1971

Interconversion of benzoxazinones in maize

Ming-Chung Wang

Iowa State University

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Interconversion of benzoxazinones in maize

by

Ming-Chung Wang

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DOCTOR OF PHILOSOPHY

Major Subject: Biochemistry

Approved:

Signature was redacted for privacy.

In Charge of Major Work

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For the Major Department

Signature was redacted for privacy.

For the Graduate College

Iowa State University
Ames, Iowa

1971
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INTRODUCTION

Since DIMBOA\(^1\) was isolated from maize in 1959, the biological significance of the compound has been investigated. DIMBOA has been shown to be largely responsible for the resistance of some strains of corn to attack by European corn borer larvae and has been correlated with resistance of corn to stalk rot, wheat to stem rust, and certain plants to 2-chloro-\(^5\)-triazine herbicides (1).

Knowledge of the mechanism of control of DIMBOA biosynthesis may be helpful to geneticists in breeding strains of corn with high concentrations of DIMBOA. Therefore it is of agronomic importance to understand the biosynthetic pathway leading to DIMBOA.

The studies in this dissertation involve:

(1) The role of a benzoxazinone, HBOA, in the biosynthesis of DIMBOA and other benzoxazinones in maize. HBOA has been isolated as the glucoside from maize and rye (2). Being the simplest of the natural benzoxazinones in chemical structure, HBOA is a possible biosynthetic precursor.

\(^1\)Abbreviations of names of the following compounds were used: DIMBOA, 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one; HMBOA, 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one; DIMBOA-glucoside, 2-O-glucosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one; HMBOA-glucoside, 2-O-glucosyl-7-methoxy-1,4-benzoxazin-3-one; HBOA, 2-hydroxy-1,4-benzoxazin-3-one; MBOA, 6-methoxy-2-benzoxazolinone; BOA, 2-benzoxazolinone; DIBOA, 2,4-dihydroxy-1,4-benzoxazin-3-one; GAP, N-glycolyl-\(^2\)-aminophenol.
(2) Metabolic interconversion of benzoxazinones, specifically DIMBOA, HMBOA, DIBOA, HBOA.

(3) Biosynthesis of DIMBOA-glucoside and HMBOA-glucoside.

(4) Purification of a 2-hydroxy-benzoxazinone reductase from maize.
REVIEW OF LITERATURE

The structure of the major benzoazinone of corn seedlings is:

\[
\text{CH}_3\text{O} - \text{C} - \text{O} - \text{OH} - \text{OH} - \text{N} - \text{O} \\
\]

Consideration of this structure and the fact that the compound is derived from anthranilic acid (1) indicates two aromatic hydroxylations and one hydroxylation on nitrogen are involved in its biosynthesis.

C-hydroxylation of aromatic compounds

In mammals

Aromatic hydroxylations can occur in the biosynthesis and degradation of normal metabolites or in the metabolism of foreign substances such as drugs.

Most of the foreign aromatic compounds are lipid-soluble and tend to accumulate in the living cells. The lipid-soluble compounds in organisms are hydroxylated into more polar compounds that are excreted or further metabolized into water-soluble derivatives by conjugation with glucuronic or sulfuric acid (3). Hydroxylation of a number of compounds have been studied in mammals. Aniline, acetanilide, 2-aminofluorene, naphthalene (4), biphenyl (5), benzoazolinone (6) and hydroxyphenylethylamine (7) are
biotransformed into phenolic derivatives in mammals (8).

Most of the foreign compounds are hydroxylated by an enzyme system occurring in the endoplasmic reticulum of various tissues. Liver has the highest content of the enzyme system (9). Because the enzyme is associated with the microsomal fraction in fractionation of the subcellular components, it is also referred to as microsomal hydroxylase or simply hydroxylase. The hydroxylations require oxygen and reduced nicotinamide dinucleotide phosphate (NADPH) (10). A study using $^{18}$O$_2$ and $^2$H$_2$O has shown that oxygen utilized in the hydroxylation of acetanilide is derived from molecular oxygen rather than water (11). The hydroxylases requiring both a reducing agent and oxygen fit into Mason's classification of oxygenases as mixed function oxidases (12) which means that the enzymes catalyze the consumption of one molecule of oxygen per molecule of substrate with one atom of oxygen appearing in the product and the other undergoing two-equivalent reduction.

It is currently believed that hydroxylation is catalyzed by a cytochrome of the microsomal hydroxylase system (13). The cytochrome P-450 is an atypical cytochrome, it can be measured spectrophotometrically only after it has been reduced and combined with carbon monoxide (CO). Therefore it is also called the CO-binding pigment. After binding with CO, cytochrome P-450 has a maximum absorption at 450 nm. Studies on the microsomal cytochrome P-450 have been extensive (14, 15). Because it has not been possible
to solubilize all of the components of the microsomes and retain enzyme activity, the evidence for the involvement of cytochrome P-450 in the hydroxylation is necessarily indirect. Cyanide does not combine with cytochrome P-450 (13), nor does it inhibit hydroxylation (8, 16). CO combines with cytochrome P-450 and it is also known to inhibit the hydroxylation (17, 18, 19). Brodie et al. have proposed a mechanism for hydroxylation. In this mechanism NADPH reduces cytochrome C reductase which in turn reduced cytochrome P-450. The reduced cytochrome P-450 then combines with oxygen to form an "active oxygen" complex which in turn oxidizes the aromatic compounds (8, 20, 21, 22).

Interestingly hydroxylations of the normal metabolites in mammals do not involve the microsomal hydroxylases. Phenylalanine hydroxylase in rat liver, for instance, is an enzyme requiring a natural cofactor 7,8-dihydropteridine which is reduced to tetrahydropteridine by NADPH dihydrofolate reductase. The tetrahydropteridine then reacts with phenylalanine and molecular oxygen in the presence of the phenylalanine hydroxylating enzyme yielding tyrosine, water and a paraquinoid, 6,7-dihydropteridine (22). Tyrosine hydroxylase partially purified from beef adrenal medulla also requires a tetrahydropteridine as cofactor (23). When tryptophan is hydroxylated to 5-hydroxytryptophan (serotonin) in mast cells a tetrahydropteridine is essential in the reaction (24). Ferrous ions seem to stimulate the hydroxylations of the three compounds. The role of the metals in these enzymes
is far from being understood. These three enzymes are all present in the soluble fraction during subcellular fractionation.

In higher plants and microorganisms

Although hydroxylation of aromatic compounds in higher plants plays an important role in the pathways of biosynthesis of some secondary plant products, enzymatic studies in this area are still immature. Enzymes catalyzing hydroxylation of phenylalanine, anthranilic acid, coumaric acid and cinnamic acid have been partially purified from spinach leaves (25), *Tecoma stans* (26), spinach beet leaves (27) and pea seedling (28), respectively. They all require NADH or NADPH or tetrahydrofolic acid (FH₄). The cinnamate hydroxylase also requires mercaptoethanol, which cannot be replaced by any other reducing agents. These enzymes have pH optima at 4.2 to 5.3 with exception of cinnamate hydroxylase for which the optimum pH is 7.5. Anthranilate hydroxylase contains ferric ion and is inhibited by metal chelating agents. Addition of ferric ion can reverse the inhibitory effect of α, α-diphenyl (26). A cinnamate hydroxylase has been highly purified from pea seedlings and characterized (28). The enzyme is specific for hydroxylation of cinnamic acid only. Results of the study have suggested that the enzyme is a monooxygenase which is located in the microsomal fraction (29). It is inhibited by CO and the inhibition can be reversed by light. Cyanide has little inhibitory effect. These properties resemble the microsomal
hydroxylase of liver. Activity of the enzyme is higher in young tissue (bud) than in old tissue (second leaves of the seedlings).

Aromatic hydroxylating enzymes in microorganisms have been better understood. Studies of hydroxylation of aromatic compounds have established that the hydroxylating enzymes are also mono-oxygenases. Using $^{18}\text{O}_2$ as the oxygen source, studies of hydroxylation of anthranilate in ergot (30) and salicylate in a pseudomonad (31) have shown the oxygen atoms of the OH groups come from $\text{O}_2$. Since salicylate hydroxylase and hydroxybenzoate hydroxylase were crystallized from two strains of pseudomonads, the properties of the hydroxylases and mechanisms of enzymatic hydroxylation have been investigated in detail (31, 32). The two hydroxylases are similar in their general properties. Both hydroxybenzoate hydroxylase and salicylate hydroxylase contain one mole of flavin adenine dinucleotide (FAD) per mole of enzyme but differ in their requirements for reduced pyridine nucleotide, salicylate hydroxylase being specific for NADH and hydroxybenzoate hydroxylase for NADPH. Furthermore the FAD in the salicylate hydroxylase is loosely bound to the apoenzyme and can be dissociated by dialysis. In contrast the enzyme-bound FAD of hydroxybenzoate hydroxylase appears to be firmly bound to the protein, and is released only under relatively drastic conditions such as acidification or heat treatment. Although the purified salicylate hydroxylase has not been analyzed for metals the results of studies with inhibitors did not seem to provide definite evidence for the participation
of iron. Neither does hydroxybenzoate hydroxylase contain iron. No evidence for involvement of a cytochrome in the two enzymatic reaction has been found (32, 33). A fluorometric study of the salicylate hydroxylase has demonstrated that apoenzyme, FAD and substrate (salicylate) can form a ternary complex in one to one to one ratio. The substrate can also form a binary complex with apoenzyme in the absence of FAD. A mechanism for the enzymatic hydroxylation has been postulated (33, 34) in which the substrate—apoenzyme—FAD ternary complex is reduced first by NADH or NADPH; the substrate in the reduced complex is then oxidized by oxygen; the oxidized substrate is finally released from the enzyme complex.

**Hydroxylation of foreign compounds in vivo by animals**

The most widely studied hydroxylation is replacement of H by OH. This type of hydroxylase occurs in the endoplasmic reticulum of the liver cells, as mentioned previously. This hydroxylation is oriented and tends to occur at the carbon atoms of the highest electron density (35, 36).

In substituted aromatic systems there is a tendency for monohydroxylation to occur in more than one position, but the extent of hydroxylation in any position appears to be related to the chemical reactivity of that position. Thus, indole is hydroxylated mainly at the 3 position, quinoline at 3 (37), pyrene at 3 (38), and 3,4-benzopyrene at 5 (38), which are the most reactive centers in these molecules. When an aromatic system contains a
substituent, the position of hydroxylation is influenced by this substitution. Aniline (39), which contains an o-p-directing NH₂ group, is extensively hydroxylated at positions o- or p- to the NH₂ group. So is acetanilide (40) which contains the o-p-directing NHCOCH₃ group. Benzoic acid, which contains the m-directing COOH group (41), is hydroxylated to a very minor extent at positions o-, m- and p- to the COOH group. Benzoxazolinone is hydroxylated at the 6 position (6).

Hydroxylation can also occur by the replacement of a substituent group by OH. F, Cl, Br, NH₂, CH₂OH and COOH groups are known to be replaced by OH in biological systems (34, 42, 43). 2,6-Dichloro-4-nitroaniline and 2,4,6-trichloroaniline are bio-transformed into 4-hydroxy-2,6-dichloroaniline, substituents at the 4 position being replaced by OH (44). Sometimes, the substituent being replaced by OH is shifted to the next carbon atom. 4-Chlorophenylalanine is also isolated from enzymatic reaction mixture. This reaction is referred to as the "NIH Shift" (since it was discovered at the National Institutes of Health) (42). The "NIH Shift" also occurs in plants during hydroxylation (45), but it is not observed in nonenzymatic reactions.

Hydroxy compounds can also be formed by reduction of quinones and epoxides in vivo. Reduction of quinone and menadione produces 1,4-dihydrobenzene and the ester sulfate of 1,4-dihydroxy 2-methylnaphthalene respectively (46, 47). In the formation of catechol from anthranilic acid and from benzoic acid, hydrolysis
of an epoxide intermediate is also suggested (48).

**Biological N-hydroxylation**

Little is known about enzymatic N-hydroxylations and their mechanism in plants and microorganisms. Glycine-\(^{14}\)C and serine-\(^{14}\)C have been shown to be precursors of a hydroxamic acid, hadacidin, in *Penicillium auranitoviolaceum* (49). Leucine and isoleucine are incorporated into aspergillic acid, a cyclic hydroxamate, by *Aspergillus flavus* (50). The results suggest that N-hydroxylations take place during the biosynthesis of the hydroxamates. The oxygen atom of the hydroxyl group in hadacidin has been shown to be derived from atmospheric O\(_2\) rather than water (49). In the biosynthesis of cyanogenic glucosides an amino acid is converted to an oxime by decarboxylation and N-hydroxylation of the amino group. The oxime is in turn dehydrated to a nitrile. That the oxime is an intermediate in the biosynthesis is evidenced by feeding experiments in which oximes are converted to nitriles (51).

In animals, the hydroxylation of nitrogen can be accomplished by direct hydroxylation of reduced N compounds or reduction of a nitrogen group (52). A variety of nitrogen-containing compounds have been shown to be hydroxylated, including 2-aminofluorene, 4-aminobiphenyl sulfanilamide, 2-acetylaminofluorene, 2-acetylaminobiphenyl, N-methylaniline, N-ethylaniline and urethane. The N-hydroxylating enzyme system, which requires NADH and
molecular oxygen, appears to belong to the group of mixed function oxidases (12) of endoplasmic reticulum. Involvement of cytochrome P-450 in the enzyme system has not been demonstrated. CO does not inhibit N-hydroxylation of N-ethylnilne (53). The properties of the enzyme indicate that it is different from the aromatic hydroxylases mentioned previously.
MATERIALS AND METHODS

Materials

Commercial materials

Whatman #3 filter paper was used for paper chromatography. Silica gel GF<sub>254</sub> was purchased from Brinkman Instruments, Inc., Westbury, N. Y., and Baker-flex, a flexible plastic sheet coated with silica gel IB-F, from J. T. Baker Chemical Co., Phillipsburg, N. J. Sephadex G-10, G-25 and G-100 were purchased from Pharmacia Fine Chemicals, Inc., Piscataway, N. J. Phenol reagent (Folin-Ciocalteau) was purchased from Fisher Scientific Co., Fair Lawn, N. J.; serum albumin (fraction V) from Pentex Incorporated, Kankakee, Ill.; glucose-6-phosphate, glucose-6-phosphate dehydrogenase and nicotinamide dinucleotide phosphate (NADP) from Sigma Chemical Co., St. Louis, Mo. For liquid scintillation 1,4-bis-2-(4-methyl-5-phenyl-oxazolyl)-benzene (dimethyl POPOP) and 2,5-di-phenyloxazole (PPO) were purchased from Packard Instrument Co., Inc., Downer Grove, Ill. Phenol-<sup>14</sup>C (U) and glucose-<sup>14</sup>C (U) were purchased from New England Nuclear, Boston, Mass. Other chemicals were reagent grade unless otherwise specified.

Seed

Maize seeds of the inbred line CI31A from Dr. W. A. Russell, Department of Agronomy, Iowa State University, Ames, Iowa were sown in moist silica sand or vermiculite and incubated at 30° in
a dark chamber for 7 to 8 days with occasional watering. The etiolated seedlings were cut above the seed and used for the experiments.

Methods

**Feeding of radioactive compounds to seedlings**

The etiolated 7- or 8-day-old seedlings (15-18 cm. high) were cut at the stem 1 cm above the seed in water. The cut stems were dipped into an aqueous solution of a radioactive compound in a small beaker. They were illuminated with one regular and one Gro-Lux fluorescent lamp, both 15 watts, at a distance of about 30 cm., at room temperature. The radioactive solution was taken up in 1 to 2 hrs., then more water was added to keep the plants alive. After 24 hrs. or other specified period the tissues were homogenized for further manipulations.

**Ultraviolet spectrophotometry**

Ultraviolet spectra were obtained using a Cary 15 spectrophotometer. Molar extinction coefficients of the following compounds were determined and used for quantitative analysis: DIMBOA, 10490 at 263 nm; HMBOA, 10440 at 258 nm; HBOA, 8950 at 250 nm; MBOA, 10140 at 232 nm; BOA, 8920 at 226 nm; DIMBOA-glucoside\(^1\), 10500 at 265 nm; HMBOA-glucoside, 10500 at 260 nm; GAP, 21410 at

\(^1\)Because DIMBOA-glucoside was not obtained in solid form its molar extinction coefficient could not be determined and the molar extinction of HMBOA-glucoside was used.
243 nm. The compounds were dissolved in 95% ethanol except DIMBOA-glucoside and HMBOA-glucoside, which were dissolved in water.

Detection and measurement of specific radioactivity

From a known volume of solution a small amount was pipetted into a cuvette and diluted with a known quantity of solvent. The solution in the cuvette was used to obtain the UV absorption spectrum. Another portion was used to measure radioactivity in a scintillation vial with 15 ml. Bray's Solution (54). The radioactivity was counted with a Tri-carb liquid scintillation counter that was adjusted to 80% counting efficiency. The radioactivity was counted to a number sufficient to make the standard deviation less than 1%. From the UV absorbance and the radioactivity, the specific radioactivity was calculated. The specific activity (S.A.) was expressed as counts per minute/μmole (cpm/μm). The data were not corrected to 100% counting efficiency.

Radioactivity on thin-layer chromatograms was detected with a radiochromatogram scanner (model 7201, Packard Instruments Co., Inc., Downers Grove, Ill.). Scan speed was 1 cm/min., 30 seconds time constant, collimator width 2 mm.

Thin-layer chromatography

Unless otherwise indicated, glass plates coated with silica gel GF$_{254}$ to a thickness of 0.5 mm. were used for thin-layer chromatography (TLC). The plates were developed with ethyl ether
saturated with water unless otherwise specified. For radiochromatogram scanning thin-layer chromatography was conducted with a 5 x 20 cm. Baker-flex sheet developed with the same solvent. Benzoxazinones and other aromatic compounds were detected under a UV lamp in a dark room.

**Column chromatography**

Sephadex G-10, G-25 and G-100 were used for column chromatography. Chromatographic column eluates were monitored by a recording analyzer, Instrumentation Specialties Co., Lincoln, Neb.

**Isolation of DIMBOA- and HMBQA-glucoside from corn seedlings**

Finely cut corn seedling leaves and stems (10 g.) were homogenized in a blender with cold acetone (-18°C). The cold acetone extract was collected by suction filtration. When the acetone extract evaporated to dryness under vacuum at 35°C, a residue was obtained to which was added 2 ml of water. There were water-insoluble substances. The aqueous solution was layered on top of a Sephadex G-10 column (2.3 x 20 cm.). The column was eluted with water at a flow rate of 100 ml/hr. Aromatic compounds in the eluate were detected with an ultraviolet (UV) monitor. After 3 minor peaks a major peak appeared at 200 ml of eluent (Figure 1). The eluent under the major peak was collected and concentrated under vacuum at 35°C to a small volume. This material was chromographed on a 40 x 50 cm #3 Whatman filter paper with a solvent system consisting of n-butanol-ethanol-ammonium hydroxide-water.
Figure 1. Elution profile of acetone extract of corn seedlings on Sephadex G-10 (90:10:1:9 V/V) for 12 hrs. UV-absorbing compounds on the paper were detected by viewing the paper under a UV light. Three bands at Rf = 0.05, 0.35 and 0.45 correspond to DIMBOA-glucoside, HMBOA-glucoside and an unidentified compound respectively. Paper strips corresponding to DIMBOA-glucoside and HMBOA-glucoside were cut off and eluted with water.

Isolation of DIMBOA, HMBOA, MBOA, HBOA and BOA from corn seedlings

DIMBOA The method of isolation of DIMBOA has been described previously (55).
HMBOA, MBOA, HBOA and BOA

Seedlings (10 g.) were homogenized with sea sand and 1 ml. of water. After 30 min. the homogenate was extracted with ethyl ether and was evaporated to dryness under vacuum. A residue was obtained to which 10 ml. water was added and refluxed for 50 min. to degrade DIMBOA and DIBOA to MBOA and BOA respectively. HMBOA and HBOA are stable in boiling water. The solutions were then evaporated to dryness under vacuum and the remaining residue was chromatographed on thin-layer plates. HMBOA, HBOA, MBOA and BOA have Rf values of 0.44, 0.55, 0.65 and 0.73 respectively. The 4 compounds were scraped from the plates and extracted with ethyl ether. The silica gel was removed by centrifugation.

Isolation of aglucones from DIMBOA-glucoside and HMBOA-glucoside

The glucosides in 1 ml. 0.2 M phosphate buffer solution (pH 7.0) were hydrolyzed enzymatically using 2 ml. of P-1 (protein preparation described later) and allowed to stand at room temperature for 30 min. The reaction mixtures were then treated as described in the preceding paragraph. The aglucones of DIMBOA-glucoside and HMBOA-glucoside were isolated as MBOA and HMBOA respectively.

Purities of DIMBOA-glucoside and HMBOA-glucoside isolated by Sephadex G-10 column chromatography and paper chromatography

When 21 um DIMBOA-glucoside and 2.4 um HMBOA-glucoside were hydrolyzed with P-1, extracted with ether, degraded and thin-
layer chromatographed, one UV absorbing spot at Rf 0.62 (XH) was found with HMBOA, another UV absorbing spot at Rf 0.47 (XM) was found with MBOA. When measured in 95% ethanol XM has an UV spectrum with 3 absorption maxima at 288 nm, 260 nm and 235 nm with similar intensities; XH has 2 absorption maxima at 280 nm and 235 nm with a shoulder at 272 nm.

Total radioactivity in XH and HMBOA were 31 cpm and 157 cpm; in XM and MBOA, 960 cpm and 12794 cpm, respectively. Therefore XH had 16% of total radioactivity and XM 5%.

**Synthesis of HBOA-\(^{14}\)C**

Phenol-\(^{14}\)C (100 mg, specific activity = 20 μcuries/mmole) was dissolved in 200 mg. glacial acetic acid and slowly added, with stirring, to a solution containing 160 mg Cu(NO\(_3\))\(_3\)·3H\(_2\)O in 500 mg glacial acetic acid. The addition required 10 min. After 30 min. 4 ml. of cold water was added. The mixture was kept in a refrigerator overnight, after which a yellowish product was obtained by decanting the supernatant solution (56).

The o-nitrophenol-\(^{14}\)C was then dissolved in 5 ml. of ethyl acetate. To the ethyl acetate solution was added 50 mg of palladium on charcoal (10% catalyst) and hydrogenation was carried out at one atmosphere H\(_2\) for 3 hrs. with vigorous stirring. The solution was filtered and the filtrate evaporated to dryness. Silver-black crystals of o-aminophenol-\(^{14}\)C were obtained.

The o-aminophenol-\(^{14}\)C was dissolved in 3 ml. of ethyl ether
and 0.1 ml. of triethylamine, then 0.1 ml. of dichloroacetyl chloride dissolved in 1 ml. ethyl ether was added dropwise. The addition lasted for 10 min. and was followed by shaking for an additional hour with a vibrating mixer. The reaction mixture was filtered and washed with ethyl ether. The filtrate was washed with 4N HCl 4 times and with water once, followed by washing with saturated NaHCO₃ solution and water once each. A brownish residue was obtained after the washed ether solution was evaporated.

The residue was dissolved in 5 ml of 0.2 M NaHCO₃ and refluxed for 15 min. The reaction mixture was then acidified with 4 N HCl and extracted with ethyl ether 8 times. The ethyl ether extract was evaporated to dryness and a light brown crystalline material was obtained which was washed with 3 ml. of ethyl ether. The HBOA-¹⁴C was purified by thin-layer chromatography and 12.5 mg. was obtained. A radioautograph of a thin-layer chromatogram of HBOA-¹⁴C (1 µm) after 41 days of exposure showed no radioactive impurity. The synthesis of HBOA was described by Wheeler (57) and modified by Dr. Phillip Carpenter (private communication). The specific radioactivity of the HBOA was 28000 µCi. The HBOA has m. p. 205-206° (lit. 201-203°). The mass spectrum shows a molecular peak at m/e 165 and the base peak at m/e 136 (Figure 2). The UV absorption spectrum in 95% ethanol has maxima at 250 nm (E = 8950) and 280 nm (E = 4470) (Figure 3). The NMR spectrum has 4 singlet peaks at δ = 3.4...
Figure 2. Mass spectrum of HBOA (All peaks less than 15% are not included)

(the hydroxyl proton), 5.5 (the C-2 proton), 6.9 (4 aromatic protons) and 7.8 (the N-4 proton). Another batch of HBOA-\(^{14}\)C with a specific activity of 36000 cpm/\(\mu\)m was synthesized by the same procedure.
Figure 3. Ultraviolet spectra of 2-hydroxy-1,4-benzoazin-3-one (HBOA) and N-glycolyl-o-aminophenol (GAP)
Preparation of extracts from corn seedlings

Corn seedlings (16 g.) were homogenized with 8 ml. of 0.2 M phosphate buffer solution (pH 7.0) in an ice-jacketed blender for 2 min. and the homogenate was quickly filtered through 6 layers of cheese cloth. The filtrate (16 ml.) was applied to a Sephadex G-25 column (2 x 40 cm.) and eluted with the same buffer solution at 4° at a flow rate of 2 ml./min. The first UV absorption peak, which came out at 80 ml. of eluate, was collected and designated P-1.

Purification of 2-hydroxy-benzoxazinone reductase

Fractionation with ammonium sulfate

P-1 (90 ml.) was prepared from 140 g. seedlings as described. Saturated ammonium sulfate solution (adjusted to pH 7.0 with ammonium hydroxide) was added and materials precipitating at 30% and 30-80% saturation were collected by centrifugation at 13,000 x g for 10 min. The precipitates, designated P_{30} and P_{30-80}, were redissolved in 0.2 M phosphate buffer, pH 7.0.

Sephadex G-100 column chromatography

P_{30-80} was applied to a Sephadex G-100 column (2 x 35 cm.) and eluted with 0.2 M phosphate buffer, pH 7.0, at a flow rate of 0.5 ml./min. Fractions (4.2 ml.) were collected automatically and UV absorbance was monitored.
Assay of 2-hydroxy-benzoxazinone reductase activity

The enzyme, in 400 μ moles phosphate buffer, pH 7.0, was incubated with 1 μ mole HBOA (in 5 μl. ethanol), 2 μ moles NADP, 6 μ moles glucose-6-phosphate and 1 unit glucose-6-phosphate dehydrogenase in a total volume of 2 ml. in a shaking incubator at 37° for a specified period. The reaction was stopped by extracting the reaction mixture with 5 ml. ethyl ether. The extraction was repeated twice. The ether extract was evaporated to a small volume and applied to a chromatographic plate. A spot at Rf 0.44 was scraped from the plate and extracted with 1.5 ml. 95% ethanol. The silica gel was removed by centrifugation in a clinical centrifuge at maximum speed for 5 min. GAP in the supernatant solution was measured spectrophotometrically at 243 nm. Recovery of authentic GAP by this procedure was 75, 73 and 78% at concentrations 7, 14 and 28 μM respectively. Data in the enzyme assays were not corrected to 100% recovery.

Determination of protein in plant materials

Protein in the plant materials was estimated by a method developed by Potty (58). Serum albumin fraction V was used as standard. A Beckman spectrophotometer, model DU equipped with a Gilford digital reading meter, was used for the measurement of the optical density.
RESULTS

Feeding of radioactive precursors to seedling

Table 1 presents summarized results of the following feeding experiments.

Glucose-\(^{14}\)C (U)

**Experiment A** Glucose-\(^{14}\)C (U) (10 μcuries, S.A. 27.55 x 10^6 cpm/μm) was fed to 10 g. seedling tissue. The radioactive solution was taken up in one hour. After 24 hrs. the seedlings were homogenized and DIMBOA-glucoside (120 μm) and HMBOA-glucoside (5.5 μm) were isolated. The glucosides were enzymatically hydrolyzed using P-1 and the aglucones isolated by other extraction. DIMBOA was then degraded to MBOA.

**Experiment B** Glucose-\(^{14}\)C (U) (15 μcuries, S.A. 27.55 x 10^6 cpm/μm) was fed to 25 gm. seedling tissue for 36 hrs. Recovery of DIMBOA-glucoside and HMBOA-glucoside were 85 μm and 6.2 μm respectively.

Glucose-\(^{14}\)C was incorporated into both the aglucone and glucosyl portions of the glucosides. The specific activities of the glucosides were considerably higher than those of the aglucones and the specific activity of MBOA was 2.7 and 3.6 times that of HMBOA in the two experiments.
Table 1. Summary of radioisotope feeding experiments

<table>
<thead>
<tr>
<th>Radioactive precursors (cpm/µm)</th>
<th>DIMBOA-glu.</th>
<th>HMBOA-glu.</th>
<th>HMBOA</th>
<th>MBOA</th>
<th>HBOA</th>
<th>BOA</th>
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<td>Glucose (B) 27.55 x 10^6</td>
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<tr>
<td>DIMBOA-glu. 5688 (1617)*</td>
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<td></td>
<td>702</td>
<td>832</td>
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<tr>
<td>HMBOA-glu. (A) 4829 (443)*</td>
<td></td>
<td></td>
<td>211</td>
<td>8</td>
<td></td>
<td></td>
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<tr>
<td>HMBOA-glu. (B) 4507 (996)*</td>
<td></td>
<td></td>
<td>82</td>
<td>947</td>
<td>546</td>
<td>45</td>
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<tr>
<td>HBOA (A)</td>
<td>28000</td>
<td></td>
<td></td>
<td>1133</td>
<td>950</td>
<td>14017</td>
</tr>
<tr>
<td>HBOA (B)</td>
<td>36000</td>
<td></td>
<td></td>
<td>1424</td>
<td>1041</td>
<td>22500</td>
</tr>
<tr>
<td>HBOA (C)</td>
<td>36000</td>
<td>1314</td>
<td>3285</td>
<td>803</td>
<td>787</td>
<td></td>
</tr>
</tbody>
</table>

*Numbers in the parentheses are S.A. (cpm/µm) of aglucone of the glucoside
**DIMBOA-glucoside\(^{14}C\)**

DIMBOA-glucoside (224,100 cpm, S.A. of aglucone 1617) was administered to 5 g. seedling tissue. The radioactive solution was taken up in 4.5 hrs. After 11 hrs. the seedlings were homogenized and HMBOA and MBOA were isolated by thin-layer chromatography. Total radioactivities in the isolated HMBOA and MBOA were 2671 cpm and 6352 cpm. The specific activity of MBOA was only 1.2 times higher than that of HMBOA at the time of isolation.

**HMBOA-glucoside\(^{14}C\)**

**Experiment A**

HMBOA-glucoside (20,320 cpm, S.A. of aglucone 443) was fed to 5 g. seedling tissue. The radioactive solution was taken up in 2 hrs. After 11 hrs. the seedlings were homogenized and HMBOA and MBOA were isolated. Total radioactivities in the isolated HMBOA and MBOA were 706 cpm and 47 cpm. The ratio of the specific activities of HMBOA and MBOA was 26 to 1.

**Experiment B**

HMBOA-glucoside (83,700 cpm, S.A. of aglucone 996) was fed to 20 g. seedling tissue. The radioactive solution was taken up in 6 hrs. After 24 hrs. of the feeding the seedlings were homogenized with cold acetone from which DIMBOA-glucoside (32.7 \(\mu\)m) and HMBOA-glucoside (7.4 \(\mu\)m) were isolated. Aglucones of the glucosides were obtained by enzymatic hydrolysis with P-1 and thin-layer chromatography. MBOA ob-
tained from DIMBOA-glucoside had 280 total cpm and HMBOA from HMBOA-glucoside, 1160 total cpm (S.A. 546). The ratio of the specific activities of HMBOA and MBOA was 12 to 1.

**HBOA-^{14}C**

*Experiment A* HBOA-^{14}C (6 μm, S.A. 28,000) was fed to 6.6 g. seedling tissue in 3 hrs. After 24 hrs. the seedlings were homogenized and extracted with ether. DIMBOA was crystallized from the ether extract; and HMBOA, HBOA, MBOA and BOA were isolated from the remaining mother liquid by thin-layer chromatography. Total isolated radioactivities in the compounds were 1690, 20815, 13210 and 14030 cpm respectively. Dilution of the specific activity of the HBOA were: HMBOA 12, MBOA 12, and BOA 8. Radioactivity of the crystalline DIMBOA was not included in the radioactivity of MBOA.

The crystalline DIMBOA was recrystallized twice. Thin-layer chromatography with Baker-flex showed no impurity. Radiochromatogram scanning of the thin-layer sheet showed no radioactive impurity (Figure 4).
Figure 4. Thin-layer chromatography and scanning radio-chromatogram of crystalline DIMBOA isolated from seedling fed with HBOA-\textsuperscript{14}C.

a. b. standard DIMBOA and HBOA
b. isolated DIMBOA
d. radioactive marker
e. radioactivity of DIMBOA
Experiment B HBOA-\(^{14}\)C (5 \(\mu\)mol, S.A. 36000) was fed to 8 g. seedling tissue. After 24 hrs. crystalline DIMBOA, HMBOA, HBOA, MBOA and BOA were isolated from the seedlings by thin-layer chromatography. Dilutions of the S.A. of the HBOA were: HMBOA 16, MBOA 22 and BOA 4. The crystalline DIMBOA was recrystallized twice. Specific activity of MBOA obtained by degradation of the crystalline DIMBOA was 992.

Experiment C HBOA-\(^{14}\)C (5 \(\mu\)mol, S.A. 36000) was fed to 8 g. seedling tissue. After 24 hrs. DIMBOA-glucoside (54 \(\mu\)mol) and HMBOA-glucoside (5.3 \(\mu\)mol) were isolated.

Time course of HBOA-\(^{14}\)C incorporation into benzoazinones

HBOA-\(^{14}\)C (28.5 \(\mu\)mol in 2.5 ml 20% ethanol, S.A. 36000) was fed to 37 seedlings (22.5 g.). Six or 7 seedlings were sampled at 2.5, 5, 10, 20, 36 and 48 hrs. The radioactive solution was completely taken up at 6 hr. HBOA, HMBOA, MBOA and BOA were isolated from each sample and specific activities of each compound were measured (Figure 5 and Table 2).

Interconversion of DIMBOA and HMBOA

DIMBOA-\(^{14}\)C and HMBOA-\(^{14}\)C were prepared from seedlings fed with glucose-\(^{14}\)C. DIMBOA-\(^{14}\)C (27000 cpm, S.A. 1825) was fed to 4 g. of seedlings; and HMBOA-\(^{14}\)C (5405 cpm, S.A. 193) to 4 g. of the seedlings. After 23 hrs. each batch of seedlings was
homogenized, and MBOA and HMBOA were isolated from the homogenate. Table 3 shows radioactivities of the isolated MBOA and HMBOA.

Figure 5. Incorporation of HBOA-$^{14}$C into the benzoxazinones at different periods
Table 2. Radioactivities of benzoxazinones from seedlings fed with HBOA-\(^{14}\)C at different periods

<table>
<thead>
<tr>
<th>Feeding time (hr.)</th>
<th>Sample weight (g)</th>
<th>Isolated compounds and their total radioactivities</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>HBOA cpm  S.A.  BOA cpm  S.A.  HMBOA cpm  S.A.  MBOA cpm  S.A.</td>
</tr>
<tr>
<td>2.5</td>
<td>2.8</td>
<td>7275  26901  2349  2728  378  963  1862  139</td>
</tr>
<tr>
<td>5.0</td>
<td>4</td>
<td>20648  29059  10619  4794  1124  1569  6507  356</td>
</tr>
<tr>
<td>10</td>
<td>3.5</td>
<td>7564  27697  24593  10443  6247  3124  16903  861</td>
</tr>
<tr>
<td>20</td>
<td>4.5</td>
<td>1445  19431  28231  10579  1288  3965  30937  1255</td>
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<tr>
<td>36</td>
<td>3.5</td>
<td>1402  23928  16470  11637  308  1201  32929  1915</td>
</tr>
<tr>
<td>48</td>
<td>3.0</td>
<td>1620  20149  8144  8097  464  1185  34652  1823</td>
</tr>
</tbody>
</table>
Table 3. Radioactivities of MBOA and HMBOA in the benzoxazinone interconversion study

<table>
<thead>
<tr>
<th>Compounds fed</th>
<th>MBOA cpm</th>
<th>S.A.</th>
<th>HMBOA cpm</th>
<th>S.A.</th>
</tr>
</thead>
<tbody>
<tr>
<td>DIMBOA</td>
<td>6604</td>
<td>310</td>
<td>202</td>
<td>297</td>
</tr>
<tr>
<td>HMBOA</td>
<td>517</td>
<td>31</td>
<td>816</td>
<td>51</td>
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</table>

Metabolism of HBOA by P-1

Isolation and identification of a metabolite in benzoxazinone metabolism

Isolation from an enzymatic reaction mixture HBOA (20 μm in 0.25 ml. ethanol), 20 μm NADP, 20 μm glucose-6-phosphate and 10 units glucose-6-phosphate dehydrogenase were incubated with 15 ml of P-1 for 36 hrs. at 37°. The reaction mixture was extracted with 30 ml of ethyl ether 3 times. The ether extract was concentrated and fractionated by thin-layer chromatography. A band with Rf 0.44 was scraped from the plate and extracted with acetone. When the acetone was evaporated a light brown solid substance (940 μg) was obtained. This substance has a UV spectrum with maxima at 243 nm and 285 nm in 95% ethanol (Figure
3). The maxima were shifted to 240 nm and 306 nm respectively in 0.1 M aq. KOH. Melting point was 101-103°C.

HBOA (5 μm) was incubated in 3 different reaction mixtures in a total volume of 7.5 ml.: #1. with NADPH generating system (NADP, glucose-6-phosphate, 10 μm each, and 4 units of glucose-6-phosphate dehydrogenase) and 6 ml. of boiled P-1, #2. with 6 ml. of the boiled P-1, #3. with 6 ml. of 0.2 M phosphate buffer, pH 7.0. The boiled P-1 was prepared by boiling P-1 solution on a hot plate for 60 min. After 22 hrs. incubation at 37°C, there was no detectable amount of GAP on the thin-layer plates. No GAP was detectable in an incubation mixture containing only 6 ml. of native P-1.

Identification of the metabolite HBOA (35 mg) was dissolved in 30 ml. glacial acetic acid and 65 mg. Zn. powder was added. The mixture was stirred with a magnetic stirrer for 15 hrs. at room temperature, then filtered and the filtrate evaporated to dryness. A compound with the same Rf as the enzymatic product (0.44) was isolated by preparative thin-layer chromatography as described in the preceding paragraph. The UV spectra of this material in 95% ethanol and in 0.1 M KOH are identical to those of the enzymatic product; m. p. 101-102°C; molecular weight (by mass spectroscopy) 167. The metabolite was identified as N-glycolyl-α-aminophenol (GAP).

\[
\begin{align*}
\text{OH} \\
\text{NHCOCH}_2\text{OH}
\end{align*}
\]
Time course

Eight tubes, each containing 0.5 μm HBOA, 1 μm NADP, 2 μm glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 1 ml. P-1 and 0.2 M phosphate buffer, pH 7.0 in a total volume of 1.2 ml, were incubated at 37°. Formation of GAP was determined in tubes taken out at 0.5, 5, 13, 20, 27 and 41 hrs. Production of GAP in the tubes at the indicated periods was 0.013, 0.038, 0.056, 0.107 and 0.111 μm respectively (Figure 6).

Optimum pH

One μm HBOA was incubated with 2 μm NADP, 2 μm glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase and 2 ml. P-1 in 0.2 M phosphate buffer in total volume of 2.4 ml, at 37° for 20 hrs. GAP formed in the reaction mixtures at pH 4.5, 5.4, 6.0, 6.5, 7.0, 7.5, 8.0, 8.5, 9.8 and 10.5 were 0.011, 0.072, 0.116, 0.119, 0.135, 0.104, 0.108, 0.069, 0.013 and 0.008 μm respectively (Figure 7).

Effect of substrate concentrations on the enzyme activity

When HBOA was incubated in the reaction mixture with P-1 at 0.25, 0.50, 1.00 and 2.50 mM concentrations for 22 hrs. at 37°, GAP formed at the respective substrate concentrations was 0.075, 0.090, 0.230 and 0.286 μm respectively (Figure 8).
Figure 6. Formation of GAP by P-1 at different length of incubation.

Figure 7. Formation of GAP by P-1 in 0.2 M phosphate buffer with different pH.
Figure 8. Effect of HBOA concentration on GAP formation by P-1

Effect of enzyme quantities on the enzyme activity

HBOA (1 µm) was incubated in a 5 ml reaction mixture with 2 µm NADP, 2 µm glucose-6-phosphate, 10 units of glucose-6-phosphate dehydrogenase, 0.2 M phosphate buffer pH 7.0 and different amounts of P-1 at 37° for 22 hrs. GAP formed by 0.5, 1.0, 2.0 and 5.0 ml. of the P-1 enzyme preparation was 0.024, 0.048, 0.045 and 0.070 µm respectively (Figure 9).

Requirements of various reductants

Seven tubes containing HBOA (1 µm) were incubated with 2 ml. of P-1 and various reductants in a total volume of 2.5 ml.
Figure 9. Formation of GAP with different volumes of P-1

Tubes 1 to 3 contained mercaptoethanol with concentrations of 10, 20 and 30 mM, respectively. Tube 4 had a NADPH-generating system consisting of NADP, glucose-6-phosphate, 4 \( \mu \text{M} \) each, and 2 units of glucose-6-phosphate dehydrogenase. Tube 5 did not have any reductant. Tubes 6 and 7 contained ascorbic acid at 0.8 and 6.7 mM concentration, respectively. After 20 hrs. of incubation at 37\(^\circ\) GAP isolated from tubes 1 to 5 were 0.070, 0.071, 0.075, 0.061 and 0.016 \( \mu \text{M} \), respectively. No detectable GAP was isolated from tube 6 or 7.
Stability of the enzyme

The enzyme activity in P-1 decreases only 30% after the enzyme solution was kept in a freezer for 73 days.

Specificity of the 2-hydroxy-benzoxazinone-reductase

HMBOA (1.5 µm) was incubated with 2 µm NADP, 6 µm glucose-6-phosphate, 1 unit glucose-6-phosphate dehydrogenase, 1 ml of 0.2 M phosphate buffer pH 7.0 and 0.5 ml. (0.83 mg protein) of the purified enzyme (#10 tube, Table 4) at 37°. After 5 hrs. incubation a product was isolated by ether extraction and thin-layer chromatography as in the isolation of GAP. The product has an Rf of 0.35 in ether saturated with water and a UV spectrum in 95% ethanol similar to that of GAP with absorption maxima at 290 nm and 250 nm.

The specificity of the purified enzyme was also tested with DIMBOA. When HMBOA was replaced with DIMBOA (1.5 µm) in a similar incubation mixture, the products isolated from the reaction mixture were MBOA and DIMBOA, which were spectrophotometrically identified.

Purification of 2-hydroxy-benzoxazinone reductase

Table 4 summarizes the procedure for purification of the enzyme in SP. Figure 10 shows profile of Sephadex G-100 gel filtration and specific activities in the various fractions. The fractionation of P-1 with ammonium sulfate slightly increased the specific activity in P_{30-80}. The gel filtration purified
Table 4. Purification of 2-hydroxybenzoxazinone reductase from corn seedlings

<table>
<thead>
<tr>
<th>Protein fraction</th>
<th>Total volume (ml)</th>
<th>Protein concentration (mg/ml)</th>
<th>Activity (unit/ml) x 10^3</th>
<th>Total unit</th>
<th>Recovery of activity (%)</th>
<th>Specific activity x 10^2</th>
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<tr>
<td>P-1</td>
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<td>2.59</td>
<td>7.65</td>
<td>689</td>
<td>100</td>
<td>3.4</td>
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<tr>
<td>P_{30}</td>
<td>20</td>
<td>3.56</td>
<td>6.48</td>
<td>130</td>
<td>18.9</td>
<td>2.1</td>
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<tr>
<td>P_{30-80}</td>
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</table>

Sephadex G100
Eluate tube #

<p>| | | | | | | |</p>
<table>
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<tr>
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<td>4.2</td>
<td>0.20</td>
<td>0</td>
<td>0</td>
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<td>0</td>
</tr>
</tbody>
</table>

^a One unit = 1 \mu m GAP formed/hr.

^b Specific activity = \mu m GAP formed/mg protein/hr.
the fraction with twice higher specific activity in the eluate between 30 ml. to 43 ml. The increase of specific activity was only twice for tube #8. However, the recovery of the enzyme activities was 52.7% from P-1. The original P\textsubscript{30-80} used for the gel filtration had 51.2% activity of the activity in P-1, therefore the recovery of the activity from the P\textsubscript{30-80} fraction was 100%.

When the enzyme activity in the P-1 fraction was regarded
as 100%, the enzyme activity in 1 g. seedling was 5 units (5 \mu \text{mum} \text{GAP formed/hr.}).

**Isolation of a HMBOA metabolite from corn seedlings**

Corn seedlings (20 g.) were homogenized with 20 ml. water. After 30 min. at room temperature the homogenate was extracted 3 times with ethyl ether. The ether was concentrated and thin-layer chromatographed. A band between Rf 0.46 and 0.55 was scraped and extracted with acetone, concentrated and re-chromatographed with a cyclohexane-iso-butanol (85:15 v/v) solvent system. A band with Rf 0.20 was scraped from the plate and extracted with 95% ethanol. After silica gel was separated by centrifugation the UV spectrum of the ethanol solution was obtained. The spectrum, with maxima at 250 nm and 290 nm, and a trough at 274 nm, is identical with that of a metabolite isolated from an enzymatic reaction of HMBOA with P-1.
DISCUSSION

Since DIMBOA and its glucoside were isolated from maize, several related compounds have been isolated from plants. HMBOA-glucoside was simultaneously isolated by Gahagan and Mumma (59) and Tipton et al. (55). The latter authors also isolated DIBOA and BOA (55). A lactam, HBAO, has been isolated as the glucoside from maize (2). The biosynthesis of these compounds has not yet been thoroughly investigated. In spite of many interesting biological properties of DIMBOA and other benzoxazinones only a single investigation of the biosynthesis of DIMBOA has been reported. Weimann and Byerrum (60) have shown that quinic acid is incorporated into the aromatic ring; the methyl group of methionine and C₂ of glycine into CH₃O⁻; the C₁ of ribose into C₂ of the oxazine ring of DIMBOA. Anthranilic acid is also incorporated into DIMBOA (1).

The glucose-¹⁴C (U) feeding experiments have shown that glucose is incorporated into DIMBOA-glucoside and HMBOA-glucoside, both in the aglucones and in the glucosyl moieties. It is not surprising that the specific activities of the glucosyl groups in DIMBOA-glucoside and HMBOA-glucoside are 2 and 3 to 10 times higher than that of DIMBOA and HMBOA respectively, since incorporation of glucose into an aromatic ring involves a long metabolic pathway (glucose A and B, Table 1). When the specific activities of DIMBOA and HMBOA are compared in the glucose
feeding experiments, that of DIMBOA is always higher than that of HMBOA. It is not certain how the hydroxamate functional group \((-N(\text{OH})\cdot\text{CO}-\) in DIMBOA is synthesized, although formation of the amide group prior to hydroxylation of the nitrogen has been suggested (60). Investigations on the biosynthesis of other hydroxamates have shown that neoaspergillic acid is formed by hydroxylation of the amide bond in flavacol (61). On the other hand the hydroxamate functional groups in hadacidin, fusarinine and ferrichromes is found to be biosynthesized through acylation after hydroxylation of the nitrogen (49, 62). In the biosynthesis of DIMBOA and HMBOA from anthranilic acid, these benzoxazinones may be precursor and product, or could arise independently. Although both of these compounds are labeled when either is fed, this may result from the following kind of relationship as well as from a precursor-product relationship:

\[
\text{Anthranilic acid} \xrightarrow{X} \text{DIMBOA} \xleftarrow{} \text{HMBOA}
\]

Feeding experiments with DIMBOA-glucoside and HMBOA-glucoside have shown they are interconverted. The re-isolation of HMBOA-glucoside from the seedlings fed HMBOA-glucoside-\textsuperscript{14}C (experiment B, Table 1) has shown the dilution of specific activity of the glucosyl group is ten times higher than that
of the aglucone, indicating the glucoside which was fed is hydrolyzed during its metabolism. In interpreting the results one should keep in mind that a small amount of DIMBOA exists as the free aglucone in intact plants (63) and that a glucosidase that hydrolyzes DIMBOA-glucoside can be demonstrated easily (1). At any rate interconversion between DIMBOA and HMBOA in maize is evidenced. These experiments did not clarify the question whether the interconversion takes place before or after hydrolysis of the glucosides. Furthermore, direct feeding of DIMBOA and HMBOA also shows the interconversion between the hydroxamate and the lactam (Table 3). This experiment also shows that a higher proportion of the total HMBOA fed is converted to DIMBOA (11%) than is DIMBOA converted to HMBOA (0.7%), although less HMBOA was fed. It is possible that an equilibrium between the lactam and the hydroxamate is favorable to the formation of the hydroxamate.

One of the problems in conducting these experiments is the difficulty in purifying small amounts of DIMBOA-glucoside and HMBOA-glucoside. In most experiments, when the isolated products were hydrolyzed enzymatically and the aglucones examined by thin-layer chromatography, no impurities were detected. However, when relatively large samples were used, impurities were found in each glucoside. The impurities, XH and XM, have UV absorption spectra overlapping those of HMBOA and MBOA.

When HBOA-\(^{14}\)C is metabolized for 24 hrs. in excised leaves
the specific activity of DIBOA (isolated as BOA) is highest, DIMBOA (isolated as MBOA) and crystalline DIMBOA lowest with HMBOA intermediate. These results suggest that HMBOA is synthesized from HBOA by hydroxylation of the benzene ring and methylation of the newly formed OH group and that DIMBOA is biosynthesized from DIBOA, which is formed by hydroxylation of the nitrogen of HBOA, by hydroxylation and methylation as in the formation of HMBOA from HBOA. DIMBOA is also possibly synthesized from HMBOA. The kinetic study of HBOA metabolism (Figure 5) suggests that HMBOA may be synthesized from DIBOA. It has shown that DIMBOA has a higher specific activity than HMBOA when HBOA was metabolized for periods longer than 30 hrs. At this time HMBOA activity decreases and DIMBOA activity continues to increase. If HMBOA is a precursor of DIMBOA, or the two compounds are inconvertible, it seems unreasonable that the specific activity of DIMBOA exceeds that of HMBOA. HMBOA has a lower concentration than DIMBOA in maize. When measuring the specific activity of HMBOA a relatively small amount of UV absorbing impurity may cause a significant error in the measurement, so the specific activities may actually converge.

The following scheme (Scheme 1) is proposed for the biosynthesis of DIMBOA, HMBOA, their glucosides and other benzoxazinones.

Glucose is converted to anthanilic acid, methionine and ribose that are in turn incorporated into DIMBOA and HMBOA. It
Scheme 1. Proposed pathways of benzoxazinone metabolism in maize
is possibly incorporated to HBOA and DIROA. In the biosynthesis of DIMBOA and HMBOA, methoxylation of the benzene ring may take place before or after formation of the oxazine ring.

DIMBOA and HMBOA can also be biosynthesized from 1,4-benzoxazinone by hydroxylation of the benzene ring and methylation of the benzenoid OH group and N-hydroxylation, in the case of DIMBOA biosynthesis. HBOA is N-hydroxylated to become DIROA and methoxylated to HMBOA. These two compounds are in turn converted to DIMBOA. HMBOA can be synthesized from DIROA as suggested by the kinetic study of HBOA metabolism.

The glucose feeding experiments (glucose A and B, Table 1) do not clarify the question whether glucosyl bond formation of DIMBOA-glucoside and HMBOA-glucoside takes place before or after completion of the oxazine ring. Direct feeding of HBOA (HBOA C, Table 1) has shown that HBOA is converted into DIMBOA-glucoside and HMBOA-glucoside with similar specific activities in DIMBOA (787 cpm/μm) and HMBOA (803 cpm/μm). The results suggest that the glucosyl bond is formed after the benzoxazine structure is completed. In addition, the similarity of the specific activities in the aglucones have shown that the two glucosides are interconverted. The other experiments have indicated that the interconversion can take place between the glucosides and their aglucones. Therefore, it is suggested that HBOA is either converted into HMBOA and DIMBOA, then into their glucosides, or into HBOA-glucoside then into their glucosides. In the course
of the biosynthesis, DIMBOA and HMBOA are interconverted as glucosides or as aglucones.

When plant tissues are disrupted phenolic compounds and phenol oxidases in the tissues are brought into contact with each other resulting in quinone formation. Quinones are powerful non-specific enzyme inhibitors. In an attempt to separate enzymes that will catalyze steps in the biosynthesis of HMBOA and DIMBOA from HBOA, a homogenate of corn seedlings was quickly passed through a Sephadex G-25 column to remove the phenols. Presumably the eluate from the column contained most of the plant protein (P-1). It has high activity for hydrolysis of the benzoxazinone glucosides. The P-1 fraction was incubated with 5 μm HBOA-^{14}C and an NADPH generating system for 3 hrs. No product was detectable under UV light after thin-layer chromatography of the reaction extract on silica gel GF_{254}. When an X-ray film was exposed to the thin-layer plate for 43 days a faint spot with an Rf value lower than that of HBOA was seen along with HBOA. When a larger quantity of HBOA was used and the incubation time increased to 24 hrs., enough product was formed for isolation and characterization. It was identified as N-glycolyl-o-aminophenol (GAP) by comparison with the product of zinc reduction of HBOA. Tu (64) has shown that glycollic acid is not utilized for DIMBOA synthesis, therefore GAP is not likely to be biosynthetic intermediate of HBOA.

The enzyme has low activity in the extracts, but is stable, and has a pH optimum of 7.0 in 0.2 M phosphate buffer solution
with slightly decreased activities in pH 6.0, 6.5, 7.5 and 8.0 (Figure 7). A crude estimate of $K_m$ from the data in Figure 8 yields a value of 1.3 mM. An NADPH generating system and 20-30 mM mercaptoethanol increase the enzyme activity, although without the reductants the enzyme is also active. Ascorbate in the concentration range of 0.8 to 6.7 mM does not affect GAP formation. Since added reductants are not essential it is not clear whether they keep the enzyme in a reduced state and increase the activity of the enzyme, or whether they act as hydrogen donors. Further studies are needed to clarify this question. In studying the kinetics and mechanism of DIBOA decomposition, Bredenberg et al. have postulated that DIBOA is in an equilibrium between the hemiacetal and aldehyde forms (65). The equilibrium is acid-base catalyzed. By analogy HBOA probably exists in an equilibrium state between hemiacetal and aldehyde. Under the conditions of the enzymatic reaction the aldehyde form is reduced and produces GAP:

![Chemical diagram]

The enzyme has been purified by Sephadex G-100 column chromatography, with a two-fold increase in the specific activity. The purified enzyme is also active in reducing HMBOA;
therefore it is termed 2-hydroxybenzoxazinone reductase (HBR). An experiment to test the HBR activity in reducing DIMBOA was not successful, because DIMBOA was not stable under the incubation conditions. Because the HBR is not highly purified, reduction of HBOA and HMBOA may be catalyzed by more than one protein. A compound, which has an identical UV spectrum with that of the product of the HMBOA enzymatic reduction, has been isolated from corn seedlings. The quantity of isolated compound was not sufficient for further chemical characterizations. An attempt to separate GAP from the corn seedlings failed, perhaps because HBOA is present in such a low concentration that its metabolite is difficult to isolate.

Klun and Robinson (66) have shown that the biosynthesis of DIMBOA and DIBOA takes place throughout the growth of the corn plants, but the overall concentration of the compounds in the whole plants decreases as the plants mature. Proliferation of the plant tissue can cause the dilution of the benzoxazinones if their biosynthetic rate is not commensurate with the growth rate, and a decrease of the biosynthetic rate at the same time can increase the dilution of the compounds. The HBR might play a role in the dilution of DIMBOA and DIBOA in the corn by decreasing HMBOA and HBOA concentrations, therefore lowering the rate of accumulation of DIMBOA and DIBOA biosynthesis. The results of these studies have shown that DIMBOA can be biosynthesized from HBOA and HMBOA; DIBOA from HBOA. If HBOA and HMBOA
concentrations are decreased DIMBOA and DIBOA formation might also be decreased. Verification of this suggestion will require further studies, including determinations of HBR activity and the rate of DIMBOA and DIBOA biosynthesis in the different stages of corn plant development.
SUMMARY

The interconversion of some benzoxazinones and their biosynthesis in maize were studied.

Feeding glucose-$^{14}$C (U) through cut stems of maize seedlings has shown that the C-14 is incorporated into the aglucone and glucosyl moieties of 2-O-glucosyl-4-hydroxy-7-methoxy-1,4-benzoxazin-3-one (DIMBOA-glucoside) and 2-O-glucosyl-7-methoxy-1,4-benzoxazine-3-one (HMBOA-glucoside). Specific activities of the aglucones (DIMBOA and HMBOA) were lower than that of the glucosyl groups.

In feeding experiment with 2-hydroxy-1,4-benzoxazin-3-one-$^{14}$C (HBOA), radioactivity was incorporated into 2,4-dihydroxy-1,4-benzoxazin-3-one (DIBOA), 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one (HMBOA) and DIMBOA; DIBOA had the highest specific activity and DIMBOA lowest. When labelled DIMBOA-glucoside, HMBOA-glucoside, DIMBOA and HMBOA were fed in separate experiments, the hydroxamates and the lactams were interconverted. These results and a kinetic study of HBOA-$^{14}$C metabolism suggest the following scheme of interconversions of the benzoxazinones:

\[
\begin{array}{c}
\text{HBOA} \\
\downarrow \quad \downarrow
\end{array}
\quad
\begin{array}{c}
\text{HMBOA} \\
\uparrow \quad \uparrow
\end{array}
\quad
\begin{array}{c}
\text{DIBOA} \\
\rightarrow \quad \rightarrow
\end{array}
\quad
\begin{array}{c}
\text{DIMBOA}
\end{array}
\]

An enzyme active in the reduction of HBOA and HMBOA has been separated from seedling homogenates. Although the enzyme
activity in the extracts is very low it is stable during extended storage in the freezer. It has a pH optimum of 7.0 in 0.2 M phosphate buffer. An NADPH generating system and mercaptoethanol at 20-30 mM increase the activity, but ascorbate does not affect the activity.
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