Catalysis of Simazine Hydrolysis by 2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one

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Hydrolysis of simazine (2-chloro-4,6-bis-ethylamino-*s*-triazine) is catalyzed by 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (I) but not by its anion. The reaction is greater than first order in I, and this material exhibits a slight increase in extinction coefficient in the uv upon dilution. The hydrolysis may be catalyzed by molecular aggregates of I.

The presence and amounts of cyclic hydroxamic acids in certain plant species have been correlated with the tolerance of these plants to 2-chloro-*s*-triazine herbicides (Hamilton, 1964; Palmer and Grogan, 1965; Shimabukuro, 1967). Roth and Knüsli (1961) showed that simazine (2-chloro-4,6-bis-ethylamino-*s*-triazine) is detoxified *in vitro* in the presence of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one (I) and related cyclic hydroxamic acids. Hamilton and Moreland (1962) showed that the product of this reaction is hydroxysimazine (2-hydroxy-4,6-bis-ethylamino-*s*-triazine) and suggested that the cyclic hydroxamic acids act as catalysts in this hydrolysis. Other important mechanisms of detoxification of *s*-triazine herbicides have been demonstrated (Shimabukuro and Swanson, 1969).

We became interested in this reaction because of our interest in the properties of the cyclic hydroxamic acids in maize (Tipton *et al.*, 1967). The degree of structural specificity required for catalysis of this reaction (Castelfranco and Brown, 1962) is not easily explained by consideration of the functional groups of I, so we have investigated some of the physical properties of the molecule in relation to the hydrolysis of simazine. The results support the previous conclusion that the action of I is catalytic and suggest further that molecular aggregates of I may be responsible for the catalysis.

MATERIALS AND METHODS

Ring-labeled ^{14}C-simazine (7.8 μCi/mg) was supplied by Geigy Chemical Corp., Ardsley, N.Y. Ring-labeled ^{14}C-hydroxysimazine, used as a chromatographic reference compound, was obtained by shaking labeled simazine (182 μg) dissolved in $CHCl_3$ (2 ml) containing conc. HCl (0.2 ml) for 12 hr at 37° C. The $CHCl_3$ solution was extracted with water (4 ml), and the aqueous solution was evaporated to dryness *in vacuo*. The residue was dissolved in 95% ethanol (100 μl). The radiochemical purity of the simazine and hydroxysimazine was evaluated by chromatographing aliquots of the two samples on Whatman No. 1 paper with 3-methyl-1-butanol saturated with 3 *N* HCl. The radioactive spots were located by radioautography, cut out, and counted in vials containing 17 ml of dioxane scintillator (Davidson, 1962). All the detectable radioactivity in the simazine sample was present at the R_f expected for simazine, and over 97% of the radioactivity in the hydroxysimazine sample was present at the R_f expected for hydroxysimazine (Hamilton and Moreland, 1962). Approximately 1% of the radioactivity was present at the origin, and 2% was found halfway between the origin and hydroxysimazine.

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2,4-Dihydroxy-7-methoxy-1,4-benzoxazin-3-one and 2-hydroxy-7-methoxy-1,4-benzoxazin-3-one were obtained from etiolated maize seedlings as described previously (Tipton *et al.*, 1967). 6-Methoxybenzoxazolinone was provided by J. A. Klun.

Radioactive samples were counted with a Packard Model 3310 Tri-Carb scintillation counter. Brays solution (Bray, 1960) was used as the scintillator and the efficiency curve was determined by the method described by Baillie (1960).

Ultraviolet spectra were recorded using a Cary Model 15 spectrophotometer equipped with a Cary-Datex SDS-1 data recording system. Spectra of I and its 2-*o*-glucosyl derivative were recorded at several pH's in the range 4 to 9. Spectra of the free acids and the anions and pK_a's for the ionization of the hydroxamic acids were computed using the program developed by Nagano and Metzler (1967). Spectra of I at concentrations from 1.76×10^{-3} *M* to 1.76×10^{-4} *M* (in water) were recorded, and the extinction coefficients at the wavelength of maximum absorption, 264 nm, were calculated.

EXPERIMENTAL

Stability of I during the Hydrolysis of Simazine. A solution containing 2 μmoles ^{14}C-simazine and 4 μmoles I in 2 ml of water was incubated at 37° C for 15 days. The initial amount of simazine present exceeded its solubility in water about 20-fold. During the incubation nearly all the simazine dissolved. At intervals of 1, 3, 6, and 15 days after the beginning of the reaction, 20 μl samples were taken and chromatographed on thin layers of silica gel GF_{254} with 1-butanol: absolute ethanol:H_2O:conc. NH_4OH (40:10:9:1, v/v). Ultraviolet absorbing spots were marked, and radioautograms were prepared to detect radioactive materials. After 15 days a 20-μl sample was also chromatographed in cyclohexane: 2-methyl-1-propanol (8 to 2, v/v).

Effect of pH. Solutions were prepared containing 10 mμmoles of ^{14}C-simazine and 2 μmoles of I in 1 ml of 0.1 *M* citrate buffer, pH 5.0, and in 1 ml of 0.1 *M* phosphate buffer, pH 6.5. Controls without I were also prepared. After incubation 4 hr at 37° C, the solutions were lyophilized. The residues were dissolved in 0.5 ml of water, and the unreacted simazine extracted with two 0.5-ml portions in $CHCl_3$. The radioactivity in the water and in the $CHCl_3$ fractions was counted, and appropriate corrections, determined by control experiments for the solubility of hydroxysimazine in $CHCl_3$ and of simazine in water, were applied. In a second experiment, the reaction mixtures were prepared in the same way except the buffer solutions were 1 mM. At the end of the reaction, the lyophilized residues were dissolved in 95% ethanol: 0.1 *N* HCl (1 to 1, v/v) and applied to Whatman No. 1 paper for chromatography, radioautography, and counting.

Dependence of Rate of Hydrolysis on Concentration of I.

Solutions containing 10 mμmoles of ^{14}C-simazine and 0, 2, 20, 200, and 2000 mμmoles of I in water were incubated for 4 hr at 37° C, then frozen and lyophilized. The residues were chromatographed and the radioactive areas counted. In a second experiment, solutions were prepared containing 20 mμmoles of ^{14}C-simazine and 0, 0.4, and 4 μmoles I in 2 ml citrate buffer, pH 4.8. Aliquots (0.2 ml) of these solutions were transferred to 15-ml vials and incubated at 37° C. At intervals of 1 hr, vials containing the higher concentration of I were removed and lyophilized. At intervals of 10 hr, vials containing the lower concentration of I and the controls without I were removed and lyophilized. The residues were dissolved in $CHCl_3$:CH_3OH:H_2O (4:4:1, v/v) without heating and transferred to sheets of Whatman No. 1 paper for chromatography and counting of the radioactive spots.

RESULTS AND DISCUSSION

Hamilton and Moreland (1962) observed that, in a mixture of I and simazine, no change in the uv spectrum that could be attributed to the decomposition of I was observed while the conversion of simazine to hydroxysimazine was taking place, and concluded from this observation that I was a catalyst for the hydrolysis. The molar ratio of I to simazine in the reaction mixture, however, was 100 to 1, so that disappearance of I in a stoichiometric reaction would be difficult to observe, even if the product had no ultraviolet absorption. Two μmoles of ^{14}C-simazine and 4 μmoles of I were allowed to react for 15 days, by which time about 90% of the simazine had been converted to products. Under these conditions, unless the aromatic ring of I was destroyed in the reaction, products derived from I should have been easily observed on thin layers of silica gel GF_{254}. No such products were seen, and the amount of I, as judged from the appearance of the thin-layer plates, was essentially undiminished. This result supports the previous conclusion that I is a catalyst for the hydrolysis of simazine.

The hydrolysis of simazine by I is accelerated in mildly acidic conditions. In the first experiment, after 4 hr at pH 5.0, 28% of the simazine was recovered, while 62% was recovered after treatment at pH 6.5. Since the presence of relatively large amounts of buffer salts prevented a clean separation of simazine and hydroxysimazine by paper chromatography, they were separated by partitioning between water and $CHCl_3$. This procedure involves rather large corrections for the solubility of simazine in water and of hydroxysimazine in $CHCl_3$. To avoid this the experiment was repeated with more diluted buffers and separation of the products by paper chromatography (Table I). In this procedure, considerable hydrolysis of simazine occurred while the samples were being dissolved in ethanolic HCl. Although close numerical agreement in these two experiments was not obtained, the results from both methods of assay show that the hydrolysis of simazine by I proceeds more rapidly in dilute acid than near neutrality.

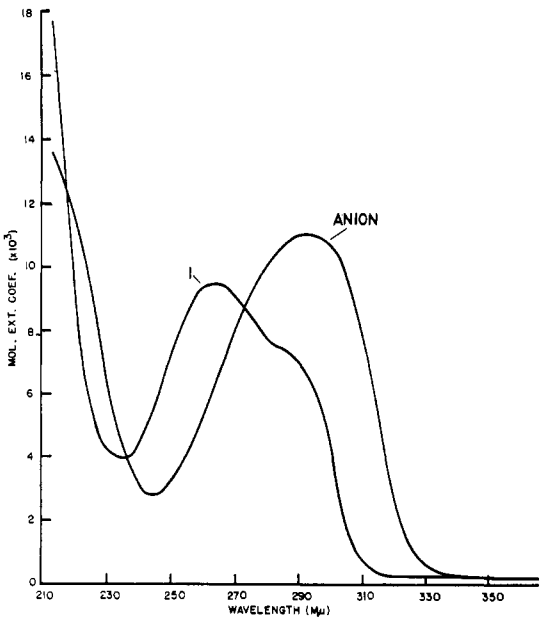
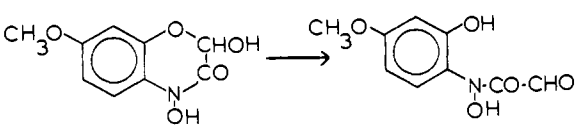


Figure 1. Absorption spectra of 2,4-dihydroxy-7-methoxy-1,4-benzoxazin-3-one and its anion

Addition of alkali to an aqueous solution of I results in a marked shift in the ultraviolet spectrum (Figure 1). The pK for this shift is 6.95. Since the 2-*o*-glucosyl derivative of I exhibits a similar shift with a pK of 6.40, the ionization responsible for the shift must be that of the hydroxamic acid group, rather than that of the phenolic group that could be formed by dissociation of the hemiacetal. Since there is



little stimulation of hydrolysis of simazine by I at a pH near the pK_a of I while at a lower pH there is marked stimulation, it seems that the anion of I does not catalyze the hydrolysis.

Incubation of simazine with I at concentrations ranging from 2 μM to 2 mM showed a dependence of the extent of simazine hydrolysis on the concentration of I (Table II), but further interpretation of the data was not possible because of extensive hydrolysis of simazine during the dissolution of the samples for chromatography. We then found that a mixture

Table I. Effect of pH on Extent of Simazine Hydrolysis by I in 1 mM Buffers

Sample	Buffer	% of Total Radioactivity: Simazine	Hydroxysimazine	Unidentified	Simazine Recovered, % of Control
Simazine[a]	...	82	15	3	100
Simazine	Citrate, pH 5.0	78	20	2	95
Simazine + I	Citrate, pH 5.0	54	45	1	66
Simazine	Phosphate, pH 6.5	70	24	6	85
Simazine + I	Phosphate, pH 6.5	71	28	1	87

[a] 10 mμmole ^{14}C-simazine dissolved and chromatographed in the same way as the lyophilized reaction mixtures.

Table II. Effect of Concentration of I on Hydrolysis of Simazine in 4 Hr

Conc. I	% Total Radioactivity: Simazine	Hydroxysimazine	Unidentified	Simazine Recovered, % of Control
0	68	25	7	100
2 μM	58	31	11	85
20 μM	60	31	9	88
0.2 mM	54	38	8	79
2 mM	27	68	5	40

Table III. Effect of Concentration of I on Rate of Hydrolysis of Simazine

Conc. I	Simazine Recovered, % of Total Radioactivity: $t \times C$, mmoles/l × hr[a] 0[b]	2	4	6	8
0	91	91	93	93	91
0.2 mM	82	68	65	72	64
2.0 mM	53	45	32	14	15

[a] Product of concentration of I and time of incubation. [b] Samples taken as soon as possible after preparation of reaction mixtures.

Table IV. Effect of Concentration on Extinction Coefficient of I

Conc. of I	Extinction Coefficient at 264 nm, 1 $mole^{-1}$ cm^{-1}
1.76×10^{-4} *M*	10,114
7.05×10^{-4}	9,759
1.06×10^{-3}	9,726
1.41×10^{-3}	9,674
1.76×10^{-3}	9,677

of $CHCl_3$, CH_3OH, and water would dissolve simazine, its hydrolysis products, and the buffer salts with much better recovery of the simazine. A study of the time-course of simazine hydrolysis by I at two concentrations differing by a factor of 10 was then performed (Table III).

The extinction coefficient of I was measured over approximately the same concentration range used in the experiment shown in Table III. As seen in Table IV, there is a small but significant increase in the extinction coefficient as the concentration of I decreases. This hyperchromism of I may be explained by analogy with that of the nucleic acids (Michelson, 1963) as resulting from the formation, at higher concentrations, of molecular aggregates with the aromatic portions of the molecules stacked like coins.

In the intact plant the cyclic hydroxamic acids occur almost entirely in the form of the 2-*o*-glucosyl derivatives (Wahlroos and Virtanen, 1964). Although we have not made any measurements on the glucoside of I, Ts'o *et al.* (1962) have shown that the nucleosides uridine and cytidine associate in aqueous solutions almost as readily as purine. This suggests that aggregates of the glucoside could be present in the intact plant.

An important aspect of the catalysis of simazine hydrolysis by I is the structural specificity involved. Castelfranco and Brown (1962) reported that simazine was not affected by a variety of phenolic materials, reducing agents, carbonyl compounds, and amines, nor by acetohydroxamic acid, the three monohydroxypyridines, and 2-hydroxy- and 8-hydroxyquinoline. Hydroxylamine and pyridine catalyzed the reaction, but no more than one-hundredth as effectively as I. A very important natural catalyst of 2-chloro-*s*-triazine hydrolysis is montmorillonite clay (Cruz *et al.*, 1968; Russell *et al.*, 1968). The triazines are adsorbed to this clay and hydrolysis results from reaction with highly dissociable adsorbed water on the clay surface. The layered structure of this clay (Buckman and Brady, 1960) may be compared with the structure that would result from extensive stacking of I in solution. Such molecular aggregates may be the catalytically active form of I, and the pH and concentration dependence of the rate of simazine hydrolysis of I may reflect the effects of these variables on the extent of association of I in solution.

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