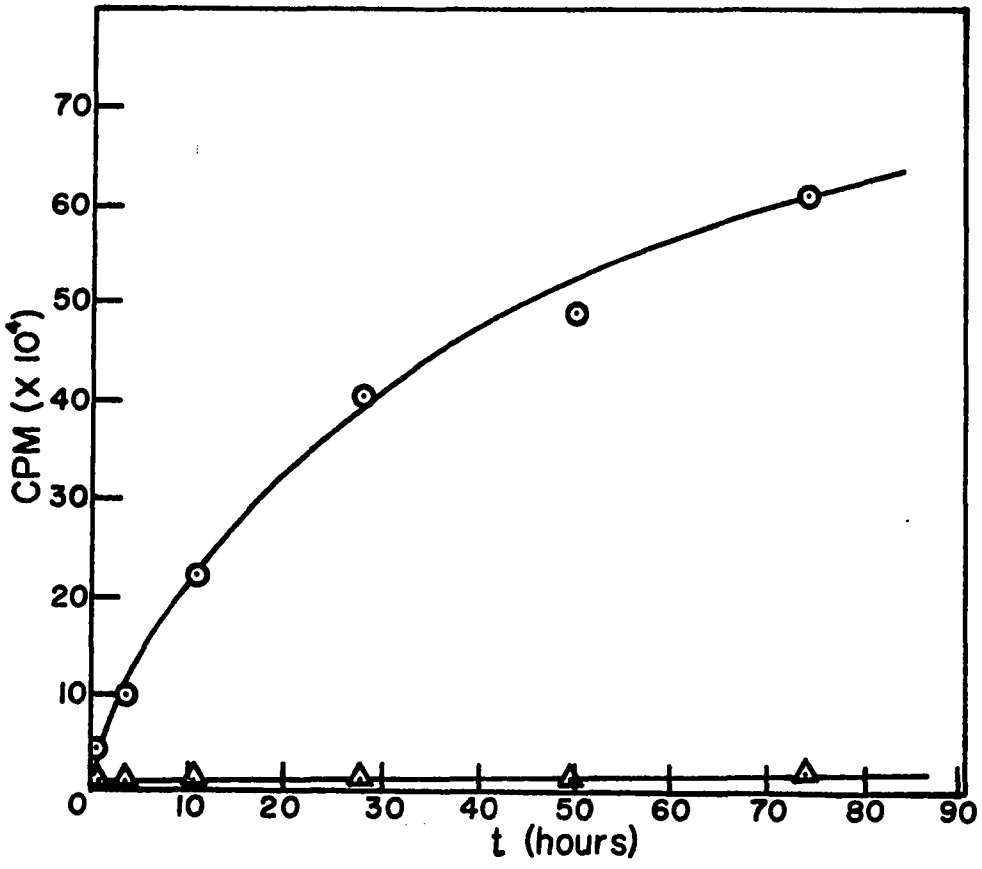


FIGURE 9. Activity of Scopolin and Scopoletin in the Roots of Tobacco Seedlings during the Continual Uptake of Scopoletin-4-C^{14}.

△ = Activity of Scopoletin in the Roots of Tobacco Seedlings

○ = Activity of Scopolin in the Roots of Tobacco Seedlings

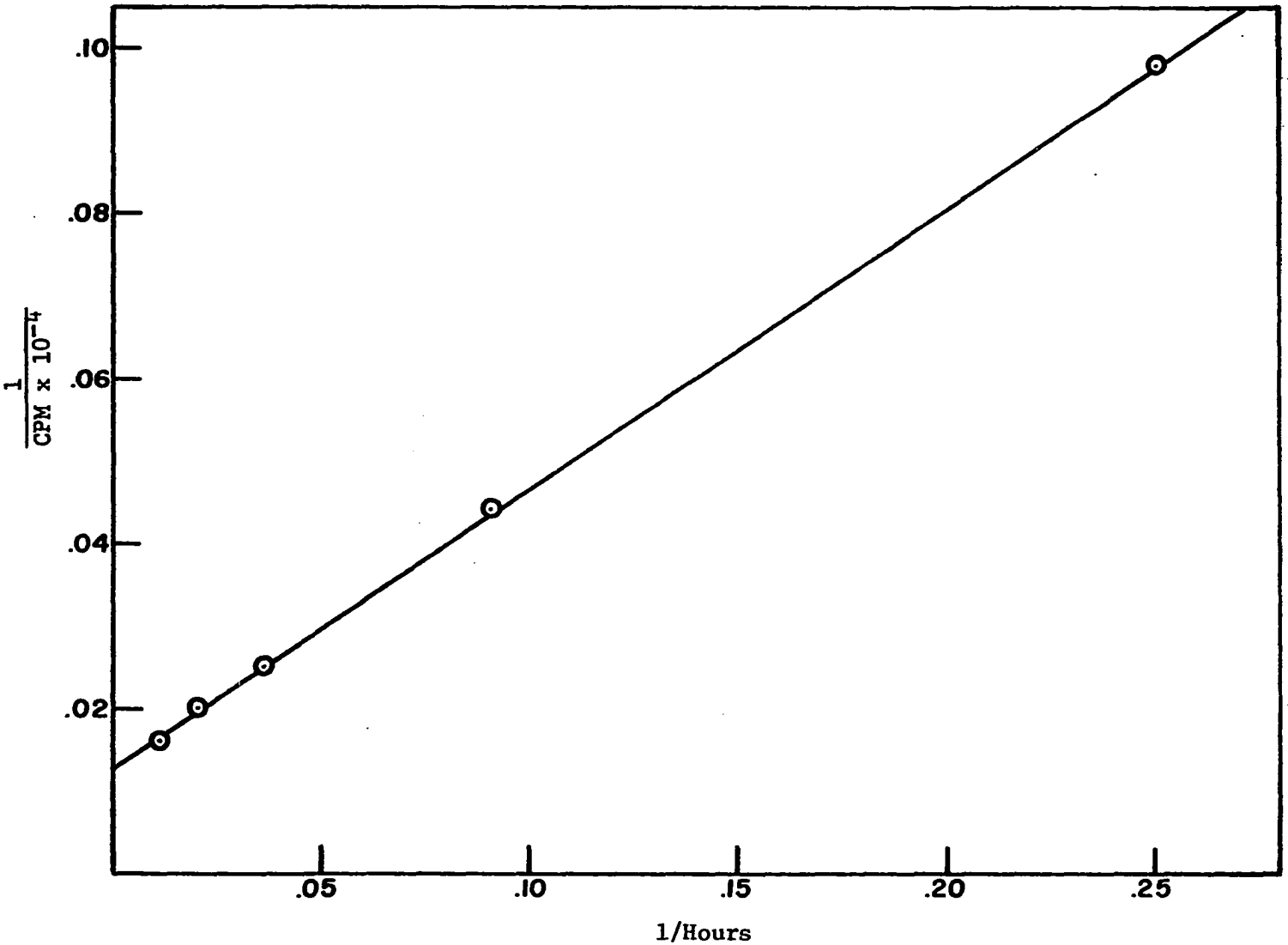


FIGURE 10. A Double Reciprocal Plot of CPM of Scopolin in Tobacco Roots vs. Time during the Continual Uptake of Scopoletin-4-C^{14}.

radioactivity in the roots was independent of the length of time the seedlings were left in the radioactive scopoletin solution. Therefore, radioactive scopoletin, scopolin or some derivative must have been very quickly translocated from the roots to the shoots.

The percent of the radioactivity in scopolin and fabiatrin as compared to the total radioactivity in the IBMW soluble portion decreased with time. This means that either scopolin or fabiatrin were rapidly converted to some other soluble compound or that some of the scopoletin was converted to compounds other than scopolin and fabiatrin.

Pulse Feeding of Scopoletin-4-C^{14}

Previous pulse-feeding experiments in this laboratory, using tritium labeled scopoletin had shown that scopolin was metabolized very slowly. Therefore, the experiment was spread over a period of sixteen days and a long pulse-feeding period was used. Scopoletin-4-C^{14} at a concentration of 1.94×10^{-4} M and a specific activity of 3.5 μ curies/μ mole was taken up by the tobacco seedlings for twenty-eight hours. The seedlings were allowed to metabolize the radioactive scopoletin for 0.3, 4, 10 and 16 days. At the end of each defined time period a group of four plants was washed, fixed and extracted as described in the experimental section.

The roots turned a dark greenish color after several hours in the scopoletin solution as was also reported by Einhellig *et al.* (1970). The growth of the plants which had absorbed the scopoletin-4-C^{14} was the same as the controls, and the fresh weight of both groups more than quadrupled during the sixteen days of the experiment.

The radioactivity of fabiatrin, scopolin, and various fractions was determined. Also, the specific activity of scopolin and fabiatrin

TABLE 6

ACTIVITIES OF FRACTIONS AND COMPOUNDS AT DIFFERENT TIMES

AFTER CESSATION OF FEEDING OF SCOPOLETIN-4-C^{14}

Days after removal of precursor	Total cpm taken up	Total cpm recovered	cpm in IBMW Insol. Roots	cpm in IBMW Sol.		Scopolin cpm		fab cpm
				Roots	Shoots	Roots	Shoots	Roots
0.3	176	119	60.1	46.6	12.6	33.9	6.51	2.62
4	178	122	71.8	38.7	11.1	28.9	6.04	4.16
10	174	110	66.7	35.5	7.48	23.4	5.43	5.88
16	175	(105)	74.1	30.6	5.00*	16.9	--	4.83

The activities are measured in cpm x 10^4
Scopoletin-4-C^{14} at a concentration of 1.94 x 10^{-4} M was taken up for 28 hours.

*estimated
( ) the estimated value was used to obtain this calculated value.

Sol. = soluble
Insol. = insoluble
IBMW = isopropanol : benzene : methanol : water, (2:1:1:1, v/v/v/v).
fab = fabiatrin

TABLE 7

ACTIVITIES OF FRACTIONS AND COMPOUNDS EXPRESSED AS PERCENT AT DIFFERENT TIMES AFTER CESSATION OF FEEDING OF SCOPOLETIN-4-C^{14}

Days after removal of precursor	% cpm recovered	% cpm in IBMW Sol.[1]	% cpm in IBMW Insol. (roots)[1]	% cpm in IBMW Sol. (roots)[1]	% cpm in IBMW Sol. (shoots)[1]	$\frac{fab}{spn} \times 100$	% cpm spn spt fab[2]	% cpm in IBMW Insol. (roots)[3]
0.3	67.6	33.6	34.1	26.4	7.2	7.7	72.7	50.4
4	68.5	28.3	40.3	21.7	6.2	14.4	78.5	58.8
10	63.1	24.7	38.3	20.4	4.3	24.6	85.6	60.7
16	(60.0)	(17.5)	42.3	17.4	2.9	26.6	--	(70.5)

[1] of total cpm absorbed
[2] of total cpm in IBMW soluble
[3] of total cpm recovered

spn = scopolin
spt = scopoletin
fab = fabiatrin
IBMW = isopropanol : benzene : methanol : water, (2:1:1:1, v/v/v/v)

Sol. = soluble
Insol. = insoluble

TABLE 8

ACTIVITIES AND SPECIFIC ACTIVITIES OF SCOPOLIN AND FABIATRIN AT DIFFERENT TIMES AFTER CESSATION OF FEEDING OF SCOPOLETIN-4-C^{14}

Days after removal of precursor	Roots				Shoots	
	Scopolin		Fabiatrin		Scopolin	
	Act.	Sp. Act.	Act.	Sp. Act.	Act.	Sp. Act.
0.3	33.9	2.8	2.62	2.8	6.51	2.1
4	28.9	2.5	4.16	2.8	6.04	2.1
10	23.4	1.8	5.88	2.4	5.43	1.3
16	16.9	1.1	4.83	2.0	--	-

Activity is measured in cpm x 10^4.
Specific activity is measured in μ curies/μ mole.

The specific activity of the original scopoletin-4-C^{14} solution was 3.5 μ curies/μ mole.

was determined. The results can be seen in Tables 6, 7 and 8. The jar containing the shoots from the sixteen day experiment was broken and the sample lost before it could be analyzed.

A plot of the log of the radioactivity of scopolin in the roots vs. days gave a straight line (Fig. 11). The biological half life of scopolin was calculated from the slope of the curve and found to be about sixteen days. In the roots, the radioactivity of fabiatrin increased from 7.7% of the radioactivity of scopolin to 26.6% in sixteen days (Table 7) while the activity of scopolin decreased. It is clear that scopolin is the precursor of fabiatrin. Also, since the specific activity of fabiatrin decreased at a slower rate than scopolin, fabiatrin is metabolized at a slower rate than scopolin. The radioactivity of scopolin in the shoots decreased at a slower rate than the activity of scopolin in the roots. The shoots have no fabiatrin which may explain the slower turnover rate of scopolin in the shoots. The percent of the radioactivity in scopolin plus fabiatrin as compared to the total radioactivity in the IBMW soluble fraction decreased with time (Table 7). Other soluble radioactive products were either oxidized to CO_2 or converted to insoluble material at a faster rate than scopolin and fabiatrin. The percent of the radioactivity in the IBMW insoluble portion (roots) of the total radioactivity recovered increased with time. Thus, some compounds in the soluble portion of the plant were slowly converted to IBMW insoluble material.

Pulse-Feeding of Scopolin-4-C^{14}

Scopolin-4-C^{14} obtained from the previous experiments was purified as described in the experimental section. Scopolin-4-C^{14} at a concentration

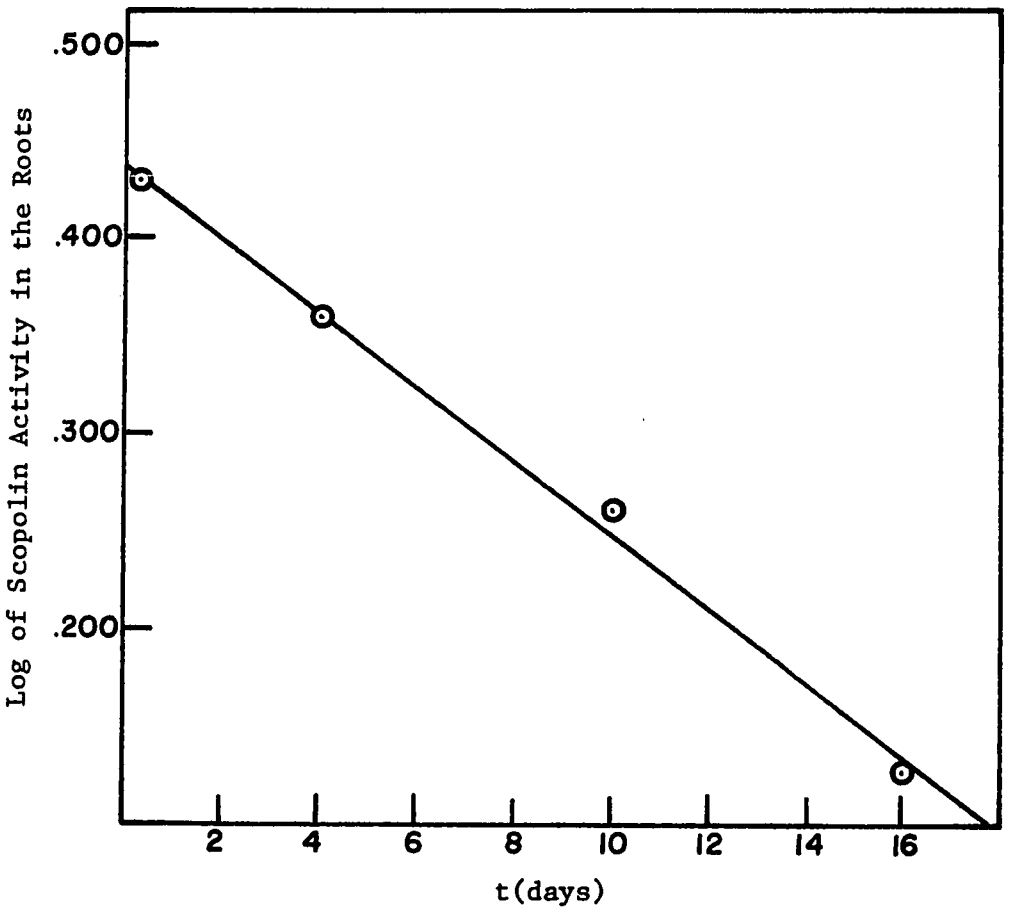


FIGURE 11. Semi-log Plot of Activity of Scopolin in Tobacco Roots vs. Time

of 1.04×10^{-4} M and a specific activity of 0.49 μ curies/μ mole was taken up by the tobacco seedlings for twenty hours. Scopoletin-4-C^{14} at a concentration of 5.57×10^{-5} M and a specific activity of 3.5 μ curies/μ mole was also taken up by one group of seedlings for twenty-eight hours. The seedlings that had absorbed the radioactive scopolin were left for 0, 3, and 8 days. At the end of each defined time period a group of three plants was washed, fixed and extracted as described in the experimental section.

The radioactivity of scopolin, scopoletin, fabiatrin, and various fractions and the specific activity of scopolin and fabiatrin were determined. The results can be seen in Tables 9, 10 and 11. In this experiment, glass extraction thimbles were used in place of paper extraction thimbles. Possibly that change accounts for the larger percent of recovered radioactivity in this experiment. A larger percent of the recovered radioactivity was found in the IBMW insoluble material than in the previous experiments.

As the concentration of precursor was decreased, a larger percent of the radioactivity was found in the IBMW insoluble portion of the plant. When the concentration of the radioactive precursor was 5.57×10^{-5} M, 89.0% of the total recovered radioactivity was found in the IBMW insoluble material (roots). At a concentration of 1.04×10^{-4} M the figure is 70.7% and at a concentration of 1.94×10^{-4} M the figure is about 50%.

A reversed trend can be seen for the percent of radioactivity in the IBMW soluble portion of the shoots. The values for the 5.57×10^{-5} M, 1.04×10^{-4} M and 1.94×10^{-4} M precursor are 0.8%, 1.4% and 6%, respectively.

The results of this experiment are very similar to those obtained

TABLE 9

ACTIVITIES OF FRACTIONS AND COMPOUNDS AT DIFFERENT TIMES

AFTER CESSATION OF FEEDING RADIOACTIVE PRECURSOR

Days after removal of precursor	Total cpm taken up	Total cpm recovered	cpm in IBMW Insol. (Roots)	cpm in IBMW Sol.		Scopolin cpm		FAB cpm
				Roots	Shoots	Roots	Shoots	Roots
Scopolin-4-C^{14}								
0	43300	34500	24400	9520	600	5500	500	425
3	37000	30800	23800	6350	600	3990	500	800
8	45450	38000	31500	6270	200	3720	166	1400
Scopoletin-4-C^{14}								
0	333000	262000	233000	26000	2700	12200	2040	1850

Scopolin-4-C^{14} at a concentration of 1.04×10^{-4} M was taken up for 20 hours.
Scopoletin-4-C^{14} at a concentration of 5.57×10^{-5} M was taken up for 28 hours.

Sol. = soluble
Insol. = insoluble
IBMW = isopropanol : benzene : methanol : water, (2:1:1:1, v/v/v/v).
FAB = fabiatrin

TABLE 10

ACTIVITIES OF FRACTIONS AND COMPOUNDS EXPRESSED AS PERCENT AT DIFFERENT TIMES AFTER CESSATION OF FEEDING OF RADIOACTIVE PRECURSOR

Days after removal of precursor	% cpm recovered	% cpm in IBMW Sol.[1]	% cpm in IBMW Insol. (roots)[1]	% cpm in IBMW Sol. (roots)[1]	% cpm in IBMW Sol. (shoots)[1]	$\frac{fab}{spn} \times 100$	% cpm spn spt fab[2]	% cpm in IBMW Insol. (roots)[3]
Scopolin-4-C^{14}								
0	79.7	23.4	56.4	22.0	1.4	7.73	66.6	70.7
3	83.2	18.8	64.4	17.1	1.6	20.0	79.3	77.6
8	83.5	14.2	69.3	11.0	0.4	26.6	84.0	82.9
Scopoletin-4-C^{14}								
0	78.7	8.6	70.0	7.8	0.8	12.7	72.9	89.0

Scopolin-4-C^{14} at a concentration of 1.04×10^{-4} M was taken up for 20 hours.
Scopoletin-4-C^{14} at a concentration of 5.57×10^{-5} M was taken up for 28 hours.

[1] of total cpm absorbed
[2] of total cpm in IBMW soluble
[3] of total cpm recovered

spn = scopolin
spt = scopoletin
fab = fabiatrin
IBMW = isopropanol : benzene : methanol : water, (2:1:1:1, v/v/v/v)

Sol. = soluble
Insol. = insoluble

TABLE 11

ACTIVITIES AND SPECIFIC ACTIVITIES OF SCOPOLIN AND FABIATRIN

AFTER CESSATION OF FEEDING RADIOACTIVE PRECURSOR

Days after removal of precursor	Roots				Shoots		Roots
	Scopolin		Fabiatrin		Scopolin		Scopoletin
	Activity	Sp. Act.	Activity	Sp. Act.	Activity	Sp. Act.	Activity
Scopolin-4-C^{14}							
0	5500	0.41	425	0.47	500	0.133	325
3	3990	0.31	800	0.43	500	0.110	225
8	3720	0.20	1400	0.38	166	0.0188	150
Scopoletin-4-C^{14}							
0	122000	1.6	1850	0.19	2040	0.103	600

The specific activity of the original scopoletin-4-C^{14} solution was 3.5 μ curies/μ mole.
The specific activity of the original scopolin-4-C^{14} solution was 0.49 μ curies/μ mole.

Specific activity is measured in μ curies/μ mole.

from pulse-feeding of scopoletin-4-C^{14}. Scopolin was converted at least in part to fabiatrin. Both fabiatrin and scopolin were metabolized very slowly with fabiatrin having a slower turnover rate. It is more apparent from Tables 9 and 10 than from Tables 7 and 8 that either fabiatrin, scopolin or some other soluble radioactive material was converted to IBMW insoluble material. The percent of the radioactivity in scopolin plus fabiatrin as compared to the total radioactivity in the IBMW soluble fraction decreased with time. Apparently some unidentified radioactive compounds in the IBMW soluble fraction were metabolized faster than scopolin and fabiatrin.

Search for Other Radioactive Compounds in the IBMW Soluble Fraction

As previously mentioned, radioactive root extracts were streaked on Whatman #1 chromatography paper. After development in KFW, different sections of the paper were counted for radioactivity. Zone D (see Figure 8) was the only zone other than the fabiatrin and scopolin bands to have significant amounts of radioactivity. Material from this zone was chromagraphed on a 1.5 cm diameter column packed to a depth of 22 cm with Polyclar AT in benzene : methanol 97:3 (v/v). The column was eluted with benzene : methanol 95:5 (v/v), and 3.5 ml fractions were taken. Aliquots of 0.5 ml from each fraction were counted for radioactivity. A plot was made of the radioactivity per fraction and seven radioactive peaks were observed. The fractions from the most radioactive peak were hydrolyzed with 1 N HCl and an aliquot of the hydrolysate was chromatographed on a column packed with Sephadex G-10. One large and one small radioactive peak were seen. The remainder of the hydrolysate was chromatographed on

a Polyclar AT column packed in methanol:water 70:30 (v/v) and eluted with the same solution. Non-radioactive scopoletin was used as a marker. Two small radioactive peaks plus a major peak were observed. The major peak was eluted just after the scopoletin peak and was observed to be non-fluorescent under ultraviolet light. The 3-hydroxy-, 4-hydroxy-, 5-hydroxy-, and 8-hydroxycoumarins are non-fluorescent (Goodwin and Kavanagh, 1950). Also, the 3,4-dihydrocoumarins are non-fluorescent (Goodwin and Pollok, 1954). One unsuccessful attempt was made to synthesize 3,4-dihydroscopoletin, but no additional work was done on this compound.

An aliquot of the 74 hour IBMW root sample (see Table 3) was chromatographed on a 1.4 cm diameter column packed to a depth of 78 cm with Sephadex G-15. The fluorescence and radioactivity of each fraction were determined. Nine percent of the total recovered activity was eluted before the fabiatrin peak. Therefore, it can be assumed that about 9% of this sample had a molecular weight greater than fabiatrin.

Compounds with free phenolic group(s) are adsorbed on aqueous Polyclar AT (Loomis and Battaile, 1966). A column packed with Polyclar AT in water was used to adsorb compounds with free phenolic groups. After all other compounds had freely passed through the column, the phenolic compounds were eluted from the column with methanol. The 28 hour IBMW root sample (see Table 3) had 9% of the total recovered radioactivity as compounds with free phenolic groups.

Translocation Experiment

In order to determine if scopolin or scopoletin or both are translocated from old leaves to new leaves, four tobacco seedlings, which had

taken up scopoletin-4-C^{14} for 28 hours, were allowed to grow for sixteen days. The leaves were separated into two groups, old leaves and new leaves. The old leaves were present at the beginning of the experiment; the new leaves had formed during the sixteen day growth period. The radioactivity and specific activity of scopolin were determined for both groups of leaves. The specific activity of the scopolin in the old leaves was about 12 times greater than in the new leaves. The radioactivity of scopolin per gram of fresh weight in the old leaves was about 18 times greater than in the new leaves.

Examination of the IBMW Insoluble Material

A summary of the operations performed on the IBMW insoluble material can be seen in Figure 12. Table 12 shows the relative amounts of radioactivity found in the various fractions from the last two IBMW root samples examined.

Klason lignin (72% sulfuric acid lignin) was prepared from IBMW insoluble samples of shoots as described in the experimental section. The 1% HCl soluble material contained 9.4% of the total radioactivity recovered. The H_2SO_4 soluble fraction contained 8.6% of the radioactivity and the Klason lignin contained 82.0% of the radioactivity.

The 1% HCl or 1% protease treatment has been used (Browning, 1967) to remove protein from alcohol insoluble extracts of annual plants. The proteins, if they are not removed, interfere with the lignin determination. An IBMW insoluble sample from roots was hydrolyzed for four hours with 1% HCl. The filtrate was evaporated to a small volume and applied to a column 3 cm in diameter that had been packed to a depth of 53 cm with Sephadex G-25 (medium). To measure the void volume, blue dextran was

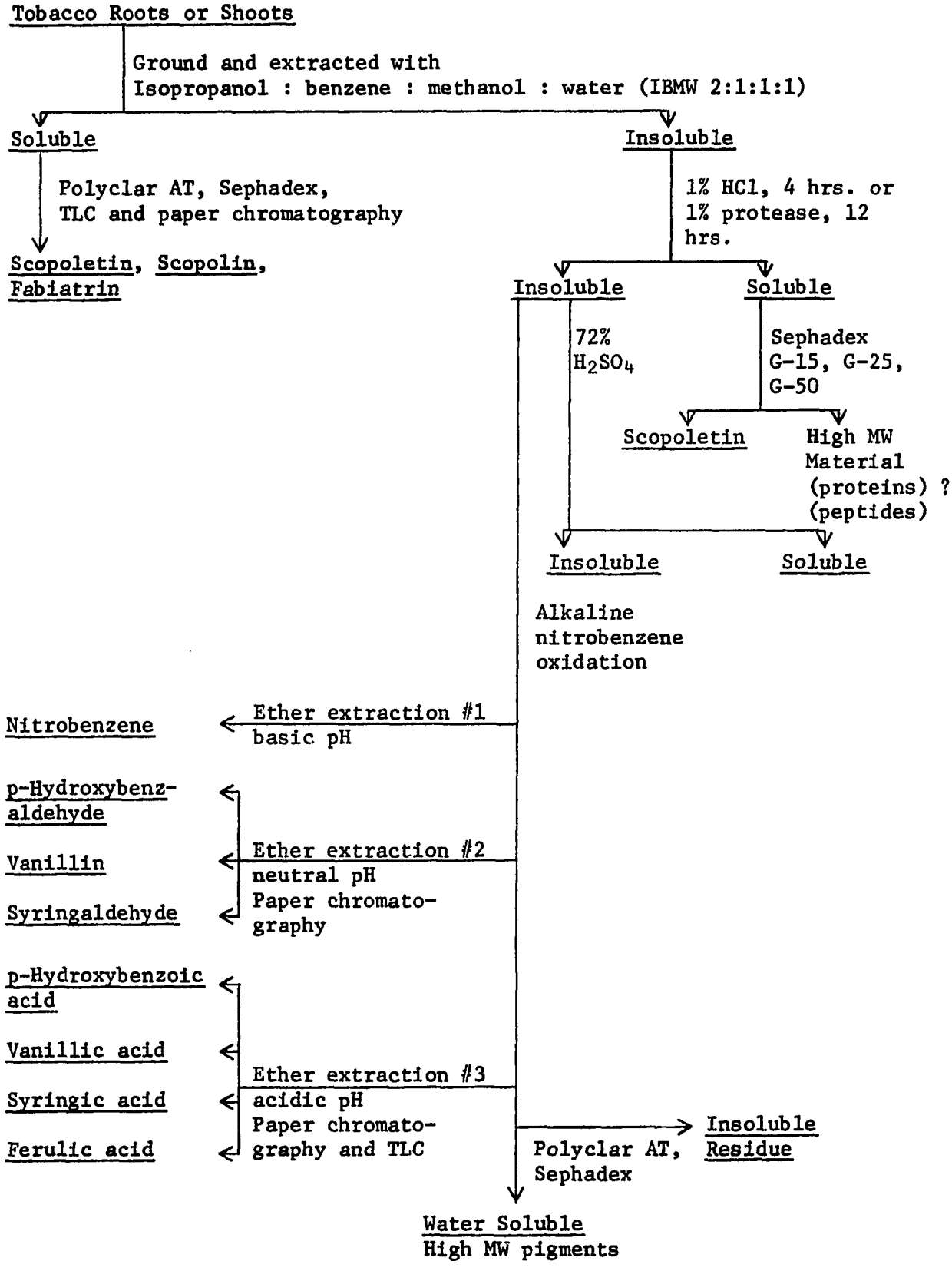


Figure 12. Flow Sheet of the Operations in the Extraction of Tobacco Seedlings Fed Scopoletin-4-C^{14} and the Separation of Different Fractions.

TABLE 12

DISTRIBUTION OF RADIOACTIVITY IN THE IBMW INSOLUBLE PORTION OF TOBACCO SEEDLINGS FED SCOPOLETIN-4-C^{14}

	% of Total Recovered	
	Sample 1	Sample 2
1% HCl, 4 hours	12.1	--
1% Protease, 12 hours	--	13.0
Ether Extractions after Nitrobenzene Oxidation		
Basic pH	0.6	0.6
Neutral pH	6.3	2.9
Acidic pH	30.1	27.6
Water Soluble Material	36.5	39.3
Insoluble Residue	14.3	16.5

applied just before the sample. The column was eluted with distilled water and eleven ml fractions were taken. One ml aliquots from each fraction were counted for radioactivity. Figure 13 shows the results. The fractions which made up the radioactive peak that eluted last from the column were reduced to a small volume, applied to Whatman No. 3 MM chromatography paper, and developed in BAW. One major blue fluorescent band was observed, and it was eluted off the paper. The eluate was spotted on Avicel-SF thin-layer plates along with a number of known phenolic compounds. The plates were developed in IFW, EPW, and BAW. The Rf of the unknown blue-fluorescing compound matched that of scopoletin, as did the color (purple) of the spot when sprayed with diazotized sulfanilic acid and Na_2CO_3 spray. Both the unknown and scopoletin exhibited the same UV spectra.

The fractions which made up the highest MW radioactive peak were evaporated to a small volume under reduced pressure. Capryl alcohol was added to prevent foaming. This sample was applied to a column 1.5 cm in diameter that had been packed to a depth of 80 cm with Sephadex G-50 (medium). The column was eluted with water. Three and a half ml fractions were collected, and blue dextran was used as described previously. The column fractions were measured for relative protein concentration (Itzhaki and Gill, 1964), fluorescence, and radioactivity. The results were plotted as shown in Figure 14. No direct correlation between the three can be seen. The radioactivity, fluorescence and relative protein concentration all seem to be spread out over a number of fractions. Both fraction A and fraction B (see Fig. 13) were chromatographed on a 1.5 x 72 cm column of Sephadex G-50 using

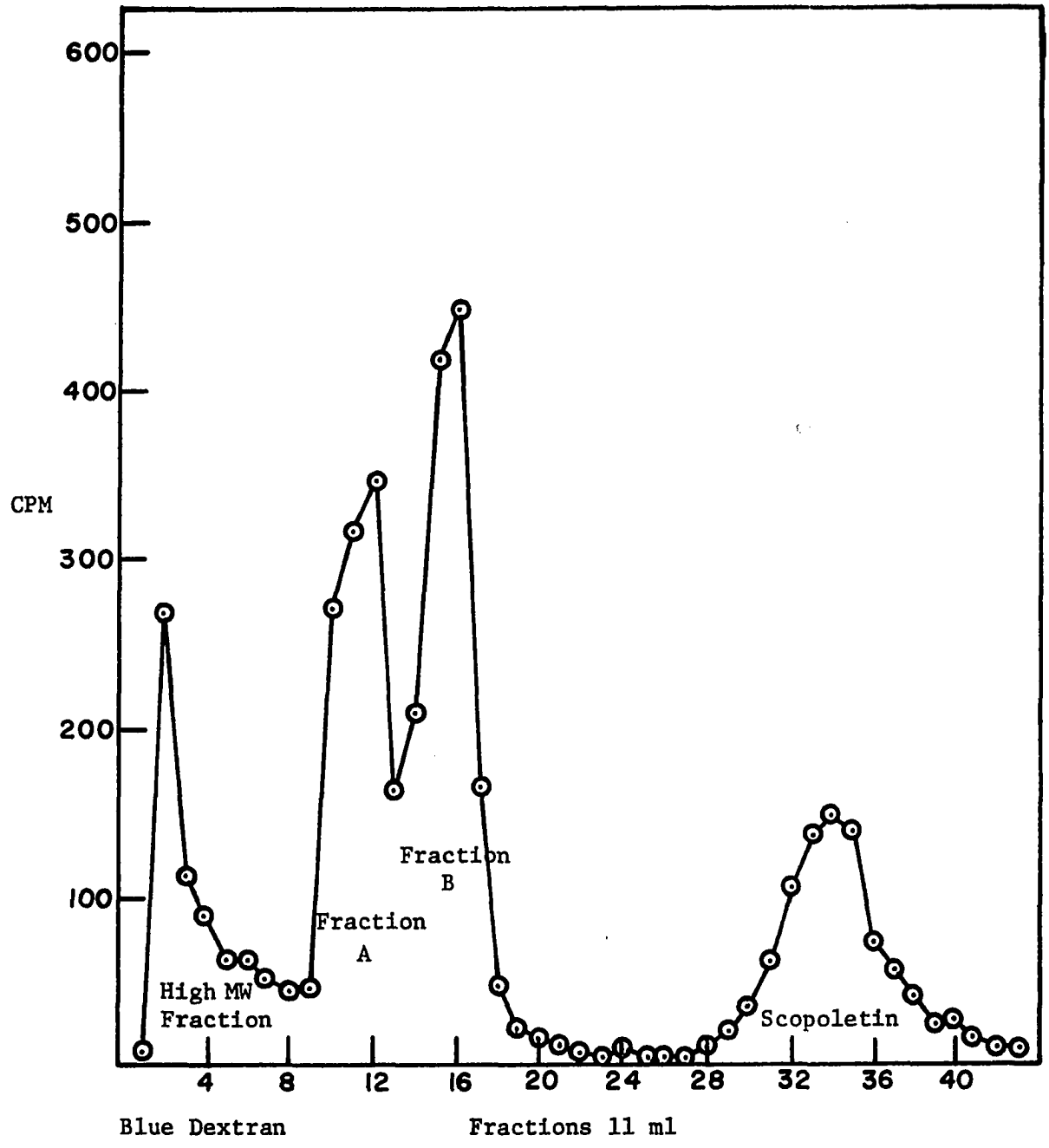


FIGURE 13. Chromatography of a 1% HCl Hydrolysate of an IBMW Insoluble Root Sample on a 3 x 53 cm Column of Sephadex G-25 Using Water as Eluting Solvent.

O = CPM per 1/11 of a fraction

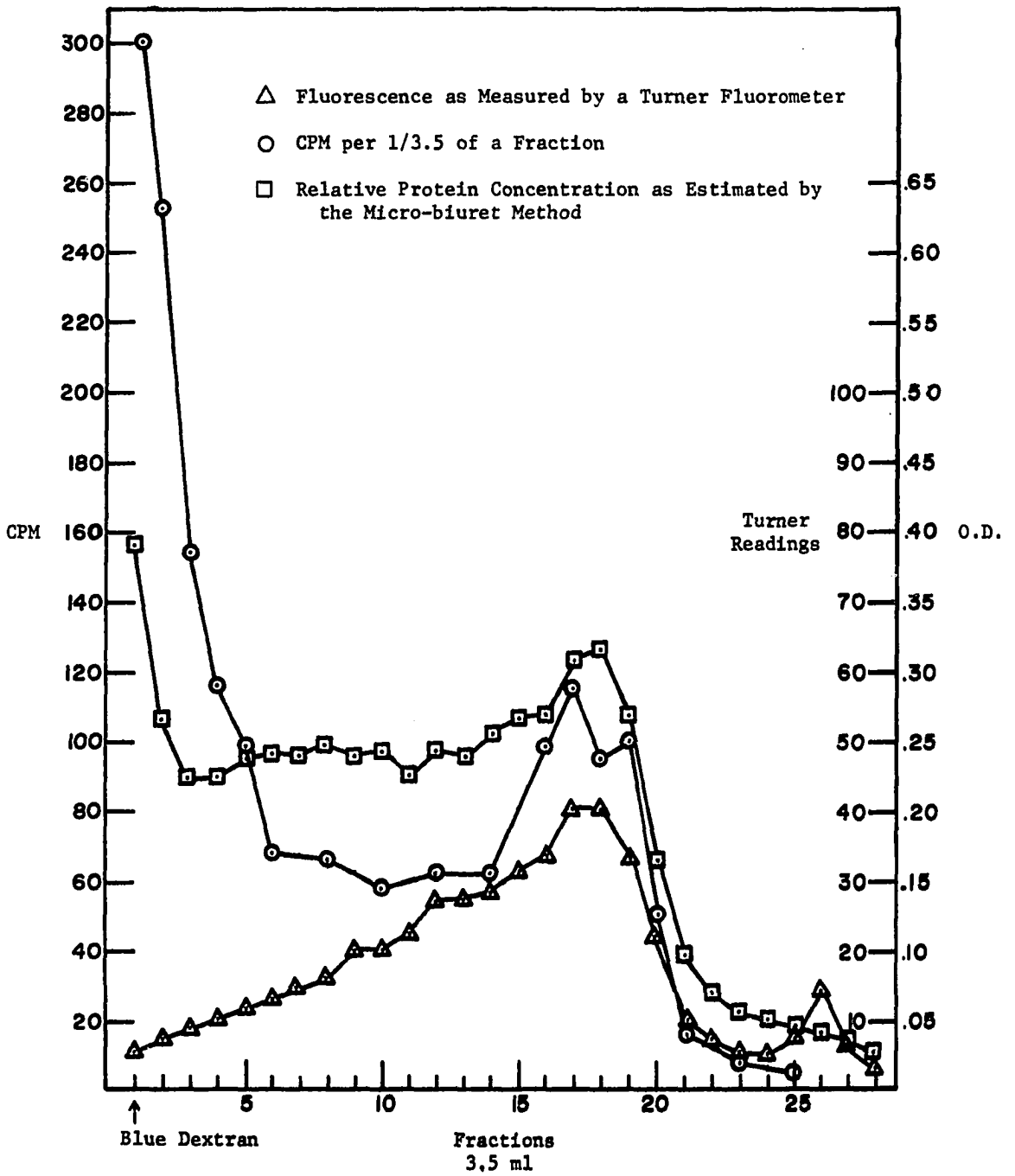


FIGURE 14. Chromatography of a High MW Fraction (Obtained from Gel Filtration Chromatography of a 1% HCl Soluble Fraction of an IBMW Insoluble Root Extract) on a 1.5 x 80 cm Column of Sephadex G-50 Using Water as Eluting Solvent.

water as the eluting solvent. The elution pattern of fraction A showed one main radioactive peak, in addition to radioactivity in the void volume. Fraction B gave one main peak with a small shoulder when chromatographed on the same column. The ultraviolet absorption spectrum of fraction A showed maxima at 265 and 213 mμ and a minimum at 240 mμ. Fraction B had a maximum at 203 mμ and a plateau minimum at 238-264 mμ.

Fraction A and B were further purified by paper chromatography on Whatman No. 3 MM chromatography paper. The solvent systems IFW, BAW, 2% acetic acid and n-butanol : formic acid : water, 15:3:2 (v/v/v) were used. The papers were cut into strips and eluted with 5% methanol. The different eluates were evaporated to a small volume and spotted on Avicel-SF thin layer plates. The plates were developed in IFW, BAW, n-butanol : acetic acid : water : pyridine, 15:3:12:10 (v/v/v/v) and pyridine : water, 4:1 (v/v). After the plates dried, they were sprayed with ninhydrin (0.3 g ninhydrin in 100 ml n-butanol mixed with 3 ml glacial acetic acid) and heated to 110°C for 10 minutes (Stahl, 1965). The ninhydrin positive spots, as well as other spots, were scraped into vials and counted for radioactivity. Radioactivity was found both in the ninhydrin positive spots and some of the other spots. Some of the fractions from the paper chromatograms had very little radioactivity in the ninhydrin positive spots, while in other fractions the majority of the radioactivity was in the ninhydrin positive spots.

Another IBMW insoluble sample from roots was hydrolyzed with 1% protease. The hydrolysate was chromatographed on a column 90 x 2.5 cm of Sephadex G-25 (medium) and was eluted with water. The elution pattern was the same as seen in Figure 13 except more radioactivity

was in the high molecular weight fraction and no scopoletin peak was seen. However, when fractions A and B were pooled, reduced in volume, streaked on paper and developed in BAW, a scopoletin band appeared on the paper. Therefore, the scopoletin must have come from one or both of these fractions.

The material obtained from the ether extraction at neutral pH (see Fig. 12) was streaked on Whatman No. 3 MM paper and developed in n-butanol saturated with 2N ammonium hydroxide. Vanillin, syringaldehyde and p-hydroxybenzaldehyde were located by spraying the chromatograms with a saturated solution of 2,4-dinitrophenylhydrazine in 2 N HCl (Dieterman, 1961). Only a very small fraction of the radioactivity was found in the lignin aldehydes. The material obtained from the ether extraction at acid pH was also streaked on Whatman No. 3 MM paper and developed in n-butanol saturated with 2N ammonium hydroxide. The papers were cut into zones, and the paper strips were eluted with 5% methanol. The different fractions were spotted on Avicel-SF and developed in butanol : pyridine : water 10:3:3 (v/v/v) along with reference phenolic acids. The plates were sprayed with diazotized sulfanilic acid + Na_2SO_3 spray. The results are shown in Table 13. The different fractions were also spotted on Avicel-SF thin layer plates and multi-developed in butanol saturated with 2N NH_4OH. The plates were again sprayed with diazotized sulfanilic acid + Na_2CO_3 spray. The results were consistent with p-hydroxybenzoic acid, ferulic acid, vanillic acid and syringic acid being present in this ether extract. The spots of the four phenolic acids were scraped off the plate and counted for radioactivity. They were only slightly radioactive (about

TABLE 13

Rf VALUES OF REFERENCE AND UNKNOWN PHENOLIC ACIDS

Compound	Rf[1]	Rf[2]	UV	DSA + Na_2CO_3
p-hydroxybenzoic acid	.64	.64	--	yellow
ferulic acid	.71	.72	blue	purple
vanillic acid	.75	.73	--	orange
syringic acid	.82	.82	--	red

The Avicel SF thin layer plates were developed in butanol : pyridine : water 10:3:3 (v/v/v). UV = ultraviolet light; (1) Reference phenolic acids; (2) unknown compounds. DSA + Na_2CO_3 = diazotized sulfanilic acid + Na_2CO_3 spray (Macek, 1963).

one to two times background). The material obtained from ether extraction at acid pH was also chromatographed on a 1.5 cm column packed to a depth of 17 cm with Polyclar AT. Non-radioactive vanillic, syringic, ferulic, and p-hydroxybenzoic acid were added. The elution was started with water then changed to methanol. Vanillic and syringic acids were in one fraction, and ferulic and p-hydroxybenzoic acids were together in another fraction. Very little of the radioactivity, however, was found in the fractions containing the phenolic acids. About half the radioactivity was not adsorbed to the column and the other half eluted from the column after the phenolic acids.

An aliquot from the water soluble material that remained after the ether extractions, was chromatographed on a 1.5 cm column packed to a depth of 10 cm with Polyclar AT. Four cm of Sephadex G-25 were layered on top of the Polyclar AT. The column was eluted first with water then

with methanol. Very little of the radioactive material was adsorbed onto the column, which demonstrates that the material had few free phenolic groups.

Another aliquot from the same sample was chromatographed on a 1.5 cm column packed to a depth of 75 cm with Sephadex G-25. Non-radioactive scopolin and blue dextran were used as markers. Two large radioactive peaks and one small radioactive peak were observed. All three peaks eluted before scopolin. Thus all of them had a molecular weight greater than scopolin.

CHAPTER IV

EXPERIMENTS WITH DOUBLE LABELED SCOPOLIN AND GLUCOSE-1-C^{14} SCOPOLIN

Introduction

In the initial experiments in search of scopolin synthetase, either the shoots or roots of whole tobacco seedlings were used as plant material. Because the cell walls from suspension tissue culture are easier to break it was hoped they would be better plant material for *in vitro* studies of scopolin synthetase. The suspension culture line WR-132 (*Nicotiana tabacum* L., var. Xanthi), however, has no scopolin (Schafer, 1970). In order to find if scopolin synthetase was present in these tissues, tritium labeled scopoletin was added to the media. Several hours after the addition of the radioactive scopoletin, the tissue was harvested and tritium labeled scopolin was found. Other studies by Schafer (1970) on the uptake of scopoletin into the cells and the conversion of scopoletin to scopolin also demonstrated that this culture line contains scopolin synthetase or at least an enzyme that would glucosylate scopoletin.

The lack of scopolin and the presence of a scopolin synthesizing enzyme(s) made this culture line an ideal vehicle for the synthetic biosynthesis of double labeled scopolin (C^{14}-glucose and tritium labeled scopoletin) and of glucose-1-C^{14} scopolin.

It was hoped that by feeding the double labeled compound to whole tobacco seedlings, evidence could be obtained for (1) a β-glucosidase that removes the glucose from scopolin giving scopoletin; (2) an equilibrium between scopolin and scopoletin as postulated by Wender (1970); and (3) the translocation of either scopoletin or scopolin.

Earlier work had indicated that radioactive scopoletin was incorporated into an IBMW insoluble fraction. Also, as the radioactivity in scopolin slowly disappeared, the IBMW insoluble fraction slowly increased in radioactivity (Table 10). In order to establish whether both scopolin and scopoletin were incorporated into the insoluble fraction, glucose-1-C^{14} scopolin was prepared. By feeding this compound to the tobacco seedlings and examining the IBMW insoluble fraction it would be possible to distinguish between scopolin and scopoletin as precursors of the insoluble material. Also, the glucose-1-C^{14} scopolin could be used to verify the presence or absence of a β-glucosidase that acts on scopolin.

Experimental

First Double Label Experiment

The original double labeled scopolin had 44% of the total cpm as C^{14} and a final concentration of 5×10^{-4} M. Three tobacco seedlings were left in the radioactive root solution for twenty-five hours. The first plant was washed, harvested, and extracted as usual after twenty-five hours. The other two plants were transferred to non-radioactive root solutions and left for an additional 69 and 167 hours before being harvested. The first step in the purification of radioactive scopolin

was used for the isolation of scopolin. The first two steps were used for the isolation of fabiatrin (see page 22).

Second Double Label Experiment

The double labeled scopolin had 41.1% of the total cpm as C^{14}. The final concentration of scopolin used in the experiment was 1.18×10^{-4} M. Tobacco seedlings were left in the scopolin solution for one, two, five, and eleven hours before they were harvested. The first two steps in the purification of radioactive scopolin were used to isolate the scopolin and fabiatrin from the crude plant extracts.

Results

First Double Label Experiment

The results of the first experiment are shown in Table 14.

It is clear from this initial experiment that most of the original glucose has been exchanged with non-radioactive glucose.

The small amount of C^{14} remaining could be due to C^{14} glucose left on the scopoletin, overlap from the tritium window to the carbon fourteen window, or C^{14} contaminants since only a one step purification was used.

Second Double Label Experiment

The results of the second experiment are shown in Table 15.

Even after one hour, over half of scopolin's C^{14}-glucose had been exchanged with non-radioactive glucose. In the roots, the per cent of the C^{14}-glucose in scopolin decreased from 16.4 to 2.3. The scopolin extracted from the shoots contains small amounts of C^{14}-

glucose at all time periods. The per cent C^{14} in the fabiatrin from the roots may also decrease with time, although the data are not clear on this.

TABLE 14

PERCENT CARBON FOURTEEN IN SCOPOLIN AND FABIATRIN ISOLATED FROM TOBACCO SEEDLINGS THAT HAD BEEN FED DOUBLE LABELED SCOPOLIN I

Hours after addition of double labeled scopolin	Hours after removal of scopolin	% C^{14} scopolin shoots	% C^{14} scopolin roots	% C^{14} fabiatrin roots
25	0	6.3%	4.5%	5.0%
94	69	3.6%	6.1%	5.4%
192	167	8.0%	3.6%	3.4%

Initial double labeled scopolin: % C^{14}-glucose 44.4%, % H^3-scopoletin 56.6%.

TABLE 15

PERCENT CARBON FOURTEEN IN SCOPOLIN AND FABIATRIN ISOLATED FROM TOBACCO SEEDLINGS THAT HAD BEEN FED DOUBLE LABELED SCOPOLIN II

Hours of uptake of double labeled scopolin	% C^{14} scopolin shoots	% C^{14} scopolin roots	% C^{14} fabiatrin roots
1	6.6	16.4	13.8
2	1.8	13.6	16.6
5	5.2	10.9	10.5
11	4.5	2.3	8.1

Initial double labeled scopolin: % C^{14}-glucose 41.1%, % H^3-scopoletin 58.9%.

Discussion

An equilibrium between scopoletin and scopolin has been assumed by Sargent and Skoog (1960), Steck (1967b) and Wender (1970). The step from scopoletin to scopolin (scopolin synthetase) has been demonstrated by radioisotopes (Steck, 1967; Fritig *et al.*, 1967) (Fig. 9). The reverse reaction has not been shown to occur. The results of this experiment indicate that the radioactive glucose is taken off and non-radioactive glucose replaces it. This indicates the likelihood of an equilibrium between scopoletin and scopolin. Either a β-glucosidase is present in tobacco which can act on scopolin, or scopolin synthetase is a reversible enzyme, or both enzymes may be present. Although limited information is available on the biosynthesis of phenolic glycosides, most reports indicate sugar nucleotides as donor molecules (Kleinhofs *et al.*, 1967; Towers, 1964). Most types of glycosyltransferases are freely reversible (Dixon and Webb, 1964). The data suggest that a large part of the radioactive glucose was taken off as the scopolin passed through the cell wall possibly by a cell wall bound β-glucosidase. After the scopolin entered the cytoplasm, the exchange between radioactive and non-radioactive glucose was slower than the rate of exchange in the first hour. Therefore, the slow rate of exchange may have been due to a reversible enzyme (scopolin synthetase). The glucose donor for scopolin synthetase will have to be found before any of the alternatives can be excluded.

It cannot be determined from the data whether it is scopolin or scopoletin that is translocated. If the small percent of C^{14} is indeed real, then some scopolin would have been translocated from the roots to the shoots. If, however, the metabolic pool for the glucose donor is relatively small and scopolin synthetase is a reversible enzyme, then

glucose donor molecules may have become radioactive and scopoletin in the shoots may have been glucosylated with radioactive glucose.

Glucose-1-C^{14} Scopolin Experiment

The final concentration of scopolin was 4.86×10^{-5} M with a specific activity of 3.08 μ curies/μ mole. A group of three plants was left in the radioactive root solution for 22 hours. The plants were harvested and extracted as described in the experimental section.

Carbon number one of glucose is oxidized to CO_2 in the pentose phosphate pathway. Therefore, less radioactivity would be found in aromatic compounds by using glucose-1-C^{14} than by using an equivalent amount of radioactive glucose labeled at any other position.

In this experiment thirty-three percent of the radioactivity absorbed was found in the IBMW insoluble fraction (roots). When scopoletin-4-C^{14} was used as the precursor at about the same initial concentration, 70% of the absorbed radioactivity was found in the IBMW insoluble fraction. Additional experiments would determine what portion of the IBMW insoluble fraction is labeled when glucose-1-C^{14} scopolin is used as the precursor. When scopolin is the precursor, the scopoletin portion may be converted to a lignin type of material and the glucose may go into cellulose.

The scopolin and fabiatrin from the soluble portion were only slightly radioactive (a few cpm above background), thus confirming the results from the double label experiment.

CHAPTER V

DISCUSSION

Two other studies of scopolin metabolism using radioisotopes have appeared. One paper deals mainly with scopolin biosynthesis in tobacco leaves (Steck, 1967b); the other paper was primarily concerned with scopolin and scopoletin metabolism in tobacco callus tissue culture, Loewenberg (1970). Before these tracer studies are compared with the present study, the differences in scopoletin derivatives normally found in the different tobacco tissues should be pointed out. Fabiatrin is found in tobacco roots but not in the shoots or callus tissue. Two unknown scopolin derivatives are found in callus tissue but normally not found in tobacco plants (Winkler, 1967). A suspension culture line of tobacco, WR-132, does not have scopolin or scopoletin. The ratio of scopolin to scopoletin (µg per g frest wt/µg per g fresh wt) has been reported as 34 in normal tobacco callus (Schafer and Wender, 1970) and in tobacco seedlings; 31 in shoots and 35 in roots (calculated from data of Einhellig, 1969).

Therefore the scopoletin derivatives differ in the various tissues, but the scopolin to scopoletin ratio is about the same.

Loewenberg (1970) incubated tobacco callus for 12, 18, 24, and 36 hours with a constant amount of uniformly labeled phenylalanine. The specific activity of both scopoletin and scopolin reached maxima at 18

hours while the concentrations remained constant. These data are in agreement with the proposal by Sargent and Skoog (1961) that normal tissues exhibit a rapid turnover of scopolin. The data from the present study with scopoletin-4-C^{14} as the precursor showed that scopolin was metabolized only very slowly. Steck (1967b) reported that scopolin, after it is biosynthesized, is further metabolized only slowly. This was true in his experiments regardless of precursor. The specific activity of scopoletin was higher than that of its glucoside over the entire time period studied, as was also found by Fritig *et al.* (1967). The usual interpretation would be that scopoletin was the precursor of scopolin. Steck's (1967a, 1967b) experiments demonstrated that scopolin can be synthesized first, as shown in Figure 1.

Possibly the metabolism of scopolin and scopoletin differ between intact plants and cultured tissues as suggested by Steck (1967b). However, the concept of compartmentation can explain the differences between the two sets of results and, perhaps, partially explain other mysteries concerning the metabolism of phenolic compounds.

Compartmentation of intermediary metabolites has been investigated in detail (Oaks and Bidwell, 1970); however, very little work has been done on the compartmentation of phenolic compounds. Assume, for example, that scopolin is compartmentalized in at least one vacuolar pool and at least one metabolic pool. Scopolin is metabolized in the metabolic pool and stored in the vacuolar pool. The compartmentation is not absolute, i.e., scopolin can pass from one compartment to another.

In the present study, scopoletin was used as the precursor of scopolin. By comparing the specific activity of the precursor, scopoletin, with that of scopolin in the tissues, it can be calculated that

most of the scopolin in the tissues was obtained from the radioactive scopoletin. Since an abnormal amount of scopolin was formed, it is proposed that the metabolic pool was glutted and the excess scopolin was stored in the vacuolar pool. In Loewenberg's experiment using phenylalanine as the precursor of scopolin, less than one percent of the total scopolin came from the radioactive phenylalanine. Therefore, the size of the metabolic pool was hardly affected, and only a small part of the radioactive scopolin was shunted into the vacuolar pool. Thus, in one study scopolin appeared to be a metabolite (Loewenberg, 1970). In two other studies it appeared to be an end product (Steck, 1967b). The vacuolar pool would have relatively little radioactive scopolin in it. When the tissues are ground up, the scopolin from the two pools would mix, and the resulting scopolin would have a specific activity lower than the scopolin from the metabolic pool, yet higher than the scopolin from the vacuolar pool. Therefore, as was shown by Fritz *et al.* (1967) and Loewenberg (1970), the specific activity of scopoletin under such experimental conditions would be higher than scopolin for all time periods. Moreover, scopoletin would have a specific activity higher than scopolin even if it is a product of scopolin and not a precursor of scopolin.

The data from the present study indicate that when scopoletin first enters the plant either it or scopolin is freely translocated, i.e., scopolin has not yet been compartmentalized. Very little scopolin, however, is translocated from old leaves to new leaves; therefore, scopolin must be in vacuolar pools.

The same hypothesis would explain the accumulation of scopolin under stress conditions. Under normal conditions, the small amount of

scopolin biosynthesized is metabolized at the same rate. When, however, the biosynthesis of phenolic compounds in general and scopolin in particular is increased, the metabolic pool is overwhelmed and the excess scopolin is shunted to the vacuolar pool.

Many of the same stress conditions that result in an accumulation of scopolin also induce the synthesis of the first enzyme in the biosynthesis of phenolic compounds from phenylalanine (see Part II) (Wender, 1970). The accumulation of scopolin under stress conditions has not been examined with respect to increased activity of phenylalanine ammonia-lyase. However, the investigations of the accumulation of many other phenolic compounds have shown increased activity of phenylalanine ammonia-lyase. Zucker (1965), for example, found a direct quantitative relationship between the light-induced increase of phenylalanine ammonia-lyase and the increased amount of chlorogenic acid in potato tubers. In his experiments, the activity of the light-induced phenylalanine ammonia-lyase was over 63 times as much as the control. Many other examples of parallel increases of certain phenolic compounds and phenylalanine ammonia-lyase have been reported (see Smith and Attridge, 1970).

The results of the present study indicate that at least some of the radioactivity in the 1% HCl hydrolysate was associated with peptides. This may be an artifact since phenolic compounds combine reversibly with proteins by hydrogen bonding, and irreversibly by oxidation followed by covalent condensation (Loomis and Battaile, 1965). Rutin and chlorogenic acid have been found in a protein-iron complex. The formation of this type of complex occurs from the oxidation of rutin and chlorogenic acid (Wright, 1960). The presence of radioactivity in the peptides may, however, be of physiological significance. El- Basyouni and Neish (1966)

found metabolically-active bound forms of cinnamic acid in acetone powders of wheat and barley plants. The elution profile of the bound cinnamic acid from a column of Sephadex G-200 showed that it had a size range somewhat below that of proteins.

Lignin, a phenolic polymer, coexists with holocellulose to form plant cell walls. The isolation of lignin can be divided into two classes: (a) those that depend on the removal of the polysaccharide portion leaving the lignin as an insoluble residue and (b) those that depend on the removal of the lignin, leaving the cellulose and other polysaccharides as an insoluble residue (Schubert, 1965). In the present study, the holocellulose was removed by a 72% sulfuric acid treatment or the lignin was removed by treatment with 2 N sodium hydroxide solution. In the former case, 82% of the radioactivity was found as the lignin fraction. In the latter case 73% of the radioactivity was found in the lignin portion.

Another characteristic of lignin is the aldehydes that are produced by alkaline nitrobenzene oxidation. Some lignins yield as much as one fourth their weight as vanillin, p-hydroxybenzaldehyde, and syringaldehyde (Browning, 1967). Phenolic acids such as p-hydroxybenzoic, vanillic, syringic and ferulic acids have also been found in the alkaline degradation products of lignin (Browning, 1967). Very little radioactivity was found in the lignin aldehydes or the phenolic acids when scopoletin-4-C^{14} was used as the precursor. Thus, the evidence indicates that scopoletin can form a lignin-like substance, but the material is not true lignin. The data and conclusions are in agreement with Loewenberg (1970). Dieterman *et al.* (1969) found that the

72% H_2SO_4-insoluble fraction contained radioactivity when C^{14}-quercetin was incorporated into tobacco leaves. No radioactivity, however, was found in the lignin aldehydes. When C^{14}-rutin was used as the precursor, no radioactivity was found in the lignin aldehydes (Dieterman, 1961). When C^{14}-chlorogenic acid was used as a precursor, radioactivity was found in the lignin fraction. After nitrobenzene oxidation, however, only a small proportion of the radioactivity in the insoluble portion was found in the lignin aldehydes (Taylor, 1968). The evidence suggests that different types of phenolic compounds can be polymerized to lignin-like material. Moreover, the lignin aldehydes are probably only obtained from lignin that was formed (in the case of tobacco) when coniferyl alcohol or sinapyl alcohol are the immediate monomeric precursors (Freudenberg and Neish, 1968; El-Basyouni *et al.*, 1964).

In order to explain the distribution and turnover rate of radioactive chlorogenic acid in Xanthium, Taylor (1968) proposed that, as chlorogenic acid enters the cell wall or passes through the plasmalemma, part of it is utilized in the synthesis of insoluble polymers, presumably lignin; and the remainder of the chlorogenic acid migrates into the cytoplasm where it is converted to 3,5-dicaffeoylquinic acid.

When scopoletin-4-C^{14} was used as the precursor, it was immediately divided between the soluble and insoluble portion of the plant (see Table 3). The scopoletin derivative that was found in the cytoplasm was for the most part scopolin. It is, therefore, proposed that as scopoletin passes through the cell wall or plasmalemma it is polymerized to a lignin-like material. The remainder of the scopoletin migrates into the cytoplasm where it is glucosylated to scopolin. Most of the scopolin is compartmentalized in a vacuolar pool; however, some of it is slowly converted to

fabiatrin and to cell wall material. As scopolin passes through the cell wall, a β-glucosidase removes most of the glucose, and the scopoletin formed is polymerized to a lignin-like material. The remainder of the scopoletin and part of the scopolin pass through the cell wall.

Other evidence from this laboratory, as well as others, also favor this hypothesis. Since the insoluble material formed was similar to lignin, it is reasonable to assume that it was formed in the same type of reaction that formed lignin. Lignin is formed from cinnamyl alcohol derivatives (El-Basyouni *et al.*, 1964) by a dehydropolymerization reaction (Freudenberg and Neish, 1968). Peroxidase is involved in the dehydrogenation reaction in the formation of lignin (Freudenberg and Neish, 1968). Lignin-like polymers have been formed from many different types of phenolic compounds by peroxidase (Stafford, 1960a,b) *in vitro*. Peroxidase will act on scopoletin (Schafer, 1970), and peroxidase has been found at the cell wall (Parish and Miller, 1968).

In spruce and other conifers, the tissues contain high concentrations of coniferin. Only the free phenolic alcohols and not their glucosides can be transformed into lignin. A β-glucosidase that splits coniferin to coniferyl alcohol and glucose has been found attached to the cell wall (Freudenberg and Neish, 1968). A β-glucosidase has been induced in tobacco leaves by *Pseudomonas syringae* (Hildebrand and Sands, 1966).

The experiments using double labeled scopolin and glucose-C^{14}-scopolin demonstrated that glucose is removed from scopolin. The β-glucosidase that splits scopolin to scopoletin and glucose is probably located at the cell wall. It is suggested, that as scopolin passes through the cell wall, a β-glucosidase removes the glucose from a portion

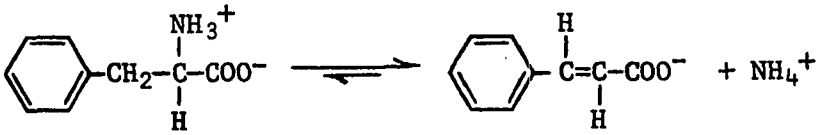
of the scopolin. The scopoletin thus formed acts as substrate for a membrane bound peroxidase. The peroxidase removes the phenolic hydrogen atom and the aroxyl radical or some mesomeric radical reacts with itself or some other phenolic compound. Thus, a lignin-like polymer is formed.

II. PHENYLALANINE AMMONIA-LYASE IN TOBACCO TISSUE CULTURE: INHIBITION STUDIES

CHAPTER I

INTRODUCTION

L-phenylalanine ammonia-lyase (E.C. 4.3.1.5) catalyses the deamination of L-phenylalanine to *trans*-cinnamic acid and ammonia (eq. 1).



equation 1

Trans-cinnamic acid is further metabolized to a large variety of phenolic compounds: hydrocinnamic acids, coumarins, flavonoids, anthocyanins, lignin, etc.

Koukol and Conn (1961) first isolated and partially purified L-phenylalanine ammonia-lyase. Since then it has been suggested (Zucker, 1969) that plant tissues appear to regulate the biosynthesis of phenylpropanoid compounds by controlling the synthesis of this enzyme. Many of the same environmental factors that have been shown to increase the concentration of phenylpropanoid compounds also induce the synthesis of L-phenylalanine ammonia-lyase. In potato tuber tissue, light increased the concentration of chlorogenic acid at the

same rate as it increased the activity of phenylalanine ammonia-lyase (Zucker, 1965). Many other reports of the stimulating effects of light on phenolic biosynthesis have been ascribed to a light induced synthesis of L-phenylalanine ammonia-lyase (Scherf and Zenk, 1967; Attridge and Smith, 1967; Engelsma, 1967). Other factors such as wounding or fungal infection (Minamikawa and Uritani, 1964, 1965b) which result in increased accumulation of phenolic compounds also increase the activity of this enzyme. Increased lignification is also correlated with increased phenylalanine ammonia-lyase activity (Cheng and Marsh, 1968; Rubery and Northcote, 1968).

Other evidence (Havir and Hanson, 1968b) indicates that L-phenylalanine ammonia-lyase could also be a regulatory or allosteric enzyme. Havir and Hanson found that the relationship between velocity and substrate concentration deviated from the Michaelis-Menten equation and that cinnamic acid inhibits the enzyme. They suggested that the inhibition of phenylalanine ammonia-lyase is a feedback control which would regulate phenylpropanoid biosynthesis. Therefore, it appeared that the biosynthesis of phenolic compounds could be controlled in part by the feedback inhibition of phenylalanine ammonia-lyase by these compounds in addition to the control afforded by the induced synthesis of this enzyme by environmental factors.

The original purpose of the experiment was to investigate the possibility that in tobacco tissue culture, phenolic end products and/or phenolic intermediates in lignin biosynthesis might be involved in feedback inhibition. Other compounds such as growth regulators were also investigated to determine if they acted as inhibitors or activators of phenylalanine ammonia-lyase.

Chapter 2

MATERIALS AND METHODS

Plant Material

The plant material used was suspension culture line WR-132 of tobacco tissue (*Nicotiana tabacum* L., var. Xanthi). The cells were grown in 125 ml erlenmeyer flasks containing 50 ml of growth medium (Linsmaier and Skoog, 1965; Kajinami, 1970). During the ten day growth period each flask of cells grew from 2 grams (weight of inoculant) to about 10 grams. The flasks of cells were constantly agitated on a reciprocal shaker (95-105 reciprocations/minute). The temperature of the growth room was 72°F and the light intensity was less than 0.5 foot candles. Since light had been found to induce synthesis of phenylalanine ammonia-lyase, 12 to 24 hours before the cells were harvested the light intensity was increased to 3 foot candles. The brighter light significantly increased the apparent level of phenylalanine ammonia-lyase. The cells were collected by suction filtration and thoroughly washed with 0.1 M imidazole buffer at pH 6.5 containing 10 mM β-mercaptoethanol and 4 mM metabisulphite.

Enzyme Preparation

About 40 g of washed cells were mixed with 40 g of glass beads, 25 g of washed Polyclar AT which had been soaked with deionized water

overnight, and 80 ml of imidazole buffer (pH 6.5) containing 10 mM β-mercaptoethanol and 4 mM metabisulphite (Kajinami, 1970). This mixture was blended for 10 minutes in a blender (Sorvall Omnimixer) at 5000 rpm. The blended mixture was centrifuged at 34,800 x g to remove the glass beads, Polyclar AT, and cell debris from the crude enzyme solution.

To the supernatant thus obtained, enough solid $(NH_4)_2SO_4$ was slowly added to form a solution that was about 28% saturated with respect to the salt. The precipitate formed was removed by centrifugation at 34,800 x g for 10 minutes and discarded. Solid $(NH_4)_2SO_4$ was slowly added to the remaining supernatant solution to give a solution that was about 58% saturated with respect to the salt. The precipitate was collected by centrifugation at 34,800 x g for 10 minutes and dissolved in about 5 ml of 0.05 M sodium borate buffer containing 5 mM β-mercaptoethanol and dialyzed about 12 hours in 500 ml of the same buffer. The enzyme was dialyzed an additional 12 hours in the same buffer without the β-mercaptoethanol. The dialyzed enzyme solution was used for all assays. In one experiment a column packed with Sephadex G-10 was used to desalt the enzyme.

Enzyme Assay

Spectrophotometric Assay

The activity of phenylalanine ammonia-lyase was determined by measurement of the cinnamic acid formed. The reaction mixture contained enzyme, various amounts of L-phenylalanine, and 150 μ moles of sodium borate buffer (pH 8.8) in a total volume of 3.0 ml. Cinnamic acid formation was followed at 290 mμ; 1 μ mole of cinnamic acid in 3 ml

has an absorbancy of 3.0 (1 cm light path) (Havir and Hanson, 1968a). The initial velocity was measured on a Beckman DB-G at 72°F every 15 minutes for 1 hour, and it was found to be linear for at least two hours.

Radioactive Assay

The reaction mixture was the same as the spectrophotometric assay except C^{14}-L-phenylalanine (Amersham/Searle) was used. Also 15 ml test tubes were used instead of cuvettes. The mixture was incubated at 72°F for 1 hour. The reaction was terminated by the addition of 0.2 ml of 50% trichloroacetic acid immediately followed by 0.1 ml of 3×10^{-2} M *trans*-cinnamic acid dissolved in 0.05 M NaOH. The unlabeled *trans*-cinnamic acid acted as a carrier of the radioactive product.

After 5 ml of benzene had been added to the acidified mixture, the benzene and aqueous layers were vigorously stirred with a spatula until an emulsion formed. After 10-15 minutes the reaction mixture was centrifuged to remove the protein from the benzene phase. A 3 ml aliquot from the benzene phase was transferred to a scintillation vial and the benzene was completely removed in a vacuum oven. To the vial containing the sample, 10 ml of dioxane-scintillation fluid was added, and the sample was counted in a Beckman DPM-100 Scintillation Counter. A reaction mixture blank, complete with enzyme and substrate, which had been taken through the entire procedure for each set of assays and/or for each different concentration of radioactive phenylalanine used, was terminated at zero reaction time. The radioactivity of the blank was subtracted from the total radioactivity of each sample. All radioactive assays were in duplicate and the averages are presented.

CHAPTER III

RESULTS

The pH optimum was found to be approximately 8.8, and the initial velocity was directly proportional to the amount of enzyme added. The plot of the substrate concentration vs. initial velocity is shown in Figure 1. The hyperbolic curve indicates Michaelis-Menten kinetics. The Michaelis constant, K_m, was determined to be 3×10^{-5} M from the double reciprocal plot of substrate concentration vs. reaction rate (Fig. 2).

In addition to cinnamic acid, it was thought that either some of the intermediates in lignin biosynthesis such as p-coumaric, caffeic and ferulic acids, or some of the phenolic compounds which accumulate in tobacco, such as rutin, scopolin, scopoletin and chlorogenic acid might be feedback inhibitors of phenylalanine ammonia-lyase. Table 1 shows the effects of the various phenolic compounds on the activity of phenylalanine ammonia-lyase. The only compound that inhibited the enzyme significantly was cinnamic acid.

The biosynthesis of both lignin (Brown, 1966) and phenolic compounds (Sargent and Skoog, 1960) is affected *in vivo* by varying concentrations of indole-3-acetic acid (IAA) and kinetin. Both were examined along with some other compounds to find whether they influence the activity of phenylalanine ammonia-lyase *in vitro*. Table 2 shows the effect of some growth regulators

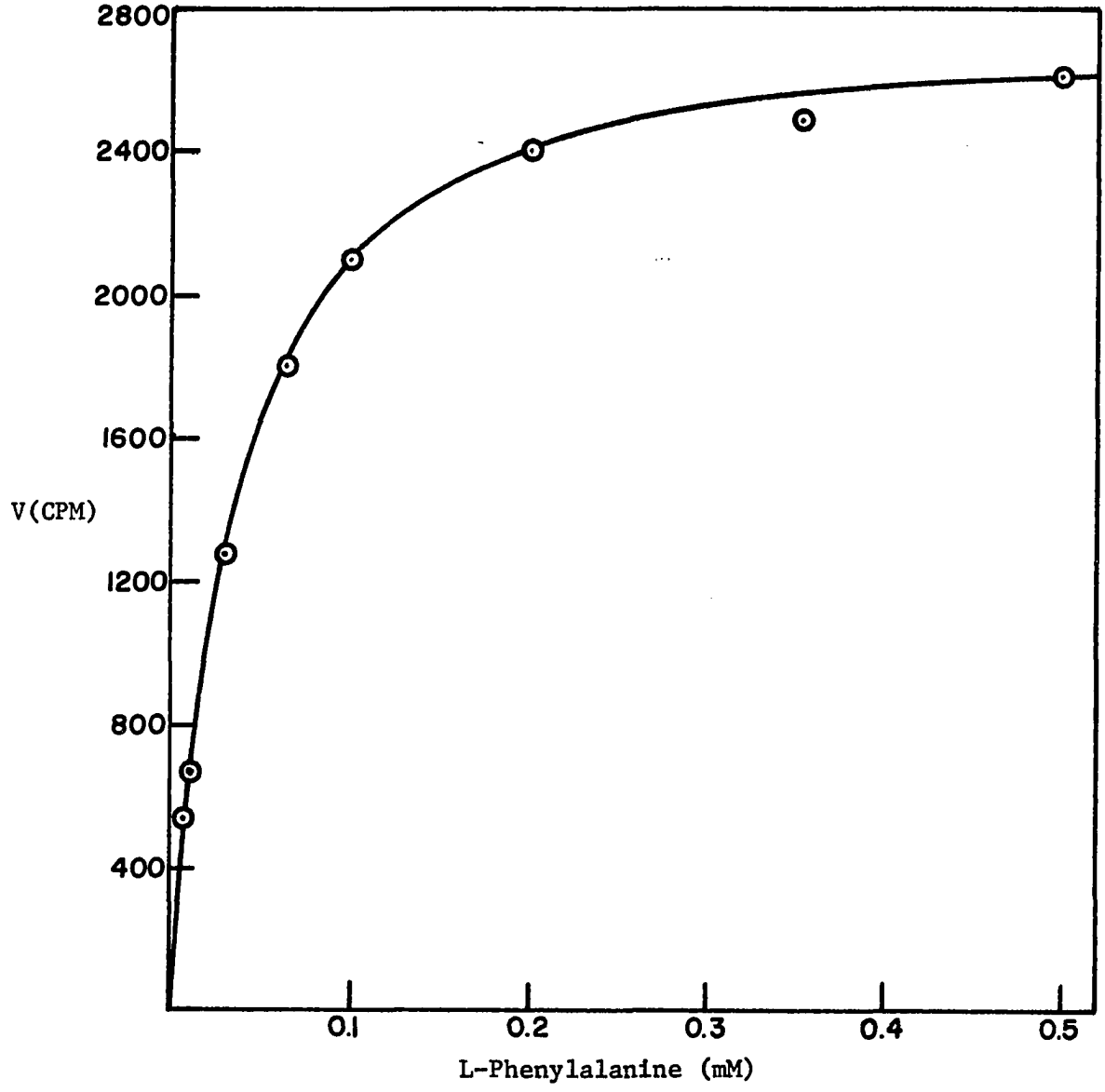


FIGURE 1. The Saturation Function of L-Phenylalanine for Phenylalanine Ammonia-lyase from Tobacco Tissue Culture

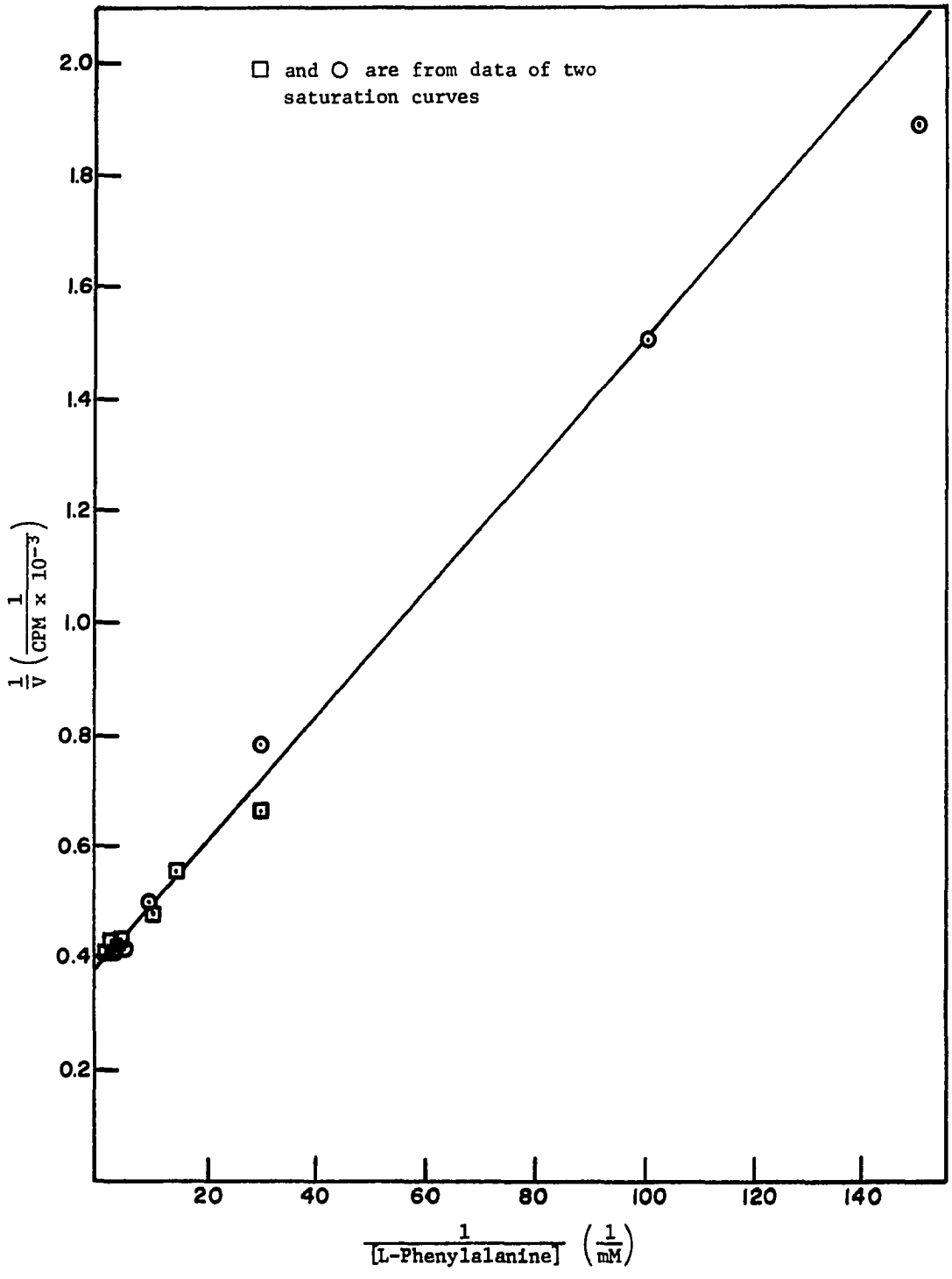


FIGURE 2. A Double Reciprocal Plot of Reaction Velocity vs. Phenylalanine Concentration for Phenylalanine Ammonia-lyase from Tobacco Tissue Culture.

TABLE 1

EFFECT OF VARIOUS PHENOLIC COMPOUNDS ON L-PHENYLALANINE AMMONIA-LYASE ACTIVITY

Compound added	Final concentration mM	Relative activity %
Scopoletin	0.1	95
Chlorogenic acid	1	114
Trans cinnamic acid	1	(24)* 66
Rutin	1	99
Scopolin	1	99
P-Coumaric acid	1	106
Ferulic acid	1	108
Caffeic acid	1	97

The radioactive assay was used as described in the text. The substrate concentration was 1 mM except for the 24% activity of cinnamic acid.

*phenylalanine concentration = .1 mM

and other miscellaneous compounds on phenylalanine ammonia-lyase. Indole-3-acetic acid was the only compound that inhibited the enzyme.

In order to determine if the inhibition of phenylalanine ammonia-lyase by IAA was due to its physiological action as an auxin, structurally similar compounds without auxin activity and structurally dissimilar compounds with auxin activity were tested for inhibition of the enzyme.

TABLE 2

EFFECT OF SOME GROWTH REGULATORS AND OTHER MISCELLANEOUS COMPOUNDS ON L-PHENYLALANINE AMMONIA-LYASE ACTIVITY

No.	Compound added	Final concentration mM	Relative activity %
1	Indole-3-acetic acid	3	39
2	Indole-3-acetic acid	1	53
3	Cyclic AMP	3	95
4	Cyclic AMP	1	99
5	IAA (1mM) with Cyclic AMP	1	47
6	Kinetin	*	103
7	Gibberellic acid	3	103
8	Proline	1	101
9	Methonine	1	95

The radioactive assay was used as described in the text except for Nos. 9 and 10 which were assayed by the spectrophotometric method. In Nos. 2, 3, 4, and 5 the substrate concentration was 1×10^{-4} M; in the rest of the samples it was 1×10^{-3} M.

*saturated solution

Phenoxy compounds were used as synthetic auxins and indole and tryptophan were indoles used that have no auxin activity. The results are shown in Table 3. The enzyme was inhibited by representatives of both groups with o-chlorophenoxyacetic acid, 2,4,5-trichlorophenoxyacetic acid and tryptophan causing the most inhibition.

It was noticed that the inhibitory effects of IAA were dependent on the concentration of β-mercaptoethanol in the assay buffer. Increasing the concentration of β-mercaptoethanol had an antagonistic effect on the inhibition of phenylalanine ammonia-lyase by IAA (Table 4). Likewise, β-mercaptoethanol inhibited phenylalanine ammonia-lyase, and increasing concentrations of IAA reduced the inhibition of phenylalanine ammonia-lyase by 30 mM β-mercaptoethanol.

At a constant concentration of 2 mM for IAA and tryptophan, the substrate concentration was varied as indicated in Fig. 3 and plots were made of the reciprocal of velocity against the reciprocal of substrate concentration. A Lineweaver-Burk plot was also made for inhibition of phenylalanine ammonia-lyase by 20 mM β-mercaptoethanol as shown in Fig. 4. The results indicate IAA, β-mercaptoethanol and tryptophan are all competitive inhibitors of phenylalanine ammonia-lyase.

Phenylalanine ammonia-lyase from barley is a sulfhydryl enzyme (Koukol and Conn, 1961) while the enzyme from potato tubers was found to be less sensitive to sulfhydryl reagents (Havir and Hanson, 1968b). As shown in Table 5, cysteine and p-chloromercuriphenyl sulfonic acid inhibit the enzyme the greatest.

In order to find the relationship between IAA concentration and activity of phenylalanine ammonia-lyase, the substrate was held constant at 0.1 mM and the concentration of IAA was varied between 5×10^{-7} and

TABLE 3

EFFECT OF SYNTHETIC AUXINS, INDOLE AND TRYPTOPHAN

ON PHENYLALANINE AMMONIA-LYASE ACTIVITY

Compound added	Final concentration mM	Relative activity %
Indole	3	84
Indole	1	105
2,4 Dichlorophen-	3	72
oxyacetic acid	1	101
2,4,5-Trichlorophen-	3	69
oxypropionic acid	1	106
o-Chlorophenoxy-	3	24
acetic acid	1	39
2,4,5-Trichlorophen-	3	45
oxyacetic acid	1	66
β-Indole 3-propionic	3	106
acid	1	104
Tryptophan	3	38

The radioactive assay was used as described in the text. The substrate concentration was 0.1 mM.

TABLE 4

ANTAGONIST EFFECT OF β-MERCAPTOETHANOL AND IAA ON THE INHIBITION OF PHENYLALANINE AMMONIA-LYASE

Concentration of β-mercaptoethanol	Concentration of IAA	Relative activity %
30 mM	6 mM	56
30 mM	3 mM	44
30 mM	1 mM	42
30 mM	0.1 mM	32
30 mM	0 mM	29
10 mM	6 mM	28
10 mM	3 mM	46
10 mM	1 mM	72
10 mM	0.1 mM	79
10 mM	0 mM	80
5 mM	6 mM	49
5 mM	3 mM	75
5 mM	1 mM	83
5 mM	0 mM	100
0.66 mM	control	100

The radioactive assay was used as described in the text. The substrate concentration was 1 mM.

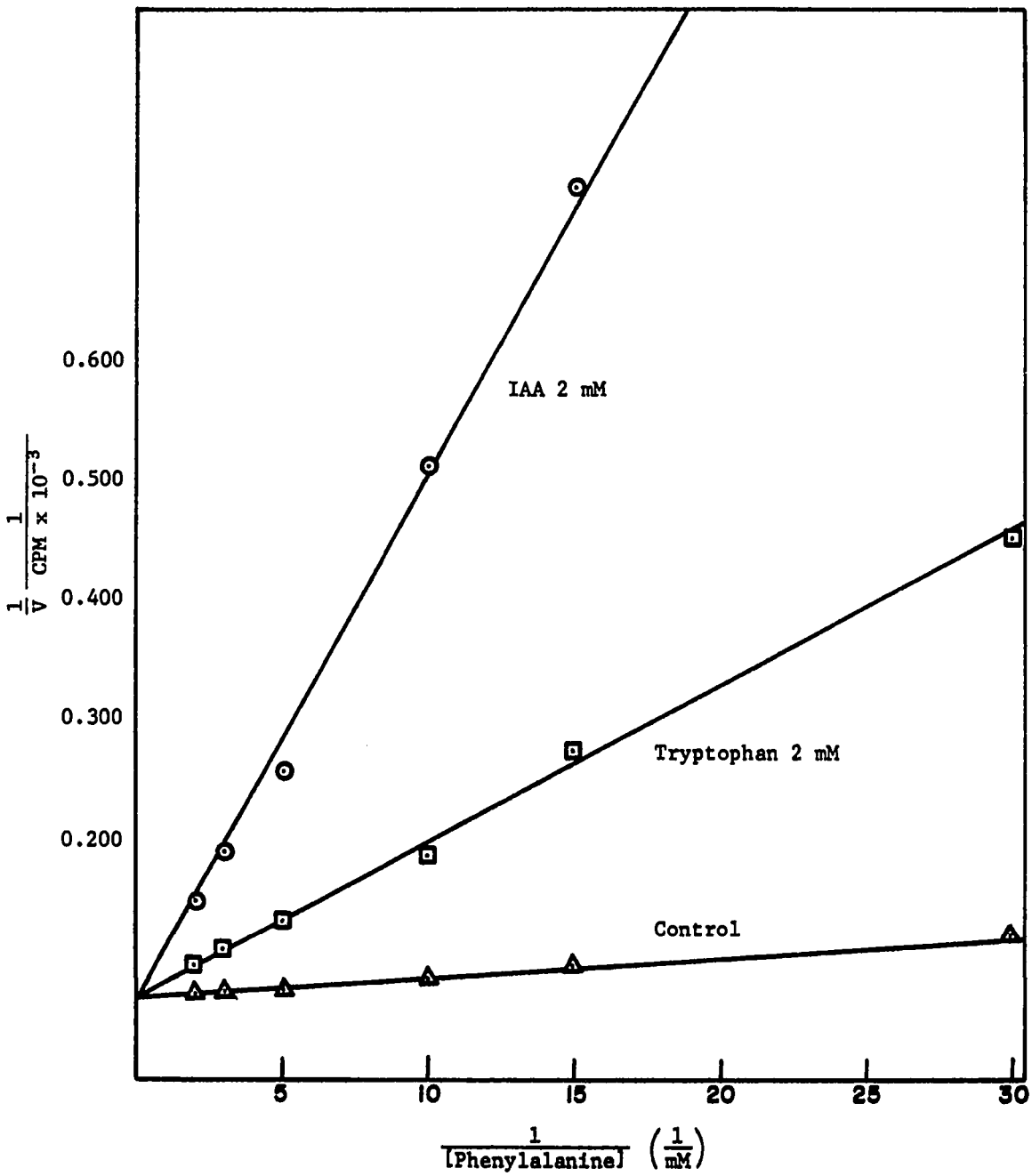


FIGURE 3. A Double Reciprocal Plot of the Inhibition of Phenylalanine Ammonia-Lyase by Indole-3-Acetic Acid and Tryptophan.

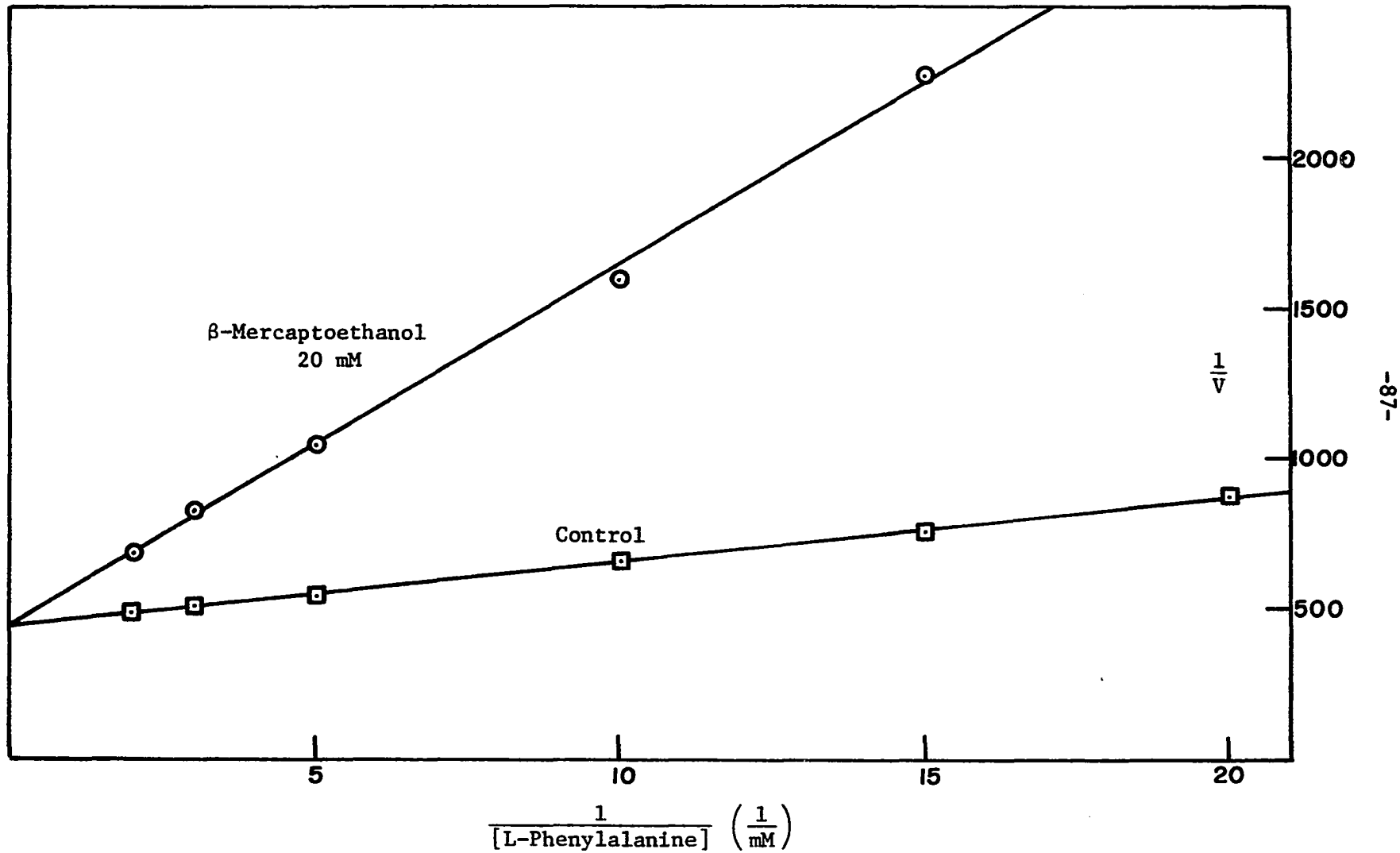


FIGURE 4. Plot of the Inhibition of the Activity of Phenylalanine Ammonia-Lyase by β-Mercaptoethanol.

TABLE 5

EFFECT OF SULFHYDRYL COMPOUNDS AND SULFHYDRYL GROUP REAGENTS ON PHENYLALANINE AMMONIA-LYASE

Compound added	Relative activity
Cysteine	1 mM, 83%
Cysteine	6 mM, 32%; 3 mM, 73%; 1.5 mM, 78%
GSH	4 mM, 106%; 2 mM, 105%
N-Ethyl maleimide	2 mM, 90%
p-Chloromercuriphenyl sulfonic acid	2 mM, 0%; 1 mM, 71%; 0.5 mM, 71%

The cinnamic acid formed was determined spectrophotometrically as described in the text. The substrate concentration was 1 mM. There was no preincubation.

7.5×10^{-3} M as shown in Fig. 5. A 50% inhibition of the enzyme was achieved at the inhibitor concentration of about 1.2 mM. At low concentrations of inhibitor (10^{-6} to 10^{-7}M IAA) the enzyme was neither inhibited nor activated.

Two experiments were performed to establish further the nature of the inhibition and the mode of binding of IAA to the enzyme. The enzyme preparation was preincubated with 1.0 mM IAA for different periods of time. Table 6 shows the results. The relative activity or the per cent inhibition of phenylalanine ammonia-lyase were independent of preincubation time for at least 6 hours. This demonstrates that it is not some product of IAA but IAA itself that inhibits phenylalanine ammonia-lyase.

To demonstrate the reversibility of the binding of IAA to phenylalanine ammonia-lyase, an enzyme solution was incubated for one hour with 1.5 M IAA, dialyzed overnight against 0.05 M sodium borate buffer (pH 8.8), and assayed for activity. The activity was found to be about the same as the controls (91%) indicating that IAA binds reversibly to the enzyme.

To demonstrate the reversibility of the binding of β-mercaptoethanol to phenylalanine ammonia-lyase, an enzyme solution was dialyzed overnight in 10 mM β-mercaptoethanol and then assayed at a final β-mercaptoethanol concentration of 0.66 mM, 5 mM, 10 mM, and 30 mM. The radioactive assay was used and the substrate concentration was 1 mM. The results can be seen in Table 4 at zero concentration of IAA.

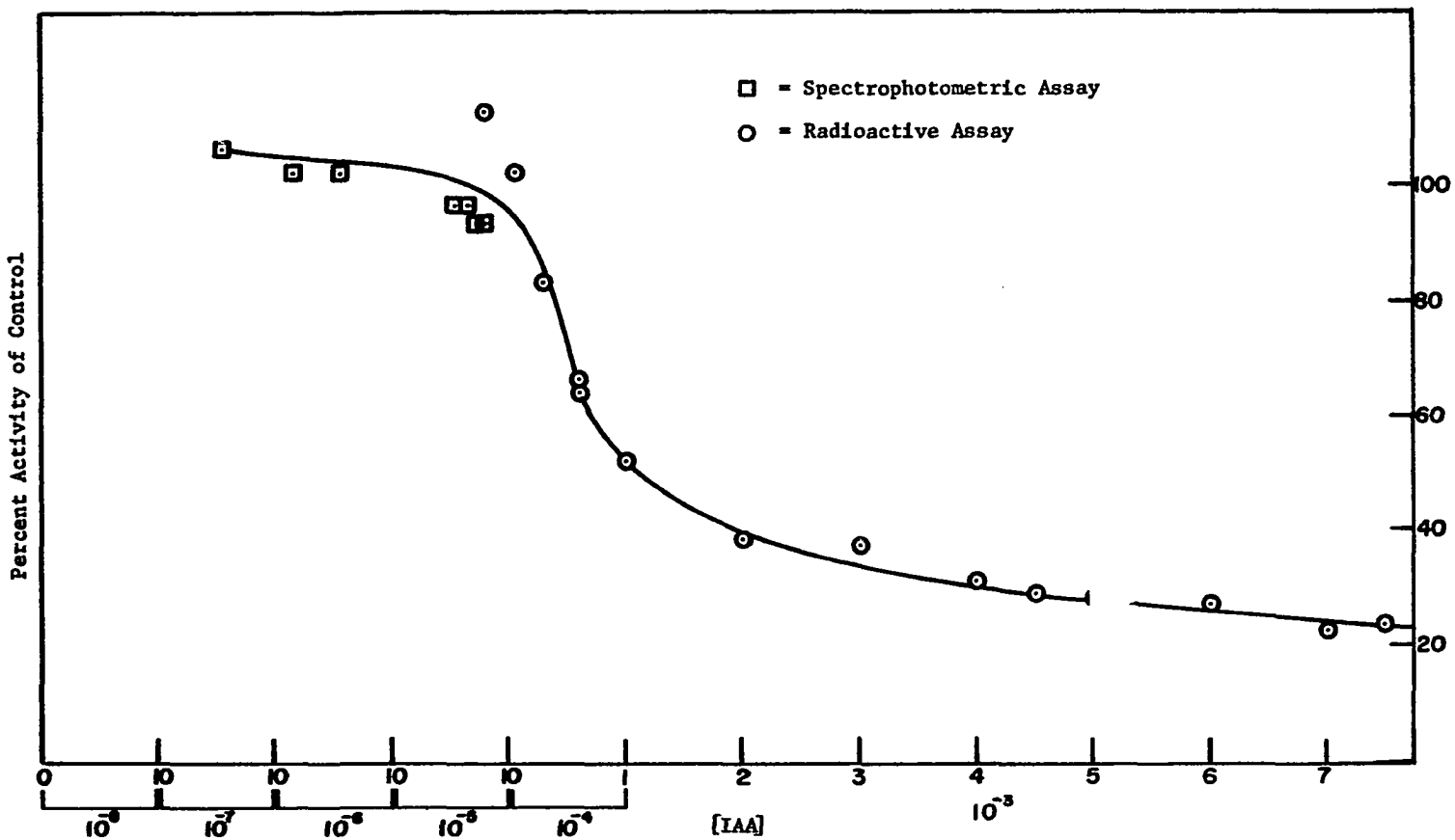


FIGURE 5. Plot of Phenylalanine Ammonia-Lyase Activity vs. Indole-3-Acetic Acid Concentration (L-Phenylalanine = 1×10^{-4} M).

TABLE 6

ACTIVITY OF L-PHENYLALANINE AMMONIA-LYASE AFTER PREINCUBATION WITH 1 mM IAA FOR DIFFERENT TIME PERIODS

Preincubation Time	Relative activity %
6 hours	59
4 hours	58
2 hours	58
0 hours	61

The reaction was started in each case by the addition of the substrate. The standard radioactive assay was used as described in the text. The substrate concentration was .1 mM.

CHAPTER IV

DISCUSSION

The K_m for L-phenylalanine ammonia-lyase from tobacco tissue culture (3×10^{-5} M) is the lowest reported for this enzyme. The K_m for the enzyme from tobacco plants at pH 8.55 was reported to be 1.6×10^{-4} M (O'Neal and Keller, 1970). β-mercaptoethanol (2.3×10^{-4} M) was used in their assays. Since β-mercaptoethanol is a competitive inhibitor of phenylalanine ammonia-lyase, a lower value for the K_m would have been obtained without it. The K_m for the enzyme from sweet potato roots was found to be 1×10^{-4} M (Minamikawa and Uritani, 1964) and from barley 1.7×10^{-3} M (Koukol and Conn, 1961). From potato tubers the apparent K_m increases from 3.8×10^{-5} M to 2.6×10^{-4} M. The enzyme isolated from potato tubers and maize seedling deviated from the Michaelis-Menten function (Havir and Hanson, 1968b; Marsh *et al.*, 1968). The kinetics of the enzyme from tobacco tissue culture and from other sources are described by the Michaelis-Menten equation.

Cinnamic acid inhibits phenylalanine ammonia-lyase from every source studied including tobacco tissue culture. p-Coumaric acid inhibits the enzyme from barley (Koukol and Conn, 1961) and from *Sporobolomyces roseus* (Camm and Towers, 1969) but not from *Ustilago hordei*. O'Neal and Keller (1970) tested many of the same phenolic compounds that were tried in the present study for inhibition of phenylalanine

ammonia-lyase from tobacco leaves. They found scopoletin inhibited the enzyme. Since the inhibition was probably competitive, the high substrate concentration used in the present study may have prevented scopoletin from inhibiting the enzyme from tobacco tissue culture.

Trans-cinnamic acid, p-coumaric acid, caffeic acid, D-phenylalanine, DL-p-fluorophenylalanine and L-tyrosine have all been shown to be competitive inhibitors of phenylalanine ammonia-lyase (Minamikawa and Uritani, 1965a; Havir and Hanson, 1968b). All the inhibitors listed have structures very similar to that of the normal substrate, L-phenylalanine. They are probably classical examples of competitive inhibition, i.e., a catalytically inactive analog of the substrate binds at the active site of the enzyme. These sites are, therefore, not available to the normal substrate molecule (Stadtman, 1966; Cohen, 1968). Kinetic analysis of the inhibition evoked by IAA on phenylalanine ammonia-lyase shows that the inhibition is competitive. The structure of IAA is somewhat similar to the normal substrate, L-phenylalanine, and IAA may inhibit at the active site; however, the differences between the two structures raise the possibility of IAA binding at an allosteric site.

A sigmoid relationship between initial velocity and substrate concentration is exhibited by most but not all enzymes subject to allosteric control. For the majority of other enzymes, this relationship is described by a rectangular hyperbola (Michaelis-Menten kinetics). As previously mentioned, phenylalanine ammonia-lyase from tobacco tissue showed Michaelis-Menten kinetics. Another characteristic of allosteric enzymes is the sigmoid relationship between inhibitor concentration and initial veolicty at constant substrate concentration (Cohn, 1968; Stadtman,

1966). As can be seen in Figure 5, there exists a sigmoid relationship between IAA concentration and the activity of the enzyme. This suggests a cooperative interaction in the binding of IAA (Stadtman, 1966).

The inhibition of phenylalanine ammonia-lyase by β-mercaptoethanol and IAA is reversible, competitive, and antagonistic. Therefore, IAA and β-mercaptoethanol must either interact with each other to form a complex that does not affect the enzyme, or they must both compete for the same site on the enzyme.

Indole-3-acetic acid has been shown to activate citrate synthase *in vitro* (Sarkissian, 1968). In the case of citrate synthase, the site affected by IAA is thought to be an allosteric site which contains a sulfhydryl group. Sarkissian (1968) suggested in the case of citrate synthase that IAA, an electron donor, transmits a signal to a sulfhydryl group on the protein molecule. A sulfhydryl group may be part of the site in which IAA binds on phenylalanine ammonia-lyase from tobacco tissue culture. Phenylalanine ammonia-lyase from potato tubers has been reported to have allosteric interactions (Havir and Hanson, 1968), and the kinetics of the binding of IAA to the enzyme in this study suggests cooperative interaction between IAA molecules. Therefore, IAA may act as an allosteric effector at an allosteric site. The evidence from physiological studies indicate that the physiological effects of IAA are related to the sulfhydryl groups of proteins. Sulfhydryl reagents inhibit the IAA induced elongation of oat coleoptile segments (Thimann, 1969), and IAA cannot produce its physiological effects in the presence of inhibitors of sulfhydryl enzymes (Sarkissian, 1968).

The experiment (Table 3) to determine whether the inhibition of phenylalanine ammonia-lyase was due to its physiological action as an auxin was inconclusive. One of the most physiologically active synthetic auxins, 2,4-dichlorophenoxyacetic acid (2,4-D), and another very active phenoxy compound, 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) inhibit phenylalanine ammonia-lyase less than a relatively inactive synthetic auxin, o-chlorophenoxyacetic acid (Thimann, 1969). Tryptophan, an indole without any auxin activity was also a good inhibitor of the enzyme, and the inhibition was found to be competitive. The evidence shows little correlation between auxin activity assayed by elongation of wheat root epidermal cells and the percent inhibition of phenylalanine ammonia-lyase.

The inhibition of phenylalanine ammonia-lyase by IAA and these other compounds may not be examples of allosteric inhibition but instead classical examples of competitive inhibition. IAA would not be classified as an analog of the substrate; however, the structures are not completely different. A thorough investigation of the specificity of phenylalanine ammonia-lyase has not been reported. The studies that have been made have shown that the enzyme is specific in terms of the substrates that will react but not in terms of the affinity of substrate analogs for the enzyme.

The concentration of IAA needed to inhibit phenylalanine ammonia-lyase *in vitro* is higher than the concentration of IAA which occurs in plants, therefore, the inhibition may not occur *in vivo*. However, since the inhibition is competitive, lower concentrations of IAA would inhibit at lower substrate concentrations.

Sarkissian (1968) believes that the primary physiological action of IAA is to transmit a signal to sulfhydryl groups of different proteins. The observed secondary effects would be diverse because of the many different sulfhydryl enzymes. Although the effects of IAA on phenylalanine ammonia-lyase differ in several aspects from the effects of IAA on citrate synthase, the results certainly fit in with Sarkissian's theory.

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