69-4244

HUSTED, Richard Rex, 1942-

BIOSYNTHESIS AND REACTIONS OF CYCLIC HYDROXAMATES IN MAIZE.

Iowa State University, Ph.D., 1968
Biochemistry

University Microfilms, Inc., Ann Arbor, Michigan

BIOSYNTHESIS AND REACTIONS OF CYCLIC HYDROXAMATES IN MAIZE

by

Richard Rex Husted

A Dissertation Submitted to the

Graduate Faculty in Partial Fulfillment of

The Requirements for the Degree of

DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

Signature was redacted for privacy.

Head of Major Department

Signature was redacted for privacy.

Dean of Graduate College

Iowa State University
Of Science and Technology
Ames, Iowa

1968

TABLE OF CONTENTS

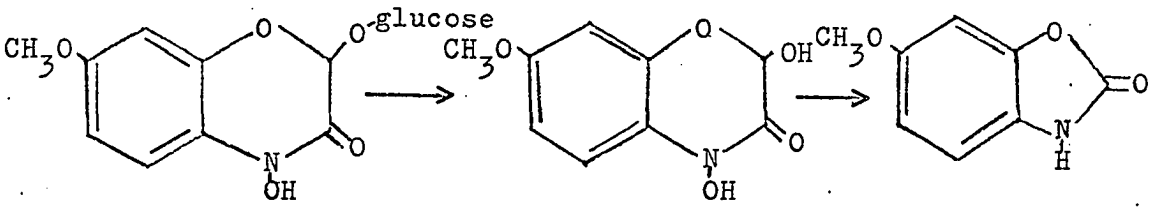
INTRODUCTION

The occurrence of a cyclic hydroxamate in maize was first reported in 1959; however, the biological significance of the cyclic hydroxamates and related compounds has yet to be established. Several correlations have been established concerning the concentration of the maize cyclic hydroxamates and the resistance of the maize to corn borers, stalk rot and 2-chloro-s-triazine herbicides. Reports on microbial hydroxamates acting variously as potent growth factors, antibiotics, antibiotic antagonists, tumor inhibitors, or cell-division factors have lent impetus to investigations of the biological significance of this functional group.

The research conducted and reported in this dissertation was conducted in order to identify a precursor to the maize cyclic hydroxamates and related compounds and to elucidate the role of the maize cyclic hydroxamates in the in vitro detoxification of simazine. The maize cyclic hydroxamates considered herein are 2,4-dihydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one (DIMBOA) and 2-O-glucosyl-4-hydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one (DIMBOA glucoside); and, the related compounds are 2-hydroxy-7-methoxy-1,4(2H)-benzoxazin-3-one (HMBOA) and 2-O-glucosyl-7-methoxy-1,4(2H)-benzoxazin-3-one (HMBOA glucoside).

REVIEW OF LITERATURE

In 1956, Virtanen et al. (1) reported the isolation of 6-methoxy-2(3)-benzoxazolinone (6MBOA) from wheat and maize. It was later reported that 6MBOA is not a constituent of the plants in vivo but is derived from DIMBOA glucoside during the isolation procedures (2). Virtanen and Wahlroos (3) suggested that when the plant tissue is disrupted a glucosidase is released which hydrolyzes the glucoside to form glucose and DIMBOA. The DIMBOA decomposes to yield formic acid and 6MBOA (4). The reaction is represented schematically:



A compound related to 6MBOA was reported isolated from rye seedlings by Virtanen and Hietala (5). The compound was 2(3)-benzoxazolinone which led to the later isolation of the 2-O-glucosyl-4-hydroxy-1,4(2H)-benzoxazin-3-one and its aglucone DIBOA (6, 7).

Although hydroxamic acids are relatively common in microorganisms (8, 9), their presence in higher plants appears to be rather limited (2, 6, 7). However, interest in their significance in higher plants has grown from reports relating the hydroxamic acid content to resistance of: 1. wheat to stem rust (10); 2. maize strains to stalk rot (11); 3. certain plants to 2-chloro-

s-triazine herbicides (12, 13); 4. various inbred strains of maize to attack by the European corn borer (14). A linear relationship has been demonstrated between log 6MBOA content and resistance of maize in the field to attack by the European corn borer (14); furthermore, the activity of DIMBOA as a feeding deterrent to the borer in bioassays on synthetic media was reported by Klun, Tipton and Brindley (15). Hamilton and Moreland (16) have reported the in vitro detoxification of simazine yielding hydroxysimazine when incubated with DIMBOA or its glucoside, and they have suggested a catalytic role for the cyclic hydroxamates in this in vitro detoxification reaction.

In 1964, Reimann and Byerrum (17) reported studies of the biosynthesis of DIMBOA. They studied the incorporation of C^{14}-labelled compounds and concluded that the aromatic ring moiety is biosynthesized by the shikimic acid pathway. The carbons in positions 2 and 3 of DIMBOA were reported to be derived from carbons in positions 1 and 2 of ribose and the 7-methoxyl carbon was incorporated from methionine.

A precursor to DIMBOA, suggested by the chemical structure of DIMBOA could be a nitrogen-containing intermediate in the shikimic acid pathway. Reimann and Byerrum (17) suggested one intermediate might be the precursor of both anthranilic acid and DIMBOA or the latter might be biosynthesized more directly from tryptophan or its degradation product, 3-hydroxyanthranilic acid. However, the results of their experiment indicated that tryptophan was an unlikely intermediate in DIMBOA synthesis.

Reimann and Byerrum also postulated a sequence of reactions for the formation of the oxazine ring of DIMBOA which was analogous to the formation of the pyrrole ring of tryptophan established by Smith and Yanofsky (18). The suggested pathway involved a ribosyl intermediate formed from the condensation of ribose, or a phosphorylated derivative, with an aromatic amine; and, the formation of a 1-deoxy-2-keto derivative by the Amadori rearrangement followed by ring closure incorporating carbons 1 and 2 of ribose in the oxazine ring. This type of condensation suggests the N-hydroxylation reaction resulting in the formation of the cyclic hydroxamate occurs subsequent to the formation of the carbon-nitrogen bond.

Anthranilic acid appears to be the only primary aromatic amine in the shikimic acid pathway (19). J. A. DeMoss (20) proposed that chorismic acid is converted to anthranilic acid by a single enzyme, "anthranilate synthetase", in Neurospora crassa. Others have suggested the possible involvement of an isolatable N-acyl anthranilic acid intermediate between chorismic acid and anthranilic acid (21, 22), but none has been isolated and identified. With this is mind, the production of anthranilic acid or its immediate precursors remains the first enzymatic step specific for the biosynthesis of tryptophan.

Anthranilic acid is a substrate required for the production of biological materials other than tryptophan. Anthranilic acid hydroxylase from the leaves of Tecoma stans (23) converts anthranilic acid to 3-hydroxyanthranilic acid and two moles of this

product are used in an oxidative dimerization catalyzed by cinnabarinic acid synthase to yield cinnabarinic acid (24). Anthranilic acid oxidase was also isolated from the same source (25) and the multienzyme system catalyzes: anthranilic acid ⟶ 3-hydroxy anthranilic acid ⟶ o-aminophenol ⟶ catechol. The oxidative dimerization catalyzed by isophenoxazine synthase (26) involves the oxidation of o-aminophenol to o-quinoneimine and the subsequent condensation of o-quinoneimine and o-aminophenol resulting in the biosynthesis of isophenoxazine.

MATERIALS AND METHODS

All chemicals used were reagent grade unless otherwise specified. Microscope slides (2.5 cm x 7.6 cm) coated with silica gel GF_{254} (Brinkman Instruments, Inc., Westbury, N. Y.) to a thickness of 250 μ were used for micro thin-layer chromatography. 20 cm x 20 cm glass plates coated with silica gel GF_{254} to a thickness of 500 μ were used for preparative thin-layer chromatography. Whatman No. 1 paper was used for paper chromatography. The following chromatographic solvents were used: 1. 1-butanol:absolute ethanol: water:concentrated ammonium hydroxide (40:10:9:1 v/v) (solvent A); 2. cyclohexane:isobutanol (80:20 v/v) (solvent B); 3. 1-butanol: concentrated hydrochloric acid:water (100:20:39 v/v) (solvent Bc20) (27); 4. isoamyl alcohol saturated with 3 N hydrochloric acid (solvent C) (16). Powdered polyamide for column chromatography was a product of M. Woelm, Eschwege, Germany (Alupharm Chemicals, New Orleans, La.). Chromatographic column eluates were monitored by recording absorption at 254 nm with a Model UA-2 ultraviolet analyzer, Instrumentation Specialities Co., Lincoln, Neb. The cyclic hydroxamates were detected by their characteristic blue color when treated with a reagent consisting of 50 grams of $FeCl_3 \cdot 6H_2O$ in 500 ml of 95% ethanol made 0.1 N in hydrochloric acid by the addition of concentrated hydrochloric acid. Elemental analyses of HMBOA and its glucoside were performed by the Ilse Beetz Laboratory for Microanalysis, 8640 Kronach, Industriestrasse 7, P.O. Box 460, Western Germany.

Infrared spectra were obtained with a Beckman IR-8 spectrophotometer equipped with a beam condenser, with samples prepared in potassium bromide pellets using a Beckman micropellet die.[1] Mass spectra were obtained with an Atlas CH 4 mass spectrometer.[2] Ultraviolet spectra were obtained using a Cary 15 equipped with the Cary-Datex SDS-1 data recording system which was used in quantitative spectrophotometric determinations. Additional equipment used consisted of a Programma 101 table model computer by Olivetti Underwood and a Packard Model 7201 radiochromatogram scanner.

Measurement of Radioactivity

The dioxane scintillator described by G. A. Bray (28) which was used for counting radioactive samples contained 60 grams of naphthalene (recrystallized from 95% ethanol), 4 grams of 2,5-diphenyloxazole (PPO), 0.2 gram of 1,4-bis-[2-(4-methyl-5-phenyloxazolyl)]-benzene (dimethyl POPOP), 100 ml of absolute methanol and 20 ml of ethylene glycol diluted to 1 liter with p-dioxane distilled at 100-101°C (atmospheric pressure) from a distillation flask containing metallic sodium. The PPO and dimethyl POPOP (scintillation grade) were obtained from Packard Instrument Company, Inc., Downers Grove, Ill. The efficiency curve

---

[1] I am indebted to Dr. J. A. Klun, Entomology Research Division, Agricultural Research Service, U.S. Department of Agriculture, Ankeny, Iowa, for obtaining the infrared spectra.

[2] I am indebted to Dr. Thomas H. Kinstle, Department of Chemistry, Iowa State University, in whose laboratory the mass spectra were obtained, for helpful discussions.

for the dioxane scintillator was determined by adding different amounts of nitromethane to the dioxane scintillator containing a known amount of radioactivity and counting the samples using the three channels of a Packard Model 3310 Tri-Carb scintillation spectrometer. One channel was used to determine the counts per minute (cpm) of the sample and the other two channels were used to establish the ratio of the cpm in the two channels for the sample in the presence of an external standard. A graph of the counting efficiency (cpm/dpm) versus the ratio of the two channels (monitoring channels) counting each sample and the external standard gave the efficiency curve which was used to determine the disintegrations per minute (dpm) from the cpm of samples containing an unknown amount of quench. The basis of this method was reported by Baillie (29). The effect of quenching is to shift and compress the spectrum to lower pulse heights so that the ratio of counts--in the monitoring channels with different but overlapping windows--is different for different amounts of quenching of the samples. With the appropriate window and gain settings (ascertained by systematic trial and error) for the monitoring channels a linear relationship was obtained for the efficiency curve over the range which was used. This greatly facilitated the required operations for the calculation of dpm from cpm:

$$\text{dpm} = \frac{\text{cpm}}{\left(\begin{matrix}\text{ratio of counts of} \\ \text{monitoring channels}\end{matrix}\right)\left(\begin{matrix}\text{slope of efficiency} \\ \text{curve}\end{matrix}\right) - \left(\begin{matrix}\text{efficiency} \\ \text{intercept}\end{matrix}\right)}$$

This calculated dpm was used in specific activity determinations and determination of the percent C^{14}-simazine recovered.

Constants Determined Spectrophotometrically

The ultraviolet spectra of DIMBOA, HMBOA and anthranilic acid were recorded for use in the spectrophotometric determinations of the amount of the compounds present. A micro balance was used to weigh out the standards. The following molar extinction coefficients were calculated: DIMBOA, ϵ = 10490 at $\lambda_{max.}$ (95% ethanol) = 263 nm; HMBOA, ϵ = 10440 at $\lambda_{max.}$ (95% ethanol) = 258 nm; anthranilic acid, ϵ = 1814 at $\lambda_{max.}$ (H_2O) = 326 nm.

The ultraviolet spectra of DIMBOA, DIMBOA glucoside and hydroxysimazine in aqueous solutions (at room temperature) were recorded over a pH range of 2.4 to 9.2 using the Cary-Datex SDS-1 data recording system at 2 nm intervals for use in computer calculation of the pK_a of the compounds and plotting of spectra of the individual ionic species (30). The pK_a for DIMBOA (6.95) and "impure" DIMBOA glucoside (6.40) involved the loss of the hydrogen on the nitrogen hydroxyl and the pK_a for hydroxysimazine (5.01) involved the loss of the hydrogen from the 2-hydroxyl. Other data obtained from these spectra and the recorded spectra of HMBOA glucoside and simazine were the $\lambda_{max.}$ (H_2O): 1. DIMBOA at 263 nm (ϵ 9500); 2. "impure" DIMBOA glucoside at 263 nm (ϵ 7400); 3. HMBOA glucoside at 263 nm (ϵ 10300); 4. hydroxysimazine at 240 nm (ϵ 24700); 5. simazine at 222 nm (ϵ 41800).

Synthesis of N^{15}-anthranilic Acid

A small scale synthesis of N^{15}-anthranilic acid from N^{15}-phthalimide (1 gram) (International Chemical and Nuclear Corp., City of Industry, Calif.) was carried out using the procedure described in Practical Organic Chemistry (31). This synthesis involves the Hoffman degradation of phthalimide using sodium hydroxide and bromine. A 25% (w/w) solution of sodium hydroxide in water was cooled to 0°C or below and bromine was added with stirring until completely reacted. This solution was cooled to 0°C or below and finely powdered N^{15}-phthalimide was added with vigorous stirring. Additional sodium hydroxide was added and the mixture was heated to 80°C for 2 minutes, cooled and neutralized with concentrated hydrochloric acid. The anthranilic acid was precipitated by the gradual addition of glacial acetic acid. The precipitate was isolated by filtration, washed with cold water and recrystallized from hot water containing decolorizing carbon. The crystals of N^{15}-anthranilic acid were dried at 100°C.

Synthesis of 1-C^{14}-anthranilic Acid

The synthesis of 1-C^{14}-anthranilic acid from 1-C^{14}-o-nitrotoluene was carried out by the oxidation of the methyl group using neutral potassium permanganate and reduction of the nitro group by catalytic hydrogenation.

The oxidation of o-nitrotoluene to o-nitrobenzoic acid adapted from the procedure reported by F. Ullmann and J. B.

Uzbachian (32) was carried out successfully. 1-C^{14}-o-nitrotoluene (0.1 mcurie) was obtained from Tracerlab, Waltham, Mass., and this sample was used without further purification in the oxidation yielding 1-C^{14}-o-nitrobenzoic acid. To 1 ml of water was added o-nitrotoluene (58.8 mg) and this mixture was stirred vigorously with a magnetic stirrer. The round-bottom flask containing the mixture was fitted with a water-cooled condenser and heated on a steam bath resting on the magnetic stirrer. Potassium permanganate (126 mg) in a minimum of water was added gradually over a period of 1.5 hours through the condenser. After 24 hours, the mixture was filtered (hot), concentrated by boiling to about 1 ml, acidified to a pH of 1 (using hydrochloric acid), allowed to cool, and placed in a refrigerator overnight for further crystallization. The crystals of 1-C^{14}-o-nitrobenzoic acid (triclinic needles) were isolated by centrifugation and removal of the supernatant by pipetting through a fine capillary. The crystals were dried on a rotary evaporator and yielded 25.6% and 17.2% of the theoretical yield on the basis of weight and radioactivity respectively.

The reduction of o-nitrobenzoic acid yielding anthranilic acid was adapted from the procedure described by J. F. Nyc and H. K. Mitchell (33) for the reduction of 2-nitro-3-methoxybenzoic acid. To the crystals of 1-C^{14}-o-nitrobenzoic acid dissolved in 10 ml of absolute ethanol contained in a hydrogenation flask was added 100 mg of 5% palladium on charcoal. The flask was flushed with hydrogen and stirred with a magnetic stirrer for 2.5 hours under 1 atmosphere pressure of hydrogen. The solution was

filtered through a fine sintered-glass filter and the filtrate was evaporated to dryness under a stream of nitrogen. After an attempt to purify the 1-C^{14}-anthranilic acid by sublimation under reduced pressure, the sublimed material was dissolved in 1 to 2 drops of hot concentrated hydrochloric acid and crystals formed upon cooling. The crystals were isolated by centrifugation and removal of the supernatant by pipetting through a fine capillary. The purity of the 1-C^{14}-anthranilic acid was ascertained by ascending paper chromatography. A crystal was dissolved in 95% ethanol and spotted on Whatman No. 1 paper, and the chromatogram was developed in solvent Bc20 (27). A fluorescent spot appeared at an R_f = 0.8 which corresponds to that of anthranilic acid. A comparison of the peak areas obtained from the radiochromatogram scanner indicated a radiochemical purity greater than 95% with the only apparent radiochemical impurity at the solvent front. The overall yield of the conversion of 1-C^{14}-o-nitrotoluene to 1-C^{14}-anthranilic acid was 7.5% and 4.9% of the theoretical yield on the basis of weight and radioactivity respectively. Some of the 1-C^{14}-anthranilic acid was dissolved in water, the concentration was determined spectrophotometrically, the radioactivity of an aliquot was measured; and, the specific activity (152 μcuries/mmole) was determined.

Synthesis of C^{14}-hydroxysimazine

Ring-labelled C^{14}-simazine [2-chloro-4,6-bis-(ethylamino)-s-triazine] (7.8 μcuries/mg) was supplied by Geigy Chemical Corpora-

tion, Ardsley, N.Y., for the study of the *in vitro* detoxification of simazine involving DIMBOA. The product of this detoxification is hydroxysimazine [2-hydroxy-4,6-bis-(ethylamino)-s-triazine] which is considered a nonphytotoxic compound (16). The ring-labelled C^{14}-hydroxysimazine used as a standard was obtained by shaking the labelled simazine (182 μg) dissolved in chloroform (2 ml) containing concentrated hydrochloric acid (0.2 ml) for 12 hours at 37°C. The chloroform solution was extracted with water (4 ml) and the aqueous solution was evaporated to dryness *in vacuo*. To the residue was added 100 μl of 95% ethanol and this was used as the C^{14}-hydroxysimazine standard. The radiochemical purity of the simazine and hydroxysimazine was checked by chromatographing the two samples on Whatman No. 1 paper using solvent C (16). The radioactive spots were located by radioautography and they were cut out and counted in scintillation vials containing 17 ml of dioxane scintillator (34). All of the detectable radioactivity for the simazine sample was present at the R_f expected for simazine (16) and better than 97% of the radioactivity for the hydroxysimazine sample was present at the R_f expected for hydroxysimazine (16). Approximately 1% of the radioactivity was present at the origin and 2% of the radioactivity occurred halfway between the origin and hydroxysimazine.

EXPERIMENTAL PROCEDURE AND RESULTS

Isolation and Characterization of HMBOA and its Glucoside

The isolation of HMBOA glucoside resulted from an attempt to isolate crystalline DIMBOA glucoside by the method of Wahlroos and Virtanen (2). The whorl portions (26.4 kg total) of corn grown in the field were processed in several batches. The leaves were cut into small pieces and placed in water maintained above 80°C. After agitation for 15 minutes, the leaves and water were transferred to a Waring Blendor and homogenized. The homogenate was squeezed through several layers of cheesecloth and the filtrate was concentrated *in vacuo* to a small volume. The sediment that appeared was removed by centrifugation and the supernatant was extracted six times with an equal volume of 1-butanol. The butanol was removed *in vacuo* and the last traces were removed by addition of water and further concentration *in vacuo*. The aqueous solution was extracted five times with equal volumes of diethyl ether. The aqueous solution was treated with charcoal as described by Wahlroos and Virtanen (2), then chromatographed on a polyamide column with water as the solvent. Ultraviolet-absorbing fractions that gave a positive ferric chloride test were combined and concentrated to a small volume. A white precipitate which formed was collected by filtration and crystallized from absolute ethanol, yielding colorless crystals which gave only a weakly positive test with $FeCl_3$: melting point 250-251°C; $\lambda_{max.\ (H_2O)}$ 263 nm (ϵ 10300); molecular

weight 357; infrared spectra 3439, 1665, 1515, 1070 and 1020 cm^{-1}. Elemental analysis. Calculated for $C_{15}H_{19}NO_9$: C, 50.42; H, 5.36; N, 3.92. Found: C, 50.12; H, 5.16; N, 3.93.

HMBOA was obtained by the enzymatic hydrolysis of its glucoside with a crude protein preparation from corn seedlings. Four plants, about 25 cm tall, were ground with sand in a mortar containing 20 ml of sodium phosphate buffer (0.2 M, pH 6.0). The mixture was filtered with suction, and the residue was washed with an additional 30 ml of the buffer. A portion (2 ml) of this filtrate was placed on a column (2.4 cm x 27 cm) of Sephadex G-25 and eluted with the same buffer. The first ultraviolet-absorbing peak was assumed to contain the protein in the sample, including the desired glucosidase. A sample (101.3 mg) of the glucoside was dissolved in the protein-containing fraction (10.2 ml) and allowed to stand at room temperature. The progress of the hydrolysis was followed by measuring the glucose released by use of glucose oxidase (35). HMBOA crystallized on the side of the container in nearly pure form. After being washed with water and air dried, the material was characterized: melting point 198.5-200°C; $\lambda_{max.\ (H_2O)}$ 260 nm (ϵ 10500); molecular weight 195; infrared spectra 3200, 1660, 1500 and 1020 cm^{-1}. Elemental analysis. Calculated for $C_9H_9NO_4$: C, 55.38; H, 4.65; N, 7.18. Found: C, 55.26; H, 5.16; N, 7.12.

Incorporation of N^{15}-anthranilic Acid into DIMBOA

N^{15}-anthranilic acid was administered to etiolated corn seedlings of CI31A (approximately 15 cm tall). N^{15}-anthranilic acid (0.00875 g) was dissolved in 2.5 ml of distilled water and the same solution was made substituting N^{14}-anthranilic acid for the N^{15}-anthranilic acid. Each of these solutions was divided into four equal parts and placed in 12-ml graduated centrifuge tubes. The roots and seeds were removed from 24 plants by cutting them under tap water; the plants were rinsed with distilled water; and, three plants were placed in each tube. The anthranilic acid concentration of the feeding solution was 25.5 mM and most of the solution was taken up after 24 hours; therefore, each plant took up approximately 5 μmoles of anthranilic acid. At this time, the seedlings were processed for the isolation of DIMBOA. The seedlings fed N^{14}-anthranilic acid and those fed N^{15}-anthranilic acid were segregated and each group of 12 seedlings was ground with 2 ml of sand and 10 ml of deionized water and allowed to stand for 1 hour to allow glucosidase action. Each sample was filtered with suction through a coarse sintered-glass filter into a 125-ml centrifuge separatory funnel and extracted three times with equal volumes of diethyl ether. The emulsions which formed were broken by centrifugation and the ethereal solution was dried over anhydrous magnesium sulfate. The ether was removed in vacuo; the residue (approximately 20 mg) was washed with minute volumes of chloroform:methanol (95:5) three times; and, the remaining residue was dissolved in a minimum volume

of hot acetone. Skelly B was added to the acetone solution until the solution became turbid. After the solution was allowed to stand for 3 hours for crystal formation, the solvent was decanted; and, the crystals were dried overnight in a vacuum desiccator. The crystals from the N^{14} feeding and N^{15} feeding appeared to decompose on melting at 159°C compared to a melting point of 156-157°C (decomposition, gas evolution) reported by Wahlroos and Virtanen (2) for pink needle-like crystals of DIMBOA. Mass spectral analysis of phthalimide, anthranilic acid, DIMBOA and the N^{15}-enriched compounds was carried out for determination of the percent N^{15} enrichment of the compounds:

$$\left(\frac{M+1}{M} \times 100\right)_{N^{15}} - \left(\frac{M+1}{M} \times 100\right)_{N^{14}} = \%\ N^{15}\ \text{enrichment}$$

M = height of molecular ion peak
M + 1 = height of molecular ion + 1 peak

The percent N^{15}-enrichment was: N^{15}-phthalimide, 39.6%; N^{15}-anthranilic acid, 38.0%; N^{15}-DIMBOA, 2.7%. This data indicated the dilution of anthranilic acid in the biosynthesis of DIMBOA in this experiment was 38.0% divided by 2.7% or approximately 14.

Incorporation of 1-C^{14}-anthranilic acid into DIMBOA and HMBOA

Administration of the 1-C^{14}-anthranilic acid to the etiolated seedings of CI31A (15 cm tall) was accomplished in two different ways: (a) the cut stem was placed in the 1-C^{14}-anthranilic acid solution as was done in the N^{15}-anthranilic acid-feeding experiment;

(b) the 1-C^{14}-anthranilic acid solution was placed (using a 50 µl syringe) in the whorl portion of the plant growing in the sand. There were four separate 1-C^{14}-anthranilic acid-feeding experiments undertaken as explained in Figure 1. The first, second and fourth feedings were carried out in the dark in an incubator at 30-35°C. The second feeding of Figure 1 was carried out in the dark in a box at 20-25°C and a hair dryer was used to blow air through the box in an attempt to accelerate the uptake of the feeding solution.

The duration of C^{14} metabolism was the time elapsed from the first exposure to 1-C^{14}-anthranilic acid until the isolation of DIMBOA and HMBOA was started. The isolation was begun by finely chopping and grinding one or two seedlings with sand and a small amount of water using a mortar and pestle. The suspension was transferred to a 50-ml Nalgene centrifuge tube to stand for 1 hour allowing enzymatic release of the aglucones. An equal volume of diethyl ether was added to the aqueous mixture and stirred vigorously. The emulsion was broken by centrifugation and the ether was removed by pipetting and was dried over anhydrous magnesium sulfate. This extraction was repeated twice. The ethereal solution was filtered and evaporated to dryness. The isolation of DIMBOA and HMBOA from the residue remaining after the evaporation of ether under a stream of nitrogen involved washing the residue with a minute quantity of chloroform:methanol (95:5) and then dissolving the residue in a small amount of hot acetone. To this solution was added Skelly B until the solution became turbid. Crystals formed as the solution cooled, and these were isolated by centrifugation

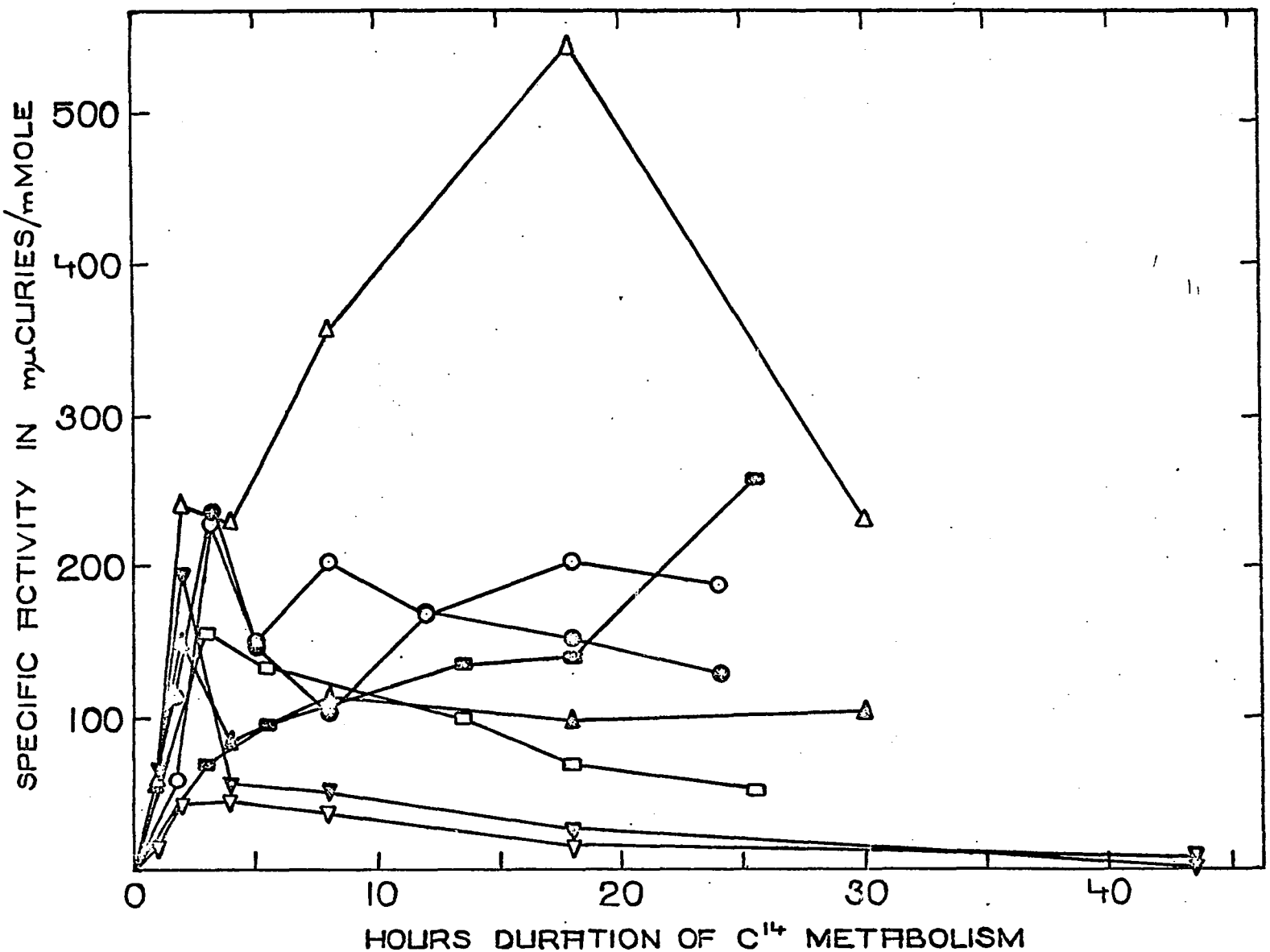
Figure 1. Specific activity of DIMBOA (unshaded symbols) and HMBOA (shaded symbols) versus duration in hours of 1-C^{14}-anthranilic acid metabolism

First (—□—) 1-C^{14}-anthranilic acid (0.216 μmole in 500 μl aqueous solution) was available to the cut stem throughout the duration of C^{14} metabolism.

Second (—○—) All of the 1-C^{14}-anthranilic acid (0.19 μmole in 50 μl aqueous solution) was absorbed by the cut stem before cessation of metabolism. After the first 50 μl of 1-C^{14}-anthranilic acid was absorbed (time required: minimum, 1.5 hours; maximum, 5 hours) 50 μl of distilled water was added and after it was absorbed 500 μl of distilled water was added for the duration of C^{14} metabolism.

Third (—▽—) 1-C^{14}-anthranilic acid (0.05 μmole in 10 μl aqueous solution) was placed in the whorl portion of the plant.

Fourth (—△—) 1-C^{14}-anthranilic acid (0.15 μmole in 10 μl aqueous solution) was placed in the whorl portion of the plant.


SPECIFIC ACTIVITY IN mμCURIES/mMOLE
500
400
300
200
100
0
10
20
30
40
HOURS DURATION OF C14 METABOLISM

and removal of the supernatant by pipetting through a fine capillary. A sample of the crystals along with standards of DIMBOA, HMBOA and 6MBOA was spotted on a micro thin-layer plate of silica gel GF_{254}. The plate was developed in solvent B which separates DIMBOA, HMBOA and 6MBOA having R_f's of 0.13, 0.40 and 0.57 respectively. The only apparent spot under UV for the crystals corresponds to that of the DIMBOA standard. For the isolation of the HMBOA, the chloroform: methanol and the acetone-Skelly B supernatants were combined and evaporated to dryness. The residue was dissolved in 95% ethanol and spotted on a preparative thin-layer plate of silica gel GF_{254} which was developed in solvent B. The spot corresponding to the HMBOA was scraped, eluted with 95% ethanol into a cuvette, diluted with 95% ethanol to 3 ml, and the concentration was determined spectrophotometrically. The solution was transferred to a scintillation vial, evaporated to dryness, and counted using dioxane scintillator in order to determine the specific activity of HMBOA. The crystals of DIMBOA were dissolved in 95% ethanol and the DIMBOA concentration was determined spectrophotometrically. The solution was treated the same as the HMBOA solution for counting, and the specific activity of the DIMBOA was determined. The results of these C^{14}-feeding experiments are shown in Figure 1.

Study of the Detoxification of Simazine

The first experiment in this series involved the incubation of 20 μmolar aqueous solutions (1 ml) of DIMBOA and C^{14}-simazine separately as controls and a solution (1 ml) 20 μmolar in both

DIMBOA and C^{14}-simazine. These solutions were incubated for 4 hours at 37°C, frozen and lyophilized. The lyophilized samples were dissolved in minute amounts of 95% ethanol and spotted on a micro thin-layer plate of silica gel GF_{254} along with standards of HMBOA and C^{14}-hydroxysimazine. The plate was developed in solvent A, air dried and subjected to radioautography for detection of C^{14}-simazine and C^{14}- hydroxysimazine. The results indicated that there was no presence of any radioactive component other than simazine for the simazine control and the solution (1:1 on a molar basis) of simazine and DIMBOA. The R_f of simazine in this system was 0.96 and the standard of hydroxysimazine streaked from the origin to an R_f = 0.65.

Another C^{14}-simazine incubation with DIMBOA was carried out using 10 μM simazine (1 ml) with 0.0, 2, 20, 200 and 2000 μM DIMBOA in the aqueous solutions. Each solution was incubated for 4 hours at 37°C and then frozen and lyophilized. Each lyophilized sample was dissolved in 95% ethanol:0.1 N hydrochloric acid (1:1) by refluxing for 0.5 minute on a steam bath and then the solution was spotted on Whatman No. 1 paper and chromatographed using solvent C (16). The chromatogram was dried and the radioactive spots were located by radioautography. The radioactive spots were cut out and counted in a scintillation vial containing 17 ml of the dioxane scintillator. The results of the simazine incubation with different amounts of DIMBOA are shown in Table 1.

Another incubation of C^{14}-simazine was carried out with 10 μM simazine (1 ml) at different hydrogen ion concentrations. The pH of each solution was adjusted using dilute hydrochloric acid and

Table 1. C^{14}-simazine incubation with different amounts of DIMBOA

Molar ratio of Simazine[b]:DIMBOA	% Total isolated radioactivity[a]				
	Origin	R_f 0.19	R_f 0.48 (Hydroxy-simazine)	R_f 0.94 (Simazine)	% Simazine recovered[c]
1:0	4.79	2.32	24.64	68.25	100
1:0.2	7.39	3.27	31.15	58.18	90
1:2	5.75	2.75	31.25	60.24	92
1:20	4.43	3.27	38.21	54.09	86
1:200	1.99	2.98	68.19	26.85	59

[a] dpm of each spot was calculated and totaled for each sample.
[b] 10 μM in water.
[c] Simazine control (1:0) corrected to 100% recovery of simazine.

dilute sodium hydroxide. The pH of each incubation mixture was measured before and after the incubation. After incubation for 4 hours at 37°C the samples were treated as described in the previous paragraph; however, only three radioactive spots for each sample were apparent on the paper chromatograms. The simazine standard (10 mμmoles) was treated in the same manner as the lyophilized samples as a control on the isolation procedure for correction of any detoxification of simazine caused by the isolation procedure. The results are shown in Table 2.

Because of the change in pH which occurred during the incubation in the previous experiment, the experiment was repeated using 0.1 M citrate buffer in the pH range 5.0-5.6 and 0.1 M phosphate buffer in the pH range 5.8-6.6. The pH remained constant at this

Table 2. Incubation of 10 μM C^{14}-simazine at various pH's

pH before incubation	pH after incubation	% Total isolated radioactivity[a]			% Simazine recovered[b]
		Origin	Hydroxysimazine	Simazine	
Simazine standard[c]		4.25	12.45	83.31	100
4.92	5.42	6.95	20.39	70.66	89
5.50	6.48	4.83	15.79	79.39	96
6.10	6.50	4.83	12.42	82.76	99
6.51	7.20	6.15	12.79	81.06	98
7.00	7.09	4.14	9.78	86.08	103
7.01[d]	7.22	1.17	9.99	88.84	106

[a] dpm of each spot was calculated and totaled for each sample.
[b] Simazine standard corrected to 100% recovery of simazine.
[c] Treated the same as the lyophilized incubation mixtures.
[d] Contained DIMBOA at a molar ratio of 200:1 (DIMBOA:Simazine).

buffer concentration, but a different method of isolation was used after lyophilization, because of the high salt concentration. To each lyophilized sample was added approximately 0.5 ml of water and this was extracted twice with 0.5-ml portions of chloroform. The two phases for each sample were separated and the chloroform solution containing most of the simazine was evaporated to dryness in a counting vial before the addition of 17 ml of dioxane scintillator, because of the high quenching caused by the presence of $CHCl_3$. The aqueous solution containing most of the hydroxysimazine was rinsed with an additional 0.5 ml of water into a counting vial and 17 ml of dioxane scintillator was added and the vials were counted. Standards of the C^{14}-simazine and C^{14}-hydroxysimazine

were pipetted into separate 1-ml portions of the two buffers, frozen, lyophilized and extracted in the same manner as the lyophilized incubation mixtures. These vials were counted for use in correcting the extraction procedure to complete separation of the C^{14}-simazine and C^{14}-hydroxysimazine. A correction was not made for the presence of other radioactive compounds in addition to the 3% present in the hydroxysimazine standard. The results are shown in Table 3 and the equations used for the correction to complete separation of simazine and hydroxysimazine in the extraction procedure were:

$$H_A = \frac{H - eS_A}{F} \qquad S_A = \frac{S - fH_A}{E}$$

H_A = actual dpm due to C^{14}-hydroxysimazine and its 3% impurities
H = dpm in aqueous phase
S_A = actual dpm due to C^{14}-simazine
S = dpm in chloroform phase

e = fraction of simazine in aqueous phase in controls lacking DIMBOA $= \frac{H}{H + S}$

E = fraction of simazine in chloroform phase in controls lacking DIMBOA $= \frac{S}{H + S}$

f = fraction of hydroxysimazine in chloroform phase in controls lacking DIMBOA $= \frac{S}{H + S}$

F = fraction of hydroxysimazine in aqueous phase in controls lacking DIMBOA $= \frac{H}{H + S}$

The values e, E, f and F were different for the citrate and phosphate buffers.

The previous experiment was repeated using more dilute phosphate and citrate buffers (1 mM), so that paper chromatography could be used in the isolation procedure. The lyophilized samples were dissolved in 95% ethanol:0.1 N hydrochloric acid (1:1)

Table 3. Incubation of 10 μM C^{14}-simazine in 1 ml of 0.1 M buffers

Sample	pH	$\frac{H}{H + S}$	$\frac{S}{H + S}$	% Simazine recovered $\frac{S_A \times 100}{H_A + S_A}$
Simazine in citrate buffer	5.0	0.0977 = e	0.9023 = E	100
Hydroxysimazine in citrate buffer	5.0	0.9787 = F	0.0213 = f	0
Simazine in phosphate buffer	7.0	0.0311 = e	0.9689 = E	100
Hydroxysimazine in phosphate buffer	7.0	0.8403 = F	0.1597 = f	0
Simazine	4.88	0.0826	0.9174	102
Simazine + 2 μmoles of DIMBOA	4.82	0.7289	0.2711	28
Simazine	5.40	0.0734	0.9266	103
Simazine	5.95	0.0550	0.9450	97
Simazine	6.50	0.1363	0.8637	87
Simazine + 2 μmoles of DIMBOA	6.58	0.3407	0.6593	62

by agitation and spotted on Whatman No. 1 paper for developing in isoamyl alcohol saturated with 3 N hydrochloric acid. A standard of simazine was treated the same as the lyophilized samples for chromatography in order to correct for detoxification of simazine caused by this isolation procedure. The results of this experiment are shown in Table 4.

Table 4. Incubation of 10 μM C^{14}-simazine in 1 ml of 1 mM buffers

pH before incubation	pH after incubation	% Total isolated radioactivity[a]			% Simazine recovered[b]
		Origin	Hydroxy-simazine	Simazine	
Simazine standard[c]		2.95	15.49	81.56	100
5.10	5.10	2.25	19.73	78.02	96
5.11[d]	4.95	1.14	45.04	53.83	72
5.55	5.55	3.41	21.14	75.45	94
5.92	6.00	5.32	24.59	70.08	89
6.40	6.35	5.63	24.26	70.11	89
6.52[d]	5.50[e]	1.13	27.66	71.21	90

[a]dpm of each spot was calculated and totaled for each sample.

[b]Simazine standard corrected to 100% recovery of simazine.

[c]Treated the same as the lyophilized incubation mixtures.

[d]Contained DIMBOA at a molar ratio of 200:1 (DIMBOA:Simazine).

[e]This value was rechecked; but, for the amount of simazine recovered and in comparison to the change in the others, it appeared erroneous.

An incubation of C^{14}-simazine with anisic acid (p-methoxybenzoic acid) and an incubation of C^{14}-simazine with DIMBOA were compared concerning the degree of detoxification caused by the two compounds. Each incubation mixture was in 0.1 M NaCl-HCl at a pH of 2.4: one incubation mixture contained 20 μM simazine; a second contained 2 mM anisic acid and 20 μM simazine; a third contained 2 mM DIMBOA and 20 μM simazine. At a pH of 2.4 most of the anisic acid was in the acid form (pK_a = 4.471 at 25°C) (36). The incubation mixtures were incubated for 3 hours at 37°C and each was extracted twice with 0.5 ml portions of chloroform. The chloroform was placed in one vial and evaporated to dryness and the aqueous solution was rinsed

with a small amount of water into another vial for each incubation mixture. Standards of C^{14}-simazine and C^{14}-hydroxysimazine were treated according to this isolation procedure and 17 ml of dioxane scintillator was added to each vial and they were counted. The treatment of the counting data was the same as that described earlier for the isolation procedure involving the chloroform extraction, and the results are given in Table 5.

Table 5. Incubation of C^{14}-simazine[a]

Sample	$\frac{H}{H+S}$	$\frac{S}{H+S}$	$\frac{H_A \times 100}{H_A + S_A}$	% Simazine recovered $\frac{S_A \times 100}{H_A + S_A}$
Simazine	0.03626 = e	0.9637 = E	0	100
Hydroxy-simazine	0.9845 = F	0.01547 = f	100	0
Simazine[b] + Anisic Acid[c]			0	100
Simazine[b] + DIMBOA[c]			47	53
Simazine[b]			0	100

[a]Calculations and symbols the same as those for Table 3.
[b]20 μM in 0.1 M NaCl-HCl (1 ml); incubated for 3 hours at 37°C.
[c]2 mM in the incubation mixture.

In an effort to detect a reaction product of DIMBOA in the detoxification of simazine yielding hydroxysimazine, 2 μmoles of C^{14}-simazine was incubated with 4 μmoles of DIMBOA in 2 ml of water. A control contained 4 μmoles of DIMBOA in 2 ml of water.

The amount of simazine in this experiment exceeded its solubility about 20-fold; therefore, the incubation period was extended to 15 days at 37°C at which time there was very little residue (simazine) remaining in the tube containing the simazine. At time intervals of 1, 3 and 6 days after the start of the incubation 20 µl from each of the incubation mixtures was spotted on a micro thin-layer plate of silica gel GF_{254} along with standards of C^{14}-simazine and C^{14}-hydroxysimazine. The plate was developed in solvent A, observed under ultraviolet light and subjected to radiochromatography. Radioactive spots were observed corresponding to simazine (R_f = 0.96) and hydroxysimazine (R_f = 0.60) for every sample obtained from the incubation mixture initially containing C^{14}-simazine and the only nonradioactive ultraviolet-absorbing spot observed for the incubation mixtures was attributed to DIMBOA (R_f = 0.25). At the end of the incubation period 20 µl from each of the incubation mixtures was spotted on a micro thin-layer plate of silica gel GF_{254} with standards of DIMBOA, HMBOA and 6MBOA in one spot, C^{14}-hydroxysimazine in another spot and C^{14}-simazine in another spot. The chromatogram was developed in solvent B, observed under ultraviolet light and subjected to radiochromatography. Radioactive spots were observed for simazine (R_f = 0.60) and hydroxysimazine (R_f = 0.0) and the only nonradioactive ultraviolet-absorbing spot observed for the incubation mixtures was streaked from the origin to an R_f = 0.13. The R_f's of DIMBOA, HMBOA, and 6MBOA were 0.13, 0.40 and 0.57 respectively. The extent of the detoxification of simazine was checked using the

chloroform extraction procedure described in the previous paragraph using the same correction values. A maximum of 9% of the simazine was recovered at the end of this incubation experiment, since the counts in the aqueous extract (hydroxysimazine) exceeded the resolution of the counter (approximately 10^6 cpm) by a factor greater than 2.

SUMMARY AND DISCUSSION

The objectives of this research were to identify precursors to the cyclic hydroxamates of maize and to elucidate the role of these cyclic hydroxamates in the in vitro detoxification of simazine.

The isolation of HMBOA and its glucoside in this laboratory (37) was done without knowledge of the nearly simultaneous isolation of HMBOA glucoside by H. E. Gahagan and R. O. Mumma (38). They reported a melting point for HMBOA glucoside of 228-232°C and reported a β-glycosidic linkage on the basis of nuclear magnetic resonance data. In this work the melting point for HMBOA glucoside was found to be slightly higher (251°C) and glucose was shown to be the carbohydrate involved in the β-glycosidic linkage. A crude protein extract from seedlings hydrolized HMBOA glucoside yielding HMBOA and glucose which was detected by the glucose oxidase assay (35). The structural relationship of HMBOA and its glucoside to DIMBOA and its glucoside raised the question concerning the product-precursor relationship between DIMBOA and HMBOA.

The feeding of N^{15}-anthranilic acid resulted in the incorporation of the N^{15} into DIMBOA with a dilution factor of 14 showing that the amino group of anthranilic acid is the source of nitrogen for the heterocyclic ring of DIMBOA. The 1-C^{14}-anthranilic acid-feeding experiment demonstrated the incorporation of the C^{14} of the aromatic ring into DIMBOA with a

minimum dilution factor of 280: (specific activity of anthranilic acid)/(specific activity of DIMBOA) = (152 μcuries/mmole)/(544 mμcuries/mmole) = 280. It is apparent that the dilution factors of the N^{15}- and C^{14}-feeding experiments differ by a factor of 20, but one must consider the much larger amount of material fed in the N^{15} experiment, since the pool size of the anthranilic acid in the plant would affect the actual specific activity of the anthranilic acid being utilized in the biosynthesis of DIMBOA. In fact the specific activity or % N^{15} enrichment of the product is a function not only of the amount of labelled anthranilic acid converted to product but of the pool sizes of the anthranilic acid, the product and all the intermediate compounds. For the following comparison all pool sizes were considered constant and the amount of anthranilic acid from its pool incorporated into DIMBOA was considered constant. The comparison is based on an adaptation of the formula used in isotopic dilution calculations:

$$X = \left(\frac{SA_1}{SA_2} - 1\right) Y$$

X = calculated quantity of compound with specific activity of 0
Y = quantity of labelled compound added
SA_1 = specific activity of labelled compound added
SA_2 = specific activity of the mixture (X + Y)

The definitions used for comparison of the dilution factors were:

X = quantity used for comparison only
Y = quantity of labelled anthranilic acid fed per plant
$\frac{SA_1}{SA_2}$ = dilution factor

Use of the equation for comparison of the dilution factors resulted in:

$$X_{\text{for } N^{15}} = (14 - 1)\ 5\ \mu\text{moles} = 65\ \mu\text{moles}$$
$$X_{\text{for } C^{14}} = (280 - 1)\ 0.15\ \mu\text{moles} = 42\ \mu\text{moles}$$

These quantities agree rather well considering the variables and the accuracy in the determination of the values used for comparison. For example, the N^{15} values were obtained from a feeding of the labelled anthranilic acid (200 μl/plant) through the cut stems of 12 plants and isolation of DIMBOA after 24 hours, while the C^{14} values were obtained from a feeding of the labelled anthranilic acid (10 μl/plant) by absorption in the whorl portion of two plants and the isolation of DIMBOA after 18 hours; consequently, the actual amount of labelled anthranilic acid available for the plant biosynthetic pathways, the rate of its availability which would affect the dilution factor and the significance of feeding only two plants for the C^{14} values used in the comparison are sources of error which would be quite significant in this comparison. It was interesting to note, however, that using the specific activity curve of the fourth C^{14}-feeding experiment (Figure 1) for estimating specific activity at the end of 24 hours (380 mμcuries/mmole) and using this value in determining the dilution factor (400), the value $X_{\text{for } C^{14}}$ would be 60 μmoles.

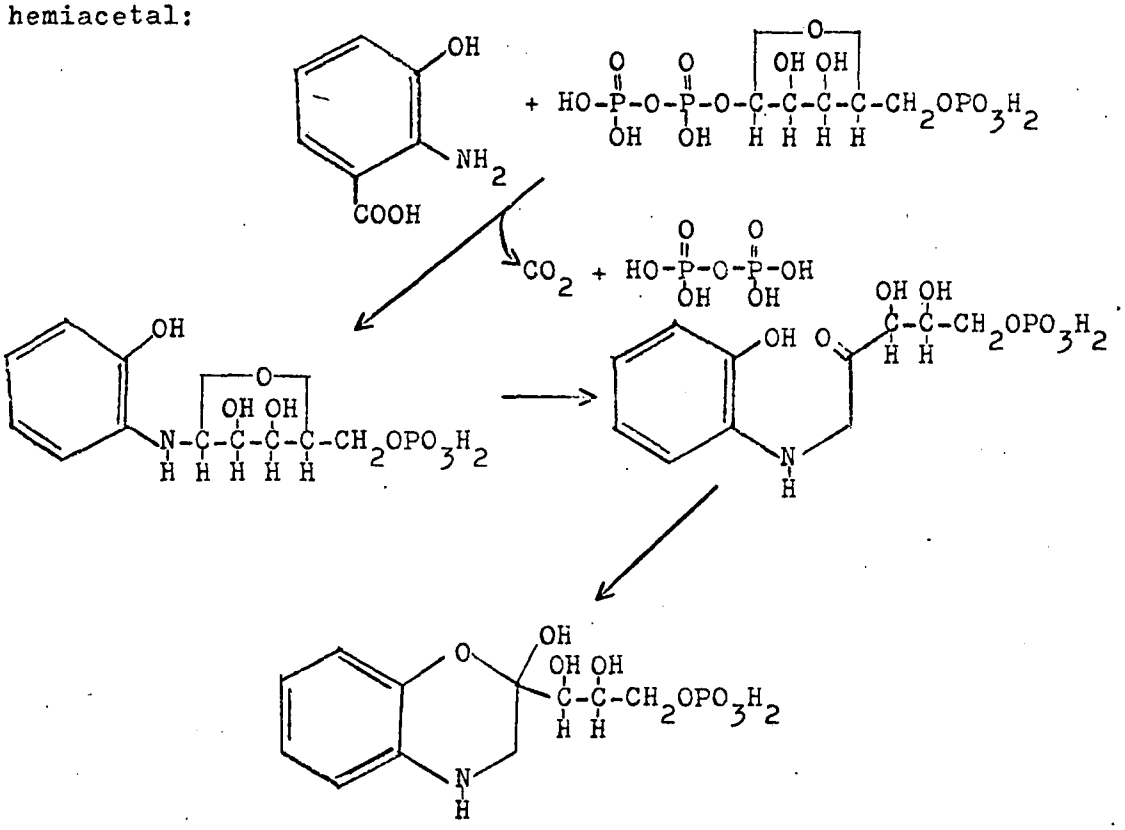
Reimann and Byerrum (17) concluded that the aromatic precursor of the ring moiety of DIMBOA is biosynthesized by the shikimic acid pathway and they suggested that a nitrogen-containing intermediate in the shikimic acid pathway might be the

precursor of both anthranilic acid and DIMBOA. The fact that anthranilic acid is the first isolatable nitrogen-containing compound in the shikimic acid pathway (18, 19) and the results of these investigations, which indicate that the nitrogen and 1-carbon of anthranilic acid are incorporated to the same extent into DIMBOA, are consistent with the conclusion that anthranilic acid is a precursor to DIMBOA. Furthermore, the dilution factor of 14 for the N^{15}-feeding experiment and 280 for the fourth C^{14}-feeding experiment (considered the most reliable due to the experience and technique gained in the earlier feeding experiments) when compared with the dilution factor of 419 reported by Reimann and Byerrum (17) for the feeding of labelled quinic acid indicates that anthranilic acid is the better precursor to DIMBOA. This means, presumably, that anthranilic acid is closer to DIMBOA biosynthetically than quinic acid and that there are fewer alternate metabolic fates for anthranilic acid than quinic acid.

In addition to establishing anthranilic acid as a precursor to DIMBOA, the C^{14}-feeding experiments were an attempt to establish a product-precursor relationship between DIMBOA and HMBOA. If the precursors equilibrate with their pools prior to being altered in the biosynthetic processes and if there are no alternate pathways for the biosynthesis of the product which circumvent the labelled precursor and utilize the labelled compound which was fed, the specific activity of the precursor will reach its maximum prior to the same for the product which will not attain a specific activity higher than the precursor. The results of the

four feeding experiments shown in Figure 1 when considered as a whole demonstrate no product-precursor relationship between DIMBOA and HMBOA. The results of the first and fourth feedings in Figure 1 would indicate that DIMBOA was the precursor to HMBOA; however, the results for the second and third feedings in Figure 1 would indicate HMBOA was the precursor to DIMBOA. If these progress curves (Figure 1) of the incorporation of C^{14} into DIMBOA and HMBOA were valid and the DIMBOA and HMBOA equilibrated with their pools prior to being altered in the biosynthetic processes, one would have to conclude that there was a branching point prior to the biosynthesis of both compounds yielding parallel pathways for the biosynthesis of the two compounds. However, it is more probable that the results in Figure 1 are indicative of the biological variance found in individuals of the same species; therefore, the specific activity of the compounds from 1 or 2 individuals at a certain time are not true progress curves of the incorporation of C^{14} into HMBOA and DIMBOA. The data in Figure 1 does demonstrate that 1-C^{14}-anthranilic acid is a precursor to both DIMBOA and HMBOA. These results are in agreement with Reimann and Byerrum's postulation (17) for the formation of the oxazine ring of DIMBOA as discussed in the literature review. Their postulation is also applicable to HMBOA. The oxazine ring suggests that 1-(o-hydroxyphenylamino)-1-deoxyribulose-5-phosphate or closely related intermediate (possibly formed by the condensation of 5-phosphoribosyl-1-pyrophosphate and 3-hydroxyanthranilic acid) participates in the formation of the oxazine by ring closure

involving the 2-keto and o-hydroxyl groups in the formation of a hemiacetal:



The successive in vitro simazine detoxification experiments were designed to answer questions left unanswered by the previous experiments and/or to answer questions arising from the previous experiments. The results of the first experiment considered with those of Hamilton and Moreland (16) suggested that the effect of DIMBOA on the in vitro detoxification of simazine was concentration dependent; therefore, the second experiment was run using 10 μM C^{14}-simazine in incubation mixtures containing molar ratios of simazine:DIMBOA of 1:0; 1:0.2; 1:2; 1:20; 1:200. The second experiment (Table 1) demonstrated the dependence on concentration of DIMBOA in the detoxification reaction; however, the pH of

similar mixtures of simazine and DIMBOA indicated a lowering of pH as the concentration of DIMBOA was increased. Since the conversion of simazine to hydroxysimazine is acid catalyzed, the significance of the change in pH was investigated in the third experiment (Table 2). This experiment suggested there might be some relevance in the change of pH to the detoxification of simazine in the range examined and definitely indicated a pH dependence of the effect of DIMBOA on the detoxification reaction although the incubation mixtures were not buffered. The fourth experiment (Table 3) was a repeat of the third using 0.1 M-buffered incubation mixtures and an added incubation with DIMBOA at a lower pH. The data was obtained by extraction procedures because of the quantity of buffer in the samples making the efficacy of paper chromatographic separation of simazine and hydroxysimazine questionable. The data for the incubations of simazine at different hydrogen ion concentrations without DIMBOA showed no significant trend; however, with DIMBOA there was definitely a pH dependence of the detoxification of simazine. The data indicated that the active form of DIMBOA for the detoxification reaction was the hydroxamic acid form as opposed to its conjugate base formed by the loss of the nitrogen-hydroxyl hydrogen (pK_a 6.95). The results of the fifth experiment (Table 4) substantiated these conclusions using the paper chromatographic separation which was applicable, since 1 mM buffers were used in the experiment instead of the 100 mM buffers used in the previous experiment. Since the

presence of the nitrogen-hydroxyl hydrogen of DIMBOA was significant, a somewhat analogous compound anisic acid (p-methoxybenzoic acid) was tested for its activity and it was inactive under conditions where only 53% of the simazine was recovered when incubated with DIMBOA (Table 5). Castelfranco and Brown (39) investigated the activity of numerous compounds including di- and tri-phenols, reducing agents, aliphatic and aromatic carbonyl compounds and amines. Of all the compounds they tested only pyridine and hydroxylamine showed any activity in the detoxification of simazine to hydroxysimazine. Their incubations were carried out in 1.0 ml of 0.1 M sodium acetate buffer, pH 5.6 with 25 μM simazine and a concentration of 200 mM for the compounds being investigated. Castelfranco and Brown concluded that DIMBOA was approximately 100 times more reactive than pyridine in the simazine detoxification to hydroxysimazine. The results of the last simazine incubation experiment indicated that there was no permanent change in the DIMBOA during the course of the in vitro detoxification of simazine. Hamilton and Moreland (16) suggested a catalytic action for the hydroxamates; however, this suggestion was based solely on the fact that they could not attribute any change in the ultraviolet spectra--used to follow the formation of hydroxysimazine from simazine--to the decomposition of the glucoside. In an incubation mixture with the molar ratio of simazine:DIMBOA of 1:100 and assuming a 1:1 stoichiometry the maximal amount of DIMBOA decomposed would be 1%. The change in spectra caused by the decomposi-

tion or transformation of 1% of a compound could be extremely hard to detect especially if the spectra were as closely related as those of DIMBOA and HMBOA and their glucosides. The chromatographic data on the 15-day incubation which converted more than 91% of the C^{14}-simazine to hydroxysimazine without any detectable loss of DIMBOA nor production of any other detectable ultraviolet-absorbing compound is more substantial evidence for the catalytic role of DIMBOA, because more than 45% of the DIMBOA would have been changed if the assumption mentioned earlier of a 1:1 stoichiometry were valid. The results of all of these investigations are consistent with the conclusion that DIMBOA acts catalytically in the in vitro detoxification of simazine yielding hydroxysimazine.

LITERATURE CITED

1. A. I. Virtanen, P. K. Hietala and O. Wahlroos, Suomen Kemistilehti B29, 143 (1956).

2. O. Wahlroos and A. I. Virtanen, Acta Chemica Scandinavica 13, 1906 (1959).

3. A. I. Virtanen and O. Wahlroos, Journal of Pharmaceutical Sciences 52, 713 (1963).

4. J. B. Bredenberg, E. Honkanen and A. I. Virtanen, Acta Chemica Scandinavica 16, 135 (1962).

5. A. I. Virtanen and P. K. Hietala, Suomen Kemistilehti B28, 165 (1955).

6. E. Honkanen and A. I. Virtanen, Suomen Kemistilehti B33, 9 (1960).

7. E. Honkanen and A. I. Virtanen, Acta Chemica Scandinavica 14, 506 (1960).

8. J. B. Neilands, Science 156, 1443 (1967).

9. T. Emery, Biochemistry 4. 1410 (1965).

10. M. A. Elnaghy and P. Linko, Physiologia Plantarum 15, 764 (1962).

11. J. N. BeMiller and A. J. Pappelis, Phytopathology 55, 1237 (1965).

12. W. Roth and E. Knusli, Experientia 17, 312 (1961).

13. R. H. Hamilton, Journal of Agricultural and Food Chemistry 12, 14 (1964).

14. J. A. Klun and T. A. Brindley, Journal of Economic Entomology 59, 711 (1966).

15. J. A. Klun, C. L. Tipton and T. A. Brindley, Journal of Economic Entomology 60, 1529 (1967).

16. R. H. Hamilton and D. E. Moreland, Science 135, 373 (1962).

17. J. E. Reimann and R. U. Byerrum, Biochemistry 3, 847 (1964).

18. O. H. Smith and C. Yanofsky, Journal of Biological Chemistry 235, 2051 (1960).

19. B. A. Bohm, Chemical Reviews 65, 435 (1965).

20. J. A. DeMoss, Journal of Biological Chemistry 240, 1231 (1965).

21. C. Ratledge, Biochimica et Biophysica Acta 156, 218 (1968).

22. R. L. Somerville, Biochemistry and Biophysics Research Communications 28, 437 (1967).

23. P. M. Nair and C. S. Vaidyanathan, Biochimica et Biophysica Acta 110, 521 (1965).

24. P. V. S. Rao and C. S. Vaidyanathan, Archives of Biochemistry Biophysics 115, 27 (1966).

25. P. M. Nair and C. S. Vaidyanathan, Phytochemistry 3, 513 (1964).

26. P. M. Nair and C. S. Vaidyanathan, Biochimica et Biophysica Acta 81, 507 (1964).

27. R. Munier, Bulletin de la Societe de Chimie Biologique 33, 857 (1951).

28. G. A. Bray, Analytical Biochemistry 1, 279 (1960).

29. L. A. Baillie, International Journal of Applied Radiation and Isotopes 8, 1 (1960).

30. K. Nagano and D. E. Metzler, Journal of the American Chemical Society 89, 2891 (1967).

31. A. I. Vogel. Practical organic chemistry. 3rd edition. New York, N.Y., Longmans, Green and Co. 1957.

32. F. Ullmann and J. B. Uzbachian, Chemische Berichte der Deutschen Chemischen Gesellschaft 36, 1797 (1903).

33. J. F. Nyc and H. K. Mitchell, Journal of the American Chemical Society 70, 1847 (1948).

34. E. A. Davidson, Packard Technical Bulletin 4, (1962).

35. I. D. Fleming and H. F. Pegler, The Analyst 88, 967 (1963).

36. J. D. Roberts and M. C. Caserio. Basic principles of organic chemistry. New York, N.Y., W. A. Benjamin, Inc. 1964.

37. C. L. Tipton, J. A. Klun, R. R. Husted and M. D. Pierson, Biochemistry 6, 2866 (1967).

38. H. E. Gahagan and R. O. Mumma, Chemistry and Industry, 1967 (1966).

39. P. Castelfranco and M. S. Brown, Weeds 10, 131 (1962).

ACKNOWLEDGMENTS

I wish to express my sincere appreciation for the guidance so ably provided by Dr. Carl L. Tipton. I wish to extend my appreciation to Dr. J. A. Klun, Dr. E. Jaworski and my father, Dr. Robert F. Husted who provided me with information which was instrumental in the initiation and completion of this research. I also wish to thank Dr. William Wildman, Dr. Dexter French, Dr. John Foss, Dr. Sam Aronoff, Dr. J. A. Klun, and Dr. John Robyt for the use of their laboratories and equipment. I am grateful for the assistance, the instruction and the moral support received from my friends and fellow graduate students including, but not limited to, James C. Linden, James R. Runyon, James Gormly, Harold B. Skrdlant, Lucy and David Ganfield, Robert J. Johnson, Gary Brammer, and Mrs. Eleanore Tarvids. Most important to me was the presence and understanding of my wife, Jean.