# A New Class of Xanthine Oxidase Inhibitors Isolated from Guanidinium Salts*

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ABSTRACT: A competitive inhibitor of xanthine oxidase was isolated from commercial preparations of guanidinium salts and was shown to be ammeline. Related derivatives of *s*-triazine were also found to be potent competitive inhibitors of this enzyme. These compounds appeared to be acting as specific analogs of xanthine since they had no effect on several enzymes which act upon nonpurine substrates.

Nitroguanidine, nitrosoguanidine, and biguanide were tested as analogs of guanidine, and like guanidine all were found to be effective competitive inhibitors of xanthine oxidase. Unlike guanidine, however, they showed no inhibitory synergism with thiocyanate.

During the course of reinvestigation of the inhibition of milk xanthine oxidase by guanidinium (Fridovich, 1964), the inhibition constant for guanidinium was found not to agree with that previously reported (Rajagopalan *et al.*, 1961). This discrepancy led to the observation that the inhibition constant for guanidinium, although reproducible for any one sample of guanidinium salt, varied markedly from preparation to preparation. It was concluded that commercial preparations of guanidinium salts must contain variable amounts of an impurity which was itself a potent competitive inhibitor of xanthine oxidase. Since guanidinium salts are frequently used as protein denaturants, the chemical identity of any contaminants of these salts is a matter of some interest. In addition there was the possibility that the inhibitory impurity might represent a new class of xanthine oxidase inhibitors ultimately informative with respect to the structure of the active site of this enzyme, and possibly a new class of purine analogs of wider biochemical significance. What follows is a report of the isolation and identification of this inhibitory impurity and a description of the inhibition of xanthine oxidase by it and by related compounds.

## Experimental Procedure

Milk xanthine oxidase was purified as described previously (Fridovich, 1962). Its activity was measured spectrophotometrically in terms of the aerobic conversion of xanthine to uric acid. All assay mixtures contained $1 \times 10^{-4}$ M EDTA (Fridovich and Handler, 1962). Assays at *p*H 5.5, 7.5, and 10.0 were performed in 0.05 M sodium acetate, sodium phosphate, and sodium carbonate buffers, respectively. Kinetic studies were performed in a Gilford absorbance indicator coupled to a Beckman DU monochromator or in a Cary Model 15 spectrophotometer. The last instrument was also used to record absorbtion spectra. In all cases cell compartments were thermostated at 26°. At *p*H 5.5 and 7.5 the low Michaelis constants for xanthine (Fridovich, 1965) made it necessary to use cuvets with a 10.0 cm light path.

Rabbit liver aldehyde oxidase was generously provided by K. V. Rajagopalan and was assayed as described previously (Rajagopalan *et al.*, 1962). Infrared spectra were recorded from KBr pellets with a Perkin Elmer Model 21 infrared spectrophotometer. Elemental analysis was performed by the Galbraith Laboratory of Knoxville, Tenn. Nitroguanidine was synthesized according to Davis *et al.* (1925). Nitrosoguanidine was prepared by reduction of nitroguanidine according to Sabetta *et al.* (1935). Ammeline, ammelide, and thioammeline were products of the American Cyanamid Corp. Cyanuric acid was obtained from Fluka AG. Chemische Fabrik and biguanide sulfate from the Chemical Procurement Laboratory. Lactic dehydrogenase was prepared from rabbit muscle as described by Racker (1950) and was assayed according to Winer and Schwert (1958) at *p*H 8.1 with $NH_2OH$ added as an aldehyde-trapping agent. Acetoacetic decarboxylase was prepared from *Clostridium acetobutylicum* and assayed as previously described (Fridovich, 1963). Histidase was prepared from *Pseudomonas fluorescens* and assayed as described by Tabor and Mehler (1955).

## Results

*An Inhibitory Impurity in Guanidinium Salts.* The $K_i$ for guandinium (as the sulfate from Eastman Organic Chemicals) was recently found to be 0.10 M at *p*H 7.8 and 0.007 M at *p*H 10.0 (Fridovich, 1965), whereas the $K_i$ reported earlier was 0.006 M at *p*H 7.8 (Rajagopalan

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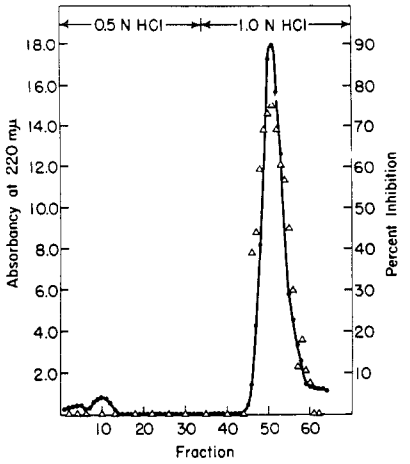


FIGURE 1: Column chromatography of insoluble residue from crude guanidinium chloride. Followed in terms of absorbancy at 220 mμ (. .) and in terms of the inhibition of xanthine oxidase at *p*H 10.0 by 0.2 ml of neutralized eluent added to 2.8 ml of reaction mixture containing $2 \times 10^{-4}$ M xanthine (Δ).

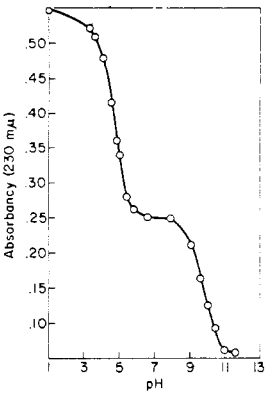


FIGURE 2: Spectrophotometric titration of the isolated inhibitor.

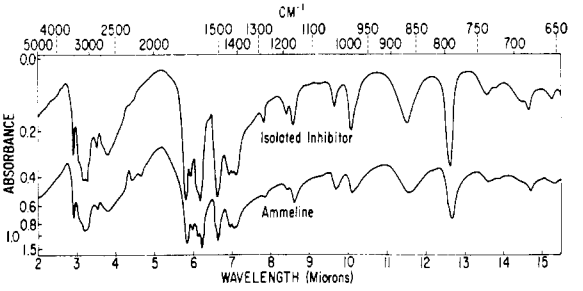


FIGURE 3: A comparison of the infrared absorption spectra of the isolated inhibitor and that of ammeline.

*et al.*, 1961). The inhibitions were of the competitive type in all cases. Since the earlier studies were done with Eastman guanidinium chloride, this same preparation was reinvestigated and the inhibition constant reported earlier (Rajagopalan *et al.*, 1961) was reproduced at *p*H 7.8 and was found to be independent of *p*H in the range 7.8–10.0.

Since 0.10 M NaCl did not enhance the inhibitory potency oft he guanidinium sulfate at *p*H 7.8 and since 0.10 M $Na_2SO_4$ did not diminish the inhibitory potency of the guanidinium chloride, it was apparent that the difference in inhibition shown by the two salts was not owing to the difference in anions but rather was caused by an impurity in the guanidinium chloride which was itself a potent competitive inhibitor of xanthine oxidase. It could further be inferred that the $K_i$ of the impurity when isolated would be found to be independent of *p*H in the range 7.8–10.0, unlike guanidinium per se, whose $K_i$ decreases by a factor of 14 in this same *p*H range.

The apparent inhibition constants for several different preparations of guanidinium salts were then determined. The richest source of the inhibitory impurity was found to be the guanidinium chloride sold by the International Chemical and Nuclear Corp. (lot 040655). This preparation was selected as the starting material for the isolation of the inhibitory impurity.

*Isolation Procedure.* Paper chromatography of the crude guanidinium chloride, using the methods of Milks and Janes (1956) and of Mazur *et al.* (1962), indicated the presence of eight components. Elution of these components from paper chromatograms followed by assaying in the enzymic system indicated that only one of these was a potent inhibitor. Attempts to dissolve 60 g of the impure guanidinium chloride in 100 ml of cold water yielded an insoluble white residue which was collected on a Buchner funnel and washed with cold water. This residue was found to be markedly enriched with respect to the inhibitory impurity and separated into only two components on paper chromatograms. The residue was dissolved in 100 ml of hot 0.5 N HCl and was run on a 2- × 20-cm column of AG-50-X8 (Bio-Rad Laboratories) which had been equilibrated with 0.5 N HCl. The column was then eluted with 680 ml of 0.5 N HCl followed by 1 liter of 1.0 N HCl. Fractions (20 ml) were assayed both in terms of their absorbtivity at 220 mμ and in terms of their ability to inhibit xanthine oxidase after careful neutralization. Figure 1 illustrates the results of this chromatographic procedure. An additional peak of ultraviolet absorbing material was eluted at higher concentrations of HCl but this was inactive in the enzymic assay and was not further investigated. Fractions 46–56 when pooled and taken to dryness *in vacuo* yielded a white powder. This was dissolved in hot 0.10 N HCl and was reprecipitated by adding ammonia to *p*H 6. After chilling overnight the white microcrystalline precipitate was collected on a Buchner funnel, washed with cold water, and then dried *in vacuo*.

*Properties of the Purified Inhibitor.* The chromatographically purified inhibitor exhibited a symmetrical absorption peak at 229 mμ in acid solution, but not in alkaline solution. A spectrophotometric titration was performed by dissolving 1.0 mg of the inhibitor in 200 ml of 0.0125 M $K_2SO_4$. Under the glass electrodel 00-ml

TABLE 1: Inhibition of Xanthine Oxidase by *s*-Triazines and Compounds of Guanidine.

| Compounds | $K_I$ | | |
|---|---|---|---|
| | *p*H 5.5 | *p*H 7.5 | *p*H 10.0 |
| Isolated inhibitor | $7.0 \times 10^{-5}$ | $1.4 \times 10^{-5}$ | $1.4 \times 10^{-5}$ |
| Ammeline | $7.0 \times 10^{-5}$ | $1.6 \times 10^{-5}$ | $1.3 \times 10^{-5}$ |
| Thioammeline | | | $1.2 \times 10^{-5}$ |
| Ammelide | $1.4 \times 10^{-5}$ | $5.6 \times 10^{-6}$ | $2.0 \times 10^{-4}$ |
| Melamine | | $3.3 \times 10^{-3}$ | $4.0 \times 10^{-3}$ |
| Cyanuric acid | | $5.2 \times 10^{-4}$ | $2.0 \times 10^{-2}$ |
| Guanidine | 0.2 | 0.1 | $7.0 \times 10^{-3}$ |
| Biguanide | | $4.0 \times 10^{-2}$ | $1.8 \times 10^{-3}$ |
| Nitroguanidine | | | $2.5 \times 10^{-5}$ |
| Nitrosoguanidine | | | $2.0 \times 10^{-4}$ |

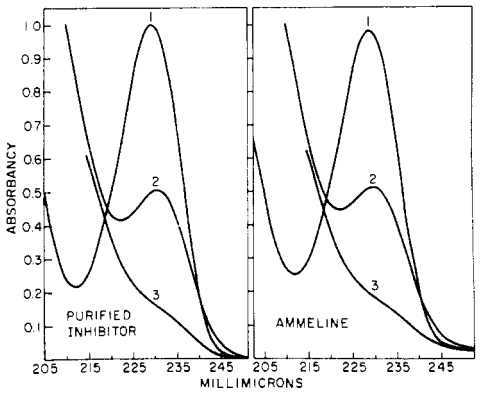


FIGURE 4: A comparison of the ultraviolet absorbtion spectra of authentic ammeline with that of the isolated inhibitor. Both at 6.67 μg/ml in 0.10 N HCl (curves 1); in 0.10 N potassium phosphate buffer, *p*H 6.5 (curves 2); and in 0.10 N KOH (curves 3).

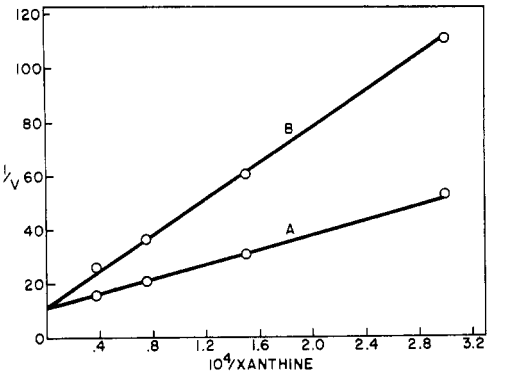


FIGURE 5: Competitive inhibition of xanthine oxidase by ammeline. Curve A, no ammeline; curve B, $2 \times 10^{-5}$ M ammeline.

aliquots of this solution were titrated with 1.197 M HCl and with 0.999 M NaOH using microburets; *p*H and absorbtion spectra were recorded after each addition of acid or alkali. Dilution effects were ignored since the maximal volume of titrant added was 0.2 ml.

Figure 2 records the effect of *p*H on the absorbancy observed at 230 mμ. It is evident from these results that the inhibitory compound bears two ionizable groups and that $pK_{a_1}$ and $pK_{a_2}$ are approximately 4.5 and 9.6, respectively. The melting point of the inhibitory compound was too high to be determined with available equipment but was estimated to be approximately 400°.

*Identification of the Inhibitory Compound.* Melamine (2,4,6-triamino-*s*-triazine), ammeline (2,4-diamino-6-hydroxy-*s*-triazine), and ammelide (2-amino-4,6-dihydroxy-*s*-triazine) are known to be produced by the procedures used in the manufacture of guanidine (Engelbrecht *et al.*, 1957). The high melting point, the infrared and ultraviolet spectra, and the solubility properties of the isolated inhibitor were consistent with its being an *s*-triazine derivative. The presence of oxygen in the isolated inhibitor was indicated by elemental analysis, and ammeline was accordingly suspected. Ammeline was prepared according to Klason (1886) and obtained from the American Cyanamid Corp. Figure 3 compares the infrared spectrum of the isolated inhibitor with that of the commerical sample of ammeline. In Figure 4 the ultraviolet absorbtion spectra of the isolated inhibitor and that of commercial ammeline are compared. Both materials were at 6.67 μg/ml in 0.10 N HCl (curve 1), 0.10 N potassium phosphate buffer, *p*H 6.5 (curve 2), and in 0.10 N KOH (curve 3). The spectra in Figure 4 agree with the known ultraviolet absorbtion spectrum of ammeline (Hirt and Schmitt, 1958). In addition the known dissociation constants for ammeline (Hirt and Schmitt, 1958) are identical with these found for the isolated inhibitor and, as shown in Table I, the inhibition constants for the isolated inhibitor were identical to those of ammeline at *p*H 5.5, 7.5, and 10.0. Elemental analysis was also consistent with the identification of the isolated inhibitor as ammeline.

*Anal.* Calcd. for $H_2NCNC(NH_2)NC(OH)N$: C, 28.3

H, 3.9; N, 55.0; O, 12.6. Found: C, 28.18, 28.29; H, 4.07, 4.10; N, 54.98, 55.10; O, 12.81, 12.83.

The slight excess of hydrogen and oxygen found over that calculated for ammeline probably relates to a small amount of tightly bound water of hydration.

*Inhibition of Xanthine Oxidase by Ammeline and Related Compounds.* Ammeline and several related compounds were investigated as inhibitors of xanthine oxidase over a wide range of *p*H. In all cases where inhibition was observed it was of the formally competitive type. Typical of the data obtained was that for ammeline at *p*H 10.0 illustrated in Figure 5. Table I summarizes all of this data. Biguanide, nitroguanidine, and nitrosoguanidine were tested as analogs of guanidine. It is of interest that whereas guanidinium per se exhibited a marked inhibitory synergism with thiocyanate (Fridovich, 1965), none of these related compounds did so. Ammeline was also competitive with respect to the oxidation of *N*-methylnicotinamide by xanthine oxidase at *p*H 10.5 (Greenlee and Handler, 1964). In contrast, the oxidation of *N*-methylnicotinamide by the rabbit liver aldehyde oxidase was not at all inhibited by ammeline or by nitroguanidine. Other enzymes found unaffected by ammeline and by nitroguanidine included the lactic dehydrogenase of rabbit muscle, the acetoacetic decarboxylase of *Cl. acetobutylicum*, and the histidase of *Ps. fluorescens*.

## Discussion

The discrepancy between the various reported inhibition constants for guanidinium (Fridovich, 1965; Rajagopalan *et al.*, 1961) has been shown to relate to the presence of an impurity in guanidinium salts which impurity is itself a potent competitive inhibitor of xanthine oxidase. This inhibitory impurity was isolated and was shown to be identical to ammeline by several lines of evidence. Thus the isolated compound and synthetic ammeline exhibited identical spectra in the ultraviolet and in the infrared regions of the spectrum, they had identical acid dissociation constants, they exhibited identical inhibition constants in the xanthine oxidase system at *p*H 5.5, 7.5, and 10.0, and as required by the behavior of the impure guanidinium chloride the $K_I$ of ammeline and of the isolated inhibitor was unaffected by *p*H in the range 7.5–10.0.

Ammeline, ammelide, thioammeline, and nitroguanidine are effective competitive inhibitors of xanthine oxidase with inhibition constants in the range $10^{-6}$–$10^{-4}$ M. Ammeline did not serve as a substrate for xanthine oxidase at *p*H 10.0, as judged by the failure of the enzyme to effect any change in the ultraviolet spectrum of ammeline. Comparison of the *p*H range of maximum effectiveness as inhibitors with the known $pK_a$ values of these compounds (Hirt and Schmitt, 1958) indicates that ammeline inhibits as the neutral or monovalent anionic form, ammelide as the monovalent anion, and cyanuric acid as the monovalent anion. It has been shown that the monovalent anion is the form of xanthine preferred by the enzyme (Fridovich, 1965). This charge similarity increases the likelihood that the *s*-triazines can act as xanthine analogs.

Since biguanide inhibited much as did guanidine, yet exhibited no synergism with thiocyanate, we may conclude that opposite charge, although probably a required condition for synergism with thiocyanate, is not a sufficient one. Several enzymes which act upon nonpurine substrates were unaffected by ammeline and related compounds. These compounds are therefore not general enzyme inhibitors and may indeed be purine analogs in a specific sense.

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