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Decomposition of 2,4-Dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one in Aqueous Solutions[1]

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ABSTRACT

Cyclic hydroxamic acids present in some species of Gramineae have been reported to be important in resistance of these plants to fungi and insects. Since the nonglucosylated forms of these acids are unstable in aqueous solution, *in vitro* methods for the measurement of their antibiotic properties have been difficult. Kinetics of the decomposition of 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one (DIMBOA), the major hydroxamate in corn (*Zea mays* L.) extracts, were studied in buffered aqueous solutions from pH 5 to 7.5 at temperatures from 20 to 80 C. Kinetics were apparently first order under all conditions tested; energies of activation (24 to 28 kcal/mol) were nearly pH-independent. DIMBOA decomposed rapidly (half-life = 5.3 hours at 28 C, pH 6.75) relative to the time required for many procedures which have been used to demonstrate the biological activity of DIMBOA. The rate of disappearance of inhibitory activity of DIMBOA toward *Erwinia carotovora* was indistinguishable from the rate of decomposition of DIMBOA. Contrary to reports, yields of 6-methoxy-2-benzoxazolinone (MBOA) were not quantitative. Gas-liquid chromatography analytical procedures were developed for quantitation of trimethylsilyl and acetyl derivatives of MBOA. As measured by ultraviolet spectroscopy and/or gas-liquid chromatography, conversion of DIMBOA to MBOA ranged from 40 to 75% of theoretical in aqueous buffers, bacterial growth medium, and ethyl acetate extracts of corn tissue resuspended in buffer. Yields varied with temperature, pH, and constituents in the medium.

The amount of DIMBOA[5] (Fig. 1) and its glucoside may total more than 1% of the dry wt of corn plants (14, 16). In aqueous solutions near neutral pH, free DIMBOA has been reported to decompose quantitatively to MBOA and formic acid (28, 29). This facile decomposition of DIMBOA to MBOA has been used for the measurement of DIMBOA content of corn by isotopic dilution (15), spectrofluorometry (3), and IR analysis (22). By each method, MBOA is the compound actually measured, and 100% conversion of DIMBOA to MBOA is assumed.

Although the physiological role of DIMBOA is unknown, various workers have postulated its involvement in: (*a*) detoxification of *s*-triazine herbicides (21, 26); (*b*) iron transport (25); (*c*) resistance to fungal infection in corn and wheat (2, 7, 8, 20); and (*d*) as a factor in insect resistance (17, 19). Because of the instability of DIMBOA, Whitney and Mortimore (30) have postulated that its degradation product, MBOA, may be involved in the resistance of corn to bacteria.

Hartman *et al.* (13) found that crude extracts of corn prolonged the lag phase of growth of various soft rotting *Erwinia* species that are nonpathogenic to corn. DIMBOA but not MBOA was shown to possess most of the inhibitory activity of the crude extract of Hartman *et al.* (5, 6).

The qualitative and quantitative differences in the biological activity of DIMBOA and MBOA to various *Erwinia* species prompted us to examine the stability of DIMBOA in aqueous solutions, particularly under the conditions used in our assay. The kinetics of the decomposition of DIBOA, an analog of DIMBOA, has been examined in aqueous solutions (4). However, ethanol was used as the solvent in the only kinetic study of the decomposition of DIMBOA (27). We have examined the kinetics of the decomposition of DIMBOA in aqueous solutions and the yield of MBOA under a variety of conditions. The results of the kinetic studies then permitted a comparison of the loss of inhibitory activity of DIMBOA in bacterial growth medium to the measured degradation rate of DIMBOA. A preliminary report of this work has been published (31).

MATERIALS AND METHODS

UV and visible spectra were obtained with a Cary 15[6] recording spectrophotometer, IR spectra with a Beckman IR-5 spectrophotometer, and NMR spectra with a Varian T-60 instrument. Mass spectra were obtained either in an MS-9 mass spectrometer (direct probe inlet) or in a Du Pont 21-491B mass spectrometer with an inlet from a Varian model 2700 gas chromatograph equipped with a glass column (1.8 m × 2 mm) packed with 3% OV-1 on 100 to 120 mesh Varaport 30. Melting points (uncorrected) were determined in a 6886-A Kofler, micro-hot-stage melting point apparatus.

Analytical GLC was performed with a Hewlett-Packard model 7620A gas chromatograph equipped with dual flame ionization detectors and fitted with glass columns (1.5 m × 2 mm) containing either 2% DC-11 or 1% OV-17 on Gas-chrom Q (80–100 mesh). The injection port was maintained at 270 C and the detector at 400 C. The He carrier gas flow rate was 60 ml/min at the detector, and H_2 and air flow rates were 26 and 400 ml/min, respectively.

Preparative GLC was performed with a Varian Aerograph model 1800 gas chromatograph fitted with glass columns (1.5 m × 2 mm) containing 2% DC-11 on 80 to 100 mesh Gas-chrom Q.

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[5] Abbreviations: BSTFA: *N,N*-bis(trimethylsilyl)-2,2,2-trifluoroacetamide; DIBOA: 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-one; DIMBOA: 2,4-dihydroxy-7-methoxy-2*H*-1,4-benzoxazin-3(4*H*)-one; DIM_2BOA: 2,4-dihydroxy-7,8-dimethoxy-2*H*-1,4-benzoxazin-3(4*H*)one; BOA: 2-benzoxazolinone; MBOA: 6-methoxy-2-benzoxazolinone; M_2BOA: 6,7-dimethoxy-2-benzoxazolinone; TMS: trimethylsilyl; E_a: energy of activation; NMR: nuclear magnetic resonance.

[6] Mention of companies or commercial products does not imply recommendation or endorsement by the U.S. Department of Agriculture over others not mentioned.

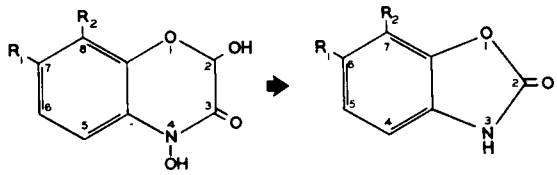

FIG. 1. Cyclic hydroxamic acids and benzoxazolinones reported to be in aqueous extracts of corn.

Nitrogen was used as the carrier gas. The column outlet was connected to an all glass stream splitter (approximately 20:1, v/v); the minor stream was diverted to a flame ionization detector, and the main stream was diverted to an adaptor into which a Pasteur pipette was inserted to recover desired fractions.

Isolation and Characterization of DIMBOA. DIMBOA was isolated by a modification of the procedure described by Klun *et al.* (17). Corn seeds of the hybrid W64A^{tcms} × W117 were planted in sand, watered with tap water, and grown in the dark at 28 C. Entire 7-day-old seedlings (238 g), from which the seeds had been removed, were blended in 480 ml of water. The macerate was allowed to stand at room temperature for 50 min. The extract was filtered through four layers of cheesecloth, heated rapidly to 65 C, cooled rapidly to 10 to 15 C, and filtered through Whatman No. 42 filter paper. The filtrate (pH 5.2) was partitioned three times against equal volumes of diethyl ether. The water phase was discarded, the ether extracts were pooled, and the ether was removed under vacuum. The viscous residue solidified during storage for 5 days at −20 C. The solid material was washed twice with 0.2 ml of a mixture of chloroform-methanol (95:5, v/v). The remaining solid material was washed quickly with 1 ml of acetone. The pink residue (150 mg) was dissolved in acetone, and DIMBOA was crystallized by addition of hexane to turbidity. The pink crystals were decolorized with activated charcoal. Recrystallization from acetone-hexane gave fine white needles (m.p. = 152–155 C). UV in absolute ethanol: λ_{max} 288 (shoulder), 262 (ϵ = 10,000), 206.5; NMR in deuterated acetone: τ 6.2 (3H-singlet), 2.8 to 3.4 (3H-multiplet), 4.28 (1H-singlet); mass spectrum: m/e 211 (parent, 6%), 195 (7%), 193 (8%), 165 (base peak), 150 (55%), 122 (18%), 109 (26%), 106 (49%).

$C_9H_9O_5N$

Calculated: C 51.19, H 4.30, N 6.63
Found: C 51.06, H 4.52, N 6.41

Data for NMR and mass spectra were the same as those reported by Gahagan and Mumma (9) and Klun *et al.* (18), respectively. DIMBOA gave a blue colored complex (λ_{max} 590 nm) with a ferric chloride reagent (50 g of $FeCl_3 \cdot 6H_2O$, 500 ml of 95% ethanol, and 5 ml of concentrated HCl).

Purity was also examined by GLC of the TMS derivative on columns of 2% DC-11 (Fig. 2) and 1% OV-17. The major peak accounted for 94% (DC-11) and 96% (OV-17) of the total area of all peaks. A purified sample of DIMBOA was obtained from C. L. Tipton and showed the same chromatographic behavior. Material corresponding to the major peak was collected by preparative GLC. A portion of the collected material, dissolved in 95% ethanol, had the same UV spectrum as authentic DIMBOA. Another portion was dissolved in methanol and dried under a stream of N_2. The mass spectra (direct probe) of the collected material and authentic DIMBOA were the same. A third portion was silylated and reexamined by GLC under the same conditions described in Figure 2. Peaks corresponding to the first four peaks in Figure 2 were observed at 6.6, 9.2, 10, and 11.5 min. Peaks 5 and 6 of Figure 2 were not observed. The major peak was shown to be a TMS derivative of DIMBOA by GLC-MS (M+ = 355).

Synthesis and Characterization of MBOA. MBOA was synthesized by the procedure of Allen and Laird (1). The orange crystalline product was decolorized with activated charcoal, and white needles (m.p. = 149–152 C) were obtained upon recrystallization from absolute ethanol-hexane. UV in absolute ethanol: λ_{max} 290 (ϵ = 5830), 232 (ϵ = 10,240) (literature: 10,100 [Hamilton *et al.*, 12] and 10,200 [Smissman *et al.*, 23]); IR: 1755 *s*, 1635 *w*, 1515 *w*, 1320 *w*, 1150 *w*, 1100 *w*, 1030 *w*, and 950 *w* cm^{-1}; NMR spectrum in $CDCl_3$: τ 6.2 (3H-singlet), 3.1 (3H-multiplet), 0.9 (1H-broad); mass spectrum: m/e 165 (parent and base peak), 150 (56%), 136 (6%), 122 (19%), 109 (27%), and 106 (56%).

$C_8H_7O_3N$

Calculated: C 58.18, H 4.27, N 8.48
Found: C 57.99, H 4.22, N 8.44

Purity of the synthetic product was also examined by GLC or TMS and acetyl derivatives (2% DC-11, temperature program from 100 C to 250 C at 8 C/min). The TMS derivatives were prepared by mixing equal volumes of BSTFA and pyridine with the crystalline material and heating the mixture to 60 to 70 C for 15 min. Two peaks were observed and identified as isomers of TMS-MBOA by GLC-MS (5). The acetyl derivative was prepared by addition of acetic anhydride to the synthetic product. GLC of this derivative showed a single peak (temperature program from 100 C to 250 C). The mass spectrum obtained by direct probe from a GLC collection of the peak has a molecular ion of 207. An authentic sample of MBOA, obtained from S. D. Beck, showed the same GLC and spectral properties as the synthesis product.

Kinetic Studies. DIMBOA decomposition rates were measured as decreases in UV absorbance at 260 nm in a Gilford model 2000 spectrophotometer equipped with a thermostatted cell compartment, and with a thermocouple mounted within the cell compartment. Rates were determined in aqueous buffers (pH 5–7.5) or in the bacterial growth medium used for studies of DIMBOA activity against *Erwinia* species (5, 6). The medium contained mineral salts, 10 g/l sucrose, 1 g/l casamino acids, and was 0.1 M in Na_2HPO_4-NaH_2PO_4 buffer (pH 6.75). In order to measure rates at high (>60 C) or low (<60 C) temperature, DIMBOA was added to the buffer or medium in two ways. Generally, for kinetic experiments below 60 C, 2 ml of buffer or medium, preequilibrated at the desired temperature, were added to a test tube containing

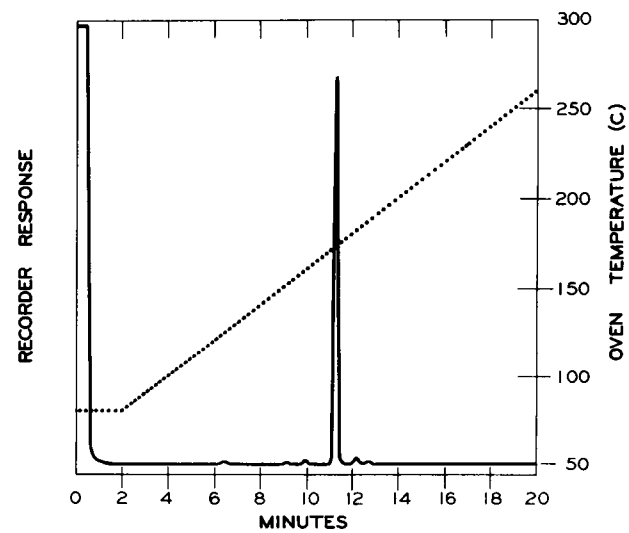

FIG. 2. Gas chromatogram of purified DIMBOA. BSTFA (60 μl) was heated to 60 to 70 C with DIMBOA (100 μg) for 20 min. Two μl were injected into a glass column containing 2% DC-11 on 80 to 100 mesh Gaschrom Q. Other operating parameters were described in the text. Peaks 1 through 6 appeared at 6.5, 9.1, 9.9, 11.4, 12.2, and 12.7 min, respectively.

50 μg of dry DIMBOA. The contents were mixed rapidly, and a 1-ml aliquot was placed in a cuvette for kinetic studies. The remaining 1 ml of solution was used immediately for determination of the UV spectrum. UV spectra were also obtained after no further change in absorbance was observed. For experiments at temperatures greater than 60 C, 1 ml of the aqueous buffer or medium in a cuvette was equilibrated in the spectrophotometer cell compartment. The reaction was started by addition of 25 μg of DIMBOA dissolved in 10 μl of ethanol. Half-lives were calculated as described by Guggenheim and Wiseman (10). Half-lives obtained at 60 C by the two procedures were identical. The inhibition of bacterial growth was measured in the above medium by the procedures described by Corcuera *et al.* (6).

Analytical Procedures for the Determination of MBOA. Aqueous buffered solutions of DIMBOA were prepared as described for the kinetic studies at temperatures below 60 C. Each 2-ml sample was incubated for 10 half-lives of DIMBOA (time as determined by the UV kinetic studies). The sample was then extracted three times with equal volumes of ethyl acetate. Each ethyl acetate phase was washed once with 1 ml of H_2O when 10 mM buffers were used or twice when 100 mM buffers were used. The three organic phases were combined and taken to dryness under vacuum. The residue was dissolved in ethanol, and samples were removed for quantitation by GLC and by UV spectrophotometry. Blanks were prepared by extraction of the appropriate buffer. Although MBOA has only a small *A* at 310 nm, samples from DIMBOA degradation reaction mixtures had significant absorbance at this wavelength (Fig. 3, ...). A small amount of material absorbing at 310 nm was carried over into the organic phase during the extraction procedure (Fig. 3, – – –). The amount of MBOA present in samples was estimated by the net *A* at 290 nm (ethanol). This value was obtained by subtraction of the *A* at 310 nm, which was not due to MBOA, from the observed value at 290 nm.

Two GLC procedures (using either the acetyl or TMS derivatives of MBOA) were developed for the quantitation of MBOA. These are referred to as the TMS-MBOA method and the acetyl-MBOA method. A known volume of an absolute ethanol solution of either pure MBOA or a sample unknown containing MBOA was added to a small vial containing an internal standard. The solvent was evaporated under a stream of N_2 60 μl of derivatizing reagent (either BSTFA or acetic anhydride) were added, the contents of the vial were mixed and heated to 60 to 70 C for 20 min before analysis. A 2-μl sample was injected into a column of 2% DC-11.

For the TMS-MBOA method, peaks eluted at 2.7, 5.2, and 6.5 min (oven temperature 100 C for 1 min, 8 C/min to 160 C, and then 30 C/min to 250 C). The first two peaks were due to isomers of TMS-MBOA and the third peak was due to TMS-myristic acid (internal standard). The relationship between response (MBOA to myristic acid ratio) and amount of MBOA injected was linear, at least from 0.2 to 1 μg of MBOA injected.

For the acetyl-MBOA method, peaks eluted at 3.5 (acetyl-MBOA) and 6.3 min (internal standard, methyl palmitate) with a temperature program (105 C for 1 min, then 10 C/min to 170 C). Response (as MBOA to methyl palmitate ratio) was linear with amount (0.1–1 μg) of MBOA injected.

Ethyl Acetate Extracts of Corn. Seeds of corn hybrid W64A × W117 and of a corn line homozygous for the absence of hydroxamates (bxbx as described by Hamilton [11]) were grown and extracts of 10-day-old seedlings were obtained as described in the preceding paper (6). An estimate of the quantity of DIMBOA and other cyclic hydroxamates present in the extract was obtained with the ferric chloride reagent (standard curve made with DIMBOA). When the extract was stored at −20 C, the total hydroxamate concentration remained unchanged for at least 6 months.

RESULTS

Kinetic Studies. DIMBOA decomposition rates were first studied in the bacterial growth medium (pH 6.75). Kinetics were first order for three half-lives. Half-lives were 5.3, 5.1, and 5 hr at 28 C for initial DIMBOA concentrations of 0.1, 0.05, and 0.025 mM, respectively. UV spectra taken after degradation of DIMBOA in medium at four temperatures showed an *A* maximum at 320 to 325 nm (Fig. 4B). The exact wavelength of the maximum varied with incubation temperature. However, none of this absorbance was due to MBOA (Fig. 4A). At 20, 25, 30, and 35 C, the apparent

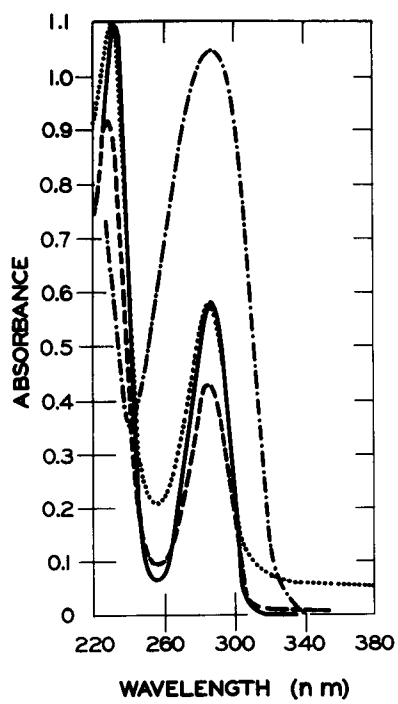

FIG. 3. UV spectra of 0.10 mM MBOA and solutions resulting from several treatments of 0.1 mM DIMBOA in 10 mM Na_2HPO_4-NaH_2PO_4 buffer (pH 7). Spectra represent: DIMBOA dissolved in buffer before (—·—) and after (·····) 31 hr at 30 C; ethyl acetate-extractable reaction products from 31 hr incubation dissolved in buffer (– –); and 0.1 mM MBOA (——).

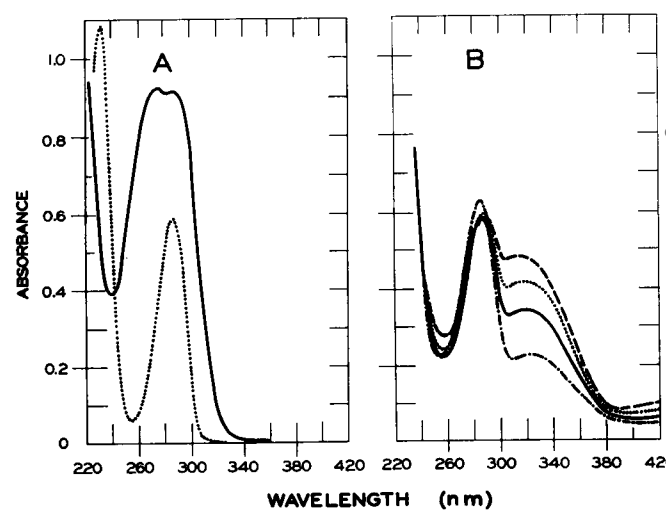

FIG. 4. UV spectra of 0.1 mM DIMBOA and 0.1 mM MBOA in bacterial growth medium. A: MBOA (·····) and DIMBOA (——) prior to degradation. B: DIMBOA maintained in medium for 47 days at 5 C (—·—), 40 hr at 30 C (——), 3.5 hr at 50 C (·····), or 0.08 hr at 80 C (– –).

first order rate constant for this increase in absorbance was the same as that for disappearance of *A* at 260 nm (DIMBOA decomposition). DIMBOA decomposition rates were then determined for temperatures up to 60 C. The relationship between the logarithm of the first order rate constant and 1/T was linear (Fig. 5). The E_a calculated from 29 individual rate determinations was 26.3 kcal/mol.

To determine if E_a had been influenced by components of the medium, a more extensive study was undertaken using 10 mM Na_2HPO_4-NaH_2PO_4 buffers (pH 6, 7, and 7.5) and 10 mM succinic acid-NaOH buffers (pH 6, 5.5, and 5). Rates of DIMBOA decomposition in these buffers were measured from 25 to 80 C (Fig. 6). Again, kinetics appeared to be first order in all cases. At pH 6, rates in the two different buffers were identical. E_a values ± SE were 28.6 ± 0.75, 27.0 ± 0.50, 26.2 ± 0.25, 23.9 ± 0.25, and 24.9 ± 0.25 kcal/mol at pH 5, 5.5, 6, 7, and 7.5, respectively.

Comparison of GLC and UV Methods for Quantitation of MBOA. In order to determine the accuracy and precision of the three analytical procedures for measuring MBOA, each GLC method was compared separately with the UV method. MBOA was stable for 48 hr at 37 C in buffer, and 97 and 95% was recovered as measured by the UV and TMS-MBOA methods, respectively. The two estimates of the yield of MBOA produced from 1 mol of DIMBOA were 0.71 and 0.74 as measured by the UV and TMS-MBOA methods, respectively (Table I, expt. 1) and 0.68 and 0.69 as determined by the UV and acetyl-MBOA methods, respectively (Table I, expt. 2).

Since all three methods gave results that were not significantly different, the simpler UV procedure was used whenever possible. When interfering absorbance made the UV method unsatisfactory, the acetyl-MBOA procedure was used.

Factors Influencing the Yield of MBOA Produced from DIMBOA in Aqueous Solution. It is apparent from the results given in Table I that the yield of MBOA was significantly less than 100%. We examined the variables of temperature and pH to determine if they affected MBOA yield. From the kinetic data obtained, the half-lives of DIMBOA were estimated at 10 C intervals from 30 to 80 C at each pH shown in Figure 6. Yields of MBOA were determined at each of the 31 pH and temperature combinations which gave a half-life of DIMBOA less than 5.5 hr. DIMBOA (206.8 ± 4.4 nmol) in ethanol was added to test tubes, the solvent was evaporated under a stream of N_2, and the tubes were refrigerated until the treatments began (within 48 hr). Tubes containing only MBOA were prepared in the same manner to correct for losses of MBOA during extraction and handling. Buffers were equilibrated at the appropriate temperatures and added to tubes containing either DIMBOA or MBOA. The tubes were then maintained for a time equivalent to 10 half-lives of DIMBOA at that pH and temperature. MBOA was extracted from the samples and yields were determined by the UV method. Under all conditions examined, the yield of MBOA from degradation of DIMBOA was less than 75% (Table II).

To determine if the initial DIMBOA concentration altered the yield of MBOA, samples with DIMBOA concentrations ranging from 0.1 to 1.6 mM were incubated at 30 C in phosphate buffer (10 mM, pH 7) for 31 hr. After extraction, the samples were analyzed by the UV procedure. The yields of MBOA (averages of three replicates) were 73.9, 72.5, 73.3, 72, and 72.3 for initial DIMBOA concentrations of 0.1, 0.2, 0.4, 0.8, and 1.6 mM, respectively. The yields were not dependent on initial DIMBOA concentration (5% level of significance).

To determine whether the per cent conversion of DIMBOA to MBOA in the bacterial growth medium differed from that in phosphate buffer alone, tubes containing 2 ml of 0.1 mM DIMBOA in medium (pH 6.75) were maintained at 30, 50, and 80 C for 40, 3.5, and 0.08 hr, respectively (times equal to 10 half-lives of

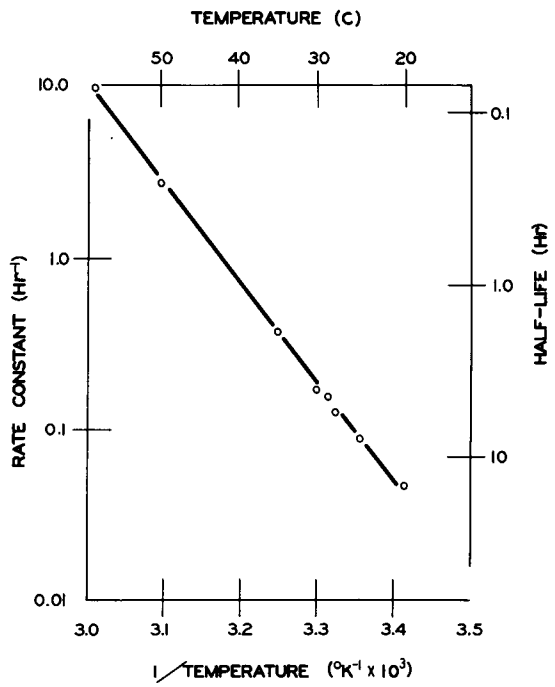

FIG. 5. Temperature dependence of DIMBOA decomposition rates in bacterial growth medium.

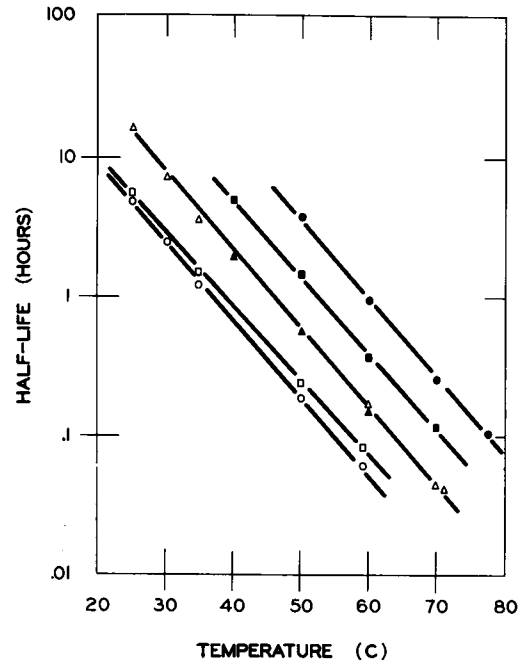

FIG. 6. Rates of DIMBOA decomposition measured at pH 5 (●), 5.5 (■), 6 (▲ and △), 7 (□), and 7.5 (○) with 10 mM succinic acid-NaOH buffers (●■▲) and 10 mM Na_2HPO_4-NaH_2PO_4 buffers (○□△).

Table I. Comparison of the GLC TMS-MBOA *vs.* UV methods (experiment 1, duplicate determinations) and the GLC acetyl-MBOA *vs.* UV methods (experiment 2, triplicate determinations) for the quantitation of MBOA.

	Starting Material (μmoles)		MBOA Found (μmoles±S.E.)	
Expt.	MBOA	DIMBOA	UV	GLC
1	0.76[a]	--	0.74 ± 0.02	0.72 ± 0.03
	0.76[b]	--	0.73 ± 0.01	0.77 ± 0.03
	--	0.95[b]	0.66 ± 0.00	0.66 ± 0.03
2	0.73[a]	--	0.72 ± 0.01	0.71 ± 0.02
	--	1.04[c]	0.70 ± 0.01	0.70 ± 0.02

[a] No incubation prior to extraction and analysis.
[b] Maintained at 37 C for 48 hr in 10 mM Na_2HPO_4-NaH_2PO_4 buffer, pH 7.0 before extraction and analysis.
[c] Held for 31 hr in 0.1 M Na_2HPO_4-NaH_2PO_4 buffer, pH 7.0, at 30 C before extraction and analysis.

Table II. Yield of MBOA (percent) from DIMBOA as affected by temperature and pH.[a]

Temp.	pH					
C	5.0[b]	5.5[b]	6.0[b]	6.0[c]	7.0[c]	7.5[c]
30	--[d]	--	--	--	72.3±0.6	73.0±0.1
40	--	62.1±0.4	68.6±1.2	68.0±0.6	70.0±0.7	70.4±0.8
50	50.0±0.6	61.4±0.4	66.6±0.7	62.2±0.4	69.2±0.4	66.8±2.8
60	51.6±0.4	61.1±0.3	64.7±1.1	63.1±0.9	67.2±0.4	67.1±0.5
70	54.6±0.7	61.0±0.1	64.0±0.9	63.4±1.4	65.3±0.5	66.8±0.4
80	56.2±0.8	60.6±2.3	64.7±1.1	61.3±1.0	64.2±0.6	64.1±0.3

a Data represent the means from 5 replicate determinations ± 2 S. E. with the appropriate correction for incomplete extraction of MBOA.
b Buffer was 10 mM succinic acid-NaOH.
c Buffer was 10 mM Na_2HPO_4-NaH_2PO_4.
d Not determined as half-life was greater than 5.5 hr.

DIMBOA at each temperature—see Fig. 5). The UV spectra of these samples taken after the appropriate incubation periods are shown in Figure 4. After extraction of the MBOA formed into ethyl acetate, the yields of MBOA, as determined by the UV procedure, were 61.2 ± 0.55, 52.5 ± 1.49, and 41 ± 1.26% at 30, 50, and 80 C, respectively. The data represent the average of three replicates (±SE). At each temperature, the molar yield of MBOA in bacterial growth medium was lower than for DIMBOA samples degraded in buffer at either pH 6 or 7 (Table II).

Effect of Ethyl Acetate Extracts of Corn on Yield of MBOA from DIMBOA. To determine the amount of MBOA present prior to degradation of DIMBOA, an ethyl acetate extract of the corn hybrid W64A × W117 was dissolved in absolute ethanol, and a portion was qualitatively analyzed for the presence of MBOA by the TMS-MBOA method. Co-chromatography showed little, if any, MBOA in the extract (less than 2% of the area of the DIMBOA peak). However, after incubation of the extract in buffer, substantial amounts of MBOA were found. A second portion of the extract was dissolved in phosphate buffer (0.1 M, pH 7), maintained at 30 C for 31 hr, extracted with ethyl acetate, and then analyzed by the acetyl-MBOA method. Peaks were observed with retention times of 3.2, 3.5, and 4.7 min. All peaks were well resolved. When a sample of acetyl-MBOA was coinjected with the acetylated corn extract, only the peak at 3.5 min (designated as peak 2) increased in size. The mass spectrum of peak 2 obtained by GLC-MS was indistinguishable from that of an acetylated sample of pure MBOA.

To determine the yield of MBOA from DIMBOA in the presence of ethyl acetate extracts of corn seedlings, portions of the extracts to which known amounts of DIMBOA had been added were resuspended in 0.1 M phosphate buffer (pH 7). The solutions were incubated for 31 hr at 30 C (≈10 half-lives). The MBOA formed was extracted into ethyl acetate and the MBOA yield was determined by the acetyl-MBOA method.

The relationship between MBOA found and DIMBOA added to corn extracts was linear (Fig. 7), with a slope of 0.744 ± 0.029 (SE). This yield of MBOA was similar to that previously found in buffer (0.723) under equivalent conditions (Table II). From the intercept in Figure 7, the amount of DIMBOA initially present in the extract was calculated to be 1.54 μmol/g fresh wt. This value was identical to that determined on the same extract by GLC of TMS derivatives of DIMBOA using an alkali flame ionization detector (5).

By the acetyl-MBOA procedure, no MBOA (less than 4 nmol/g fresh wt) was detected in extracts from the bxbx plants. Samples of these extracts to which DIMBOA was added were carried through the entire procedure, and the per cent conversion was calculated to be 70% based on the average of three replicates.

Thus as determined in buffer, yields of MBOA from added DIMBOA were 0.70 and 0.74 in the presence of ethyl acetate extracts of two lines of corn. These yields were not significantly different from the yield of MBOA in buffer alone (0.72) under the same conditions of pH, temperature, and reaction time.

Effect of Degradation on the Biological Activity of DIMBOA. If DIMBOA in corn extracts is responsible for a large portion of the inhibitory activity of these extracts toward *Erwinia* species (6), then degradation of DIMBOA prior to the addition of the bacteria should substantially reduce the inhibition. For this experiment, flasks containing 0.8 mM DIMBOA in bacterial growth medium were incubated at 28 C for 0, 5.3, 10.5, 16, and 53 hr (0, 1, 2, 3, and 10 half-lives, respectively). The contents of each flask were then filter-sterilized and diluted with fresh, sterile bacterial medium to give concentrations equivalent to 0.1, 0.2, 0.4, 0.6, and 0.8 mM DIMBOA (based on the concentration of DIMBOA prior to degradation). A suspension of bacteria was then added and the inhibition determined (Fig. 8). As expected, decreased inhibitory activity was observed in treatments with increasing preincubation time. For example, a Δ lag of 45 hr was observed for the 0.8 mM treatment with no preincubation whereas a Δ lag of only 2.4 hr was observed for the comparable 0.8 mM DIMBOA treatment which had received a 53-hr (10 half-life) preincubation.

The nature of the material(s) responsible for this residual inhibitory activity after 10 half-lives remains unknown. The activity is not due to DIMBOA, however, since <80 μM DIMBOA is not inhibitory to *E. carotovora* under these conditions (32).

Rate of Disappearance of Inhibitory Activity. For each concentration of DIMBOA, and preincubation time, a corrected inhibition (Δ lag*) was obtained by subtracting the appropriate residual (10 half-life) inhibition. The values thus obtained for the 0.4, 0.6, and 0.8 mM concentrations (DIMBOA equivalents as degraded) were then plotted (Fig. 9). The kinetics of the loss of inhibition with time was apparently first order and the apparent half-life of the inhibitory activity was about 5 hr—nearly identical to the half-life of DIMBOA (5.3 hr) actually measured under those conditions in medium without added bacteria (Fig. 5).

DISCUSSION

Those analytical procedures that measure amounts of 2,4-dihydroxy-2*H*-1,4-benzoxazin-3(4*H*)-ones by conversion to and measurement of the corresponding 2-benzoxazolinones (3, 15, 22)

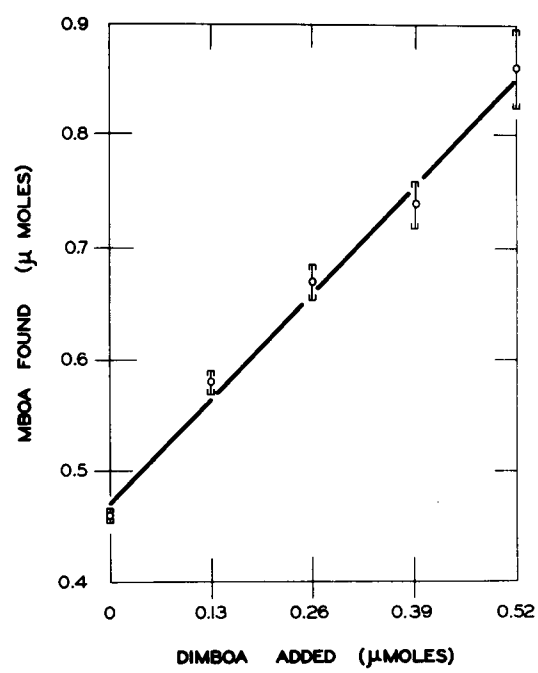

FIG. 7. Determination of MBOA yield in an ethyl acetate extract of corn. Various amounts of DIMBOA (0.0, 0.13, 0.26, 0.39, and 0.52 μmol—abscissa) were incubated with a constant amount of an extract of corn line W64A × W117 (equivalent to 410 mg fresh wt) in 2 ml of 0.1 M Na_2HPO_4-NaH_2PO_4 buffer (pH 7), at 30 C for 31 hr. MBOA was measured by the GLC acetyl-MBOA method (ordinate). Slope of the line (0.744) represents the fraction of DIMBOA converted to MBOA. Each point represents the mean of three replicates ± 2 SE (vertical lines).

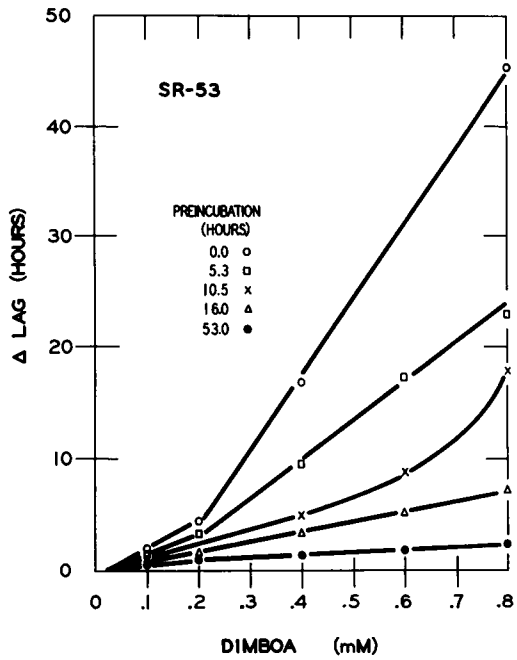

FIG. 8. Effect of preincubation of DIMBOA on the inhibition of *E. carotovora* (SR-53). Concentrations shown on the abscissa are those for the treatment prior to (or without) preincubation. Inhibition was measured as Δ lag, the difference between the time for treatments and controls to reach a cell density which gave a 100 unit reading on a Klett-Summerson colorimeter. The time required for the control to attain a turbidity of 100 Klett units was 5.4 hr.

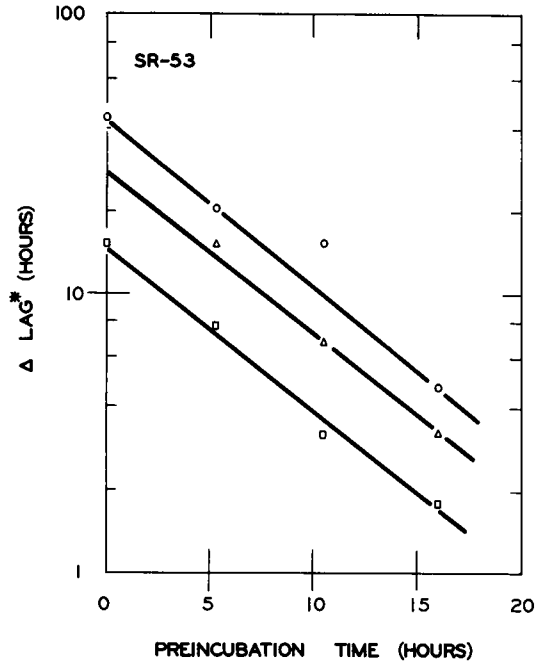

FIG. 9. Disappearance of inhibitory activity of DIMBOA as a function of preincubation time. Initial DIMBOA concentration (prior to preincubation) were: 0.8 (○), 0.6 (△), and 0.4 (□) mM. The Δ lag* values for each DIMBOA concentration are the actual values of Δ lag for that concentration less the Δ lag value after 53 hr (10 half-life) preincubation for that concentration.

are based on the assumption that the yields are quantitative (28, 29). In our studies, the yield of MBOA from DIMBOA in aqueous solution varied with temperature, pH, and the presence of uncharacterized solutes. However, under no condition that we tried was more than 75% yield of MBOA from DIMBOA obtained.

When DIMBOA decomposition was examined in various 10 mM buffers at several temperatures, the yields ranged between 50 and 73% depending on the conditions used (Table II). Yields of MBOA decreased with increasing temperature but were not strongly pH-dependent (from pH 6 to 7.5). However, yields of MBOA appeared to be pH-dependent below pH 6. When DIMBOA was incubated in bacterial growth medium (pH 6.75), the yield of MBOA decreased sharply as the temperature was increased. Since temperature dependence was much more apparent in the bacterial growth medium than in buffer, it appears that the concentration of one or more solutes altered the yield of MBOA. Initial concentrations of DIMBOA (0.1–1.6 mM) incubated in buffer (pH 7) at 30 C for 31 hr did not alter the yield of MBOA. Similarly, changing buffer concentration from 10 mM to 100 mM did not alter MBOA yield (Table I). Under all conditions examined, yields of MBOA were less than 75% of the theoretical value.

We have not tested the conditions used for conversion of DIMBOA to MBOA in other published procedures for determination of DIMBOA. We would caution, however, that estimates of DIMBOA based on the assumption of 100% conversion of DIMBOA to MBOA may be low. Tang *et al.* (24) attempted to correct for losses of MBOA incurred in their procedure, as well as for the possibility of a nonquantitative yield, by converting DIMBOA to MBOA and using the MBOA obtained in this way as the standard in their analyses for DIMBOA. Modification of their procedure to correct for degradation of standards and unknowns in the presence of the same solutes at a controlled pH may increase the accuracy and reproducibility of the method.

The UV spectra shown in Figure 3 suggest a possible reason for why Wahlroos and Virtanen (28, 29) described the conversion of DIMBOA to MBOA as quantitative. Based only on the *A* at 286 nm (absorbance maximum in water), one would conclude that 1 mol of DIMBOA yields 1 mol of MBOA (compare degraded DIMBOA [· · · · ·] to MBOA [——] in Fig. 3). However, the two spectra are not identical. Ethyl acetate extraction of the MBOA from the degradation mixture showed that the actual yield was less than 75% (compare extracted material [– – –] with MBOA [——] in Fig. 3). The residual UV absorbing material (not MBOA) remained in the aqueous phase (spectrum not shown).

The conversion of DIBOA (Fig. 1) to BOA also has been reported as quantitative (4). However, since this result was obtained only by UV spectroscopy, the conversion of DIBOA to BOA may require reexamination if it is to be used analytically for measurement of DIBOA.

Determination of cyclic hydroxamates in corn by conversion to other compounds is tedious, and, at least for DIMBOA, yields are nonquantitative (Fig. 7). For these reasons, a procedure for direct determination of 1,4-benzoxazin-3-ones would be desirable. We have performed GLC of the TMS derivative of DIMBOA (Fig. 2) and have developed a GLC procedure to measure five 1,4-benzoxazin-3-ones in corn extracts (33).

The parallel loss of DIMBOA and of inhibitory activity toward *E. carotovora* (compare Figs. 5 and 9) is taken as evidence that DIMBOA, and not some degradation product of it, is the primary inhibitor in our system. Compared to the activity of DIMBOA, the residual inhibition after 10 half-lives is relatively unimportant (Fig. 8). This may serve as a clear illustration of the importance of knowing the stability of the compound under study in the determination of its biological activity.

Prior to our study, precise information on the rate of DIMBOA degradation has not been available. Since DIMBOA and MBOA have qualitatively and quantitatively different activities in some bioassay systems, estimates of DIMBOA degradation rates can be important for interpretation of assay results, particularly in those cases where the half-life of DIMBOA is substantially less than the duration of the bioassay. In the following paper (32) we present evidence of additional factors that affect the inhibition of *Erwinia* spp. by DIMBOA.

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