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STUDIES ON THE MECHANISM OF INHIBITION OF XANTHINE OXIDASE BY 5-DIAZOIMIDAZOLE-4-CARBOXAMIDE AND RELATED THIOAZOIMIDAZOLE CARBOXAMIDES

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Abstract—The mechanism of inhibition of milk xanthine oxidase by 5-diazoimidazole-4-carboxamide (diazo-ICA) and by five related thioazoimidazole carboxamides (thioazo-ICAs) was studied. The extent of inhibition of xanthine oxidase by diazo-ICA and thioazo-ICAs decreased greatly when these compounds were preincubated in a buffer before the addition of substrate and enzyme. In 0·1 M Tris–HCl buffer, pH 7·5, thioazo-ICAs were converted to 2-azahypoxanthine, a cyclized product of diazo-ICA, which inhibits xanthine oxidase slightly. The inhibition of xanthine oxidase by thioazo-ICAs is probably due to this diazo-ICA. With xanthine as a variable substrate, diazo-ICA caused uncompetitive, irreversible inhibition. The inhibition of xanthine oxidase by diazo-ICA was reduced by simultaneous addition of a sulfhydryl compound, such as cysteine, cysteamine or reduced glutathione, but not other amino acids. Diazo-ICA inactivated the enzyme more significantly and rapidly than other sulfhydryl reagents. The inhibitory activity of diazo-ICA was potentiated strongly by Fe^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+}, and slightly by Mo^{5+}. Treatment of milk xanthine oxidase with diazo-ICA changed the absorption spectrum of the enzyme.

ALTHOUGH 5-diazoimidazole-4-carboxamide (diazo-ICA) is unstable in an aqueous solution,[1] it is reported to have various types of biological activity. It has been reported to have antineoplastic activity, inhibiting human epidermoid carcinoma (H.Ep.-2) cells in culture,[1] subcutaneous Walker carcinoma 256,[1] and Ehrlich ascites carcinoma.[1,2] It has also some antimicrobial activity on *Mycobacterium tuberculosis*.[3] Moreover, it completely inhibits the growth of *Escherichia coli in vitro*[4,5] at low concentrations by inhibiting DNA synthesis and is highly inhibitory to growth of *Bacillus subtilis*.[6,7] In the third type of action, both the diazo compound and thioazo derivatives prepared from it, markedly inhibit the activities of xanthine oxidase (xanthine–oxygen oxidoreductase, EC 1.2.3.2) and uricase (urate–oxygen oxidoreductase, EC 1.7.3.3).[8,9] This report describes the mechanism of inhibition of xanthine oxidase by diazo-ICA and related thioazo compounds.

MATERIALS AND METHODS

Materials. Bovine milk xanthine oxidase was prepared by the method of Klenow and Emberland.[10] Diazo-ICA and 2-azahypoxanthine monohydrate were synthesized by the method of Shealy *et al.*[1,11] 5-Thioazoimidazole-4-carboxamides (thioazo-ICAs) were prepared by reactions coupling diazo-ICA with the corresponding mercapto compounds. Allopurinol was kindly supplied by Mr. M. Takahashi of Kojin Co., Ltd. Other reagents were obtained from commercial sources.

Enzyme assay. Xanthine oxidase activity was determined as previously reported,[9] assay mixtures contained 0·1 M Tris–HCl buffer (pH 7·5); 60 μM xanthine (monosodium salt); 0·1 mM EDTA (disodium salt); various concentrations of drugs (when appropriate), and 0·4 ml of the enzyme (15 μg protein; sp. act. 0·68 U/μg protein) in a final volume of 4·0 ml. Diazo-ICA and its thioazo derivatives are unstable in an aqueous solution, so reactions were started by addition of the enzyme immediately after adding a freshly prepared solution of the test drug. Enzyme activity was measured by the increase in E_{293} due to formation of uric acid from xanthine during a 10-min period at 25° using a Shimadzu QV-50 spectrophotometer. One unit of enzyme activity is defined as that catalyzing the synthesis of 1 nmole of uric acid/min at 25°. Xanthine was omitted from blanks. Protein was determined by the method of Lowry *et al.*[12] using bovine serum albumin as a standard.

Absorption spectra of thioazo-ICAs and milk xanthine oxidase. Ultraviolet absorption spectra of thioazo-ICAs and the visible absorption spectrum of milk xanthine oxidase were recorded with a Shimadzu MPS-50L recording spectrophotometer.

RESULTS

Active forms of thioazo-ICAs for inhibition of xanthine oxidase. As reported previously,[8,9] diazo-ICA and five thioazo derivatives from it, inhibited milk xanthine oxidase more effectively than allopurinol *in vitro*. In an aqueous solution, over a wide range of pH-values, diazo-ICA cyclized readily to 2-azahypoxanthine,[1] which is a weak inhibitor of xanthine oxidase.[8,9,13] Therefore, the inhibitory activity of diazo-ICA decreased rapidly with time on preincubation in buffer before addition of the substrate and enzyme (unpublished data). As illustrated in Fig. 1, the inhibitory acti-

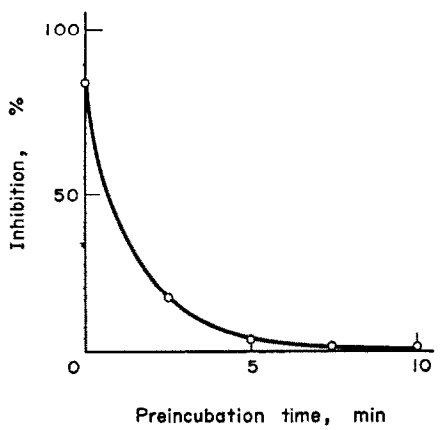

FIG. 1. Effect of preincubation on inhibition of xanthine oxidase by aminocarboxyethylthioazo-ICA. Aminocarboxyethylthioazo-ICA, $2{\cdot}12 \times 10^{-6}$ M, was preincubated at 25° in a total volume of 3·2 ml containing 0·125 M Tris–HCl buffer (pH 7·5) and 0·125 mM EDTA. Then residual inhibitory activity of this compound on xanthine oxidase was measured by addition of 0·4 ml of substrate (0·6 mM) and 0·4 ml of enzyme (15 μg protein) at the indicated times.

vity of 5-(2-amino-2-carboxyethylthioazo)imidazole-4-carboxamide (aminocarboxyethylthioazo-ICA), a thioazo-ICA, also decreased with time on preincubation and the percentage inhibition reached zero after preincubation for 5 min. The inhibitory

activities of four other thioazo-ICAs also showed a similar decrease on preincubation. Figure 2 shows the conversion of aminocarboxyethylthioazo-ICA to 2-azahypoxanthine in 0·1 M Tris–HCl buffer (pH 7·5) in light at 25°. This compound decomposed

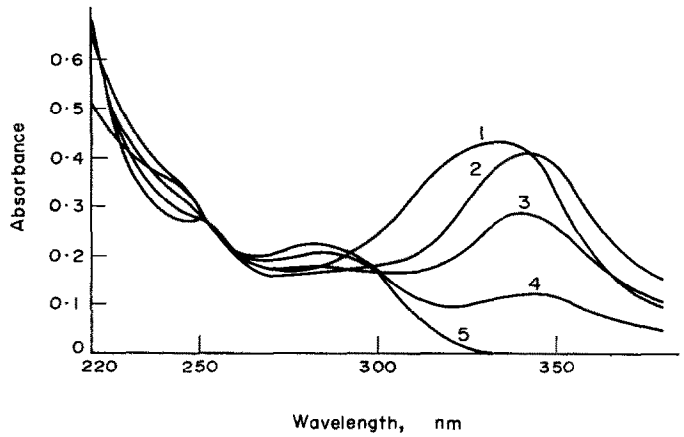

FIG. 2. Conversion of aminocarboxyethylthioazo-ICA to 2-azahypoxanthine. The absorption of 5×10^{-5} M aminocarboxyethylthioazo-ICA in 0·1 M Tris–HCl buffer (pH 7·5) was recorded against buffer at 25°. Cysteine or cystine has only slight absorption below 290 nm. Curve 1, 1 min; curve 2, 30 min; curve 3, 2 hr; curve 4, 6 hr; curve 5, 24 hr after addition of solvent to the compound. Curve 5 was identical with the absorption spectrum of 2-azahypoxanthine.

with time and after 24 hr its spectrum became identical to that of 2-azahypoxanthine. This observation seems to indicate that aminocarboxyethylthioazo-ICA is first converted to diazo-ICA, which then cyclizes to 2-azahypoxanthine. Other thioazo-ICAs also decomposed in a similar manner. These findings suggest that the inhibition of xanthine oxidase by thioazo-ICAs is probably due to the formation of diazo-ICA. In the following experiments we used diazo-ICA in studies on the mechanism of inhibition of the enzyme by these compounds.

Type of inhibition of xanthine oxidase by diazo-ICA. Diazo-ICA caused a 30 per cent

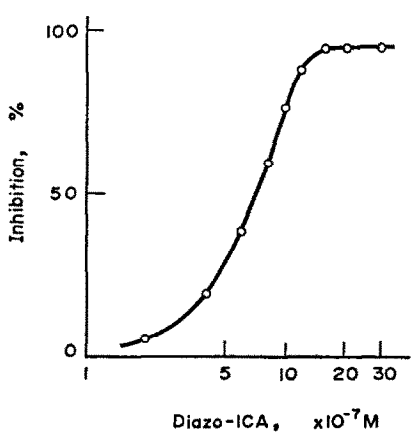

FIG. 3. Inhibition of xanthine oxidase by diazo-ICA.

inhibition of milk xanthine oxidase at a concentration of 5×10^{-7} M, 79 per cent at 1×10^{-6} M and nearly 100 per cent at above $1{\cdot}5 \times 10^{-6}$ M (Fig. 3).

Lineweaver–Burk plots[14] showed that diazo-ICA caused uncompetitive inhibition, in which the inhibitor attacked the enzyme–substrate complex, with xanthine as a variable substrate (Fig. 4). The K_m-value for xanthine and the K_i-value for diazo-ICA

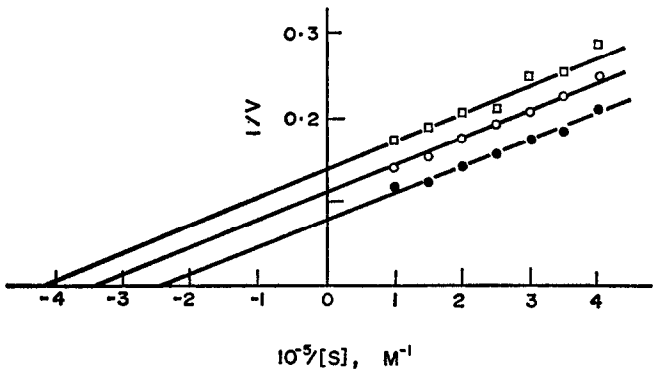

FIG. 4. Lineweaver–Burk plots of inhibition of xanthine oxidase by diazo-ICA. Enzyme activity was assayed as described in Materials and Methods except that the reaction time was 1·5 min. ●, No inhibitor; ○, 4×10^{-7} M diazo-ICA; □, 6×10^{-7} M diazo-ICA.

were calculated to be $4{\cdot}2 \times 10^{-6}$ M and $9{\cdot}3 \times 10^{-7}$ M, respectively. We constructed Ackermann–Potter plots[15] of the data to examine the reversibility of inhibition of xanthine oxidase by diazo-ICA. As shown in Fig. 5, the inhibition was irreversible.

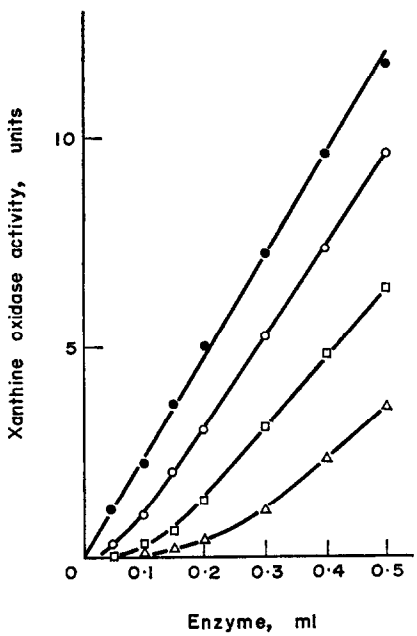

FIG. 5. Ackermann–Potter plots of inhibition of xanthine oxidase by diazo-ICA. Enzyme activity was assayed as described in Materials and Methods except that the amount of enzyme added was varied. ●, No inhibitor; ○, 4×10^{-7} M diazo-ICA; □, 7×10^{-7} M diazo-ICA; △, 1×10^{-6} M diazo-ICA.

This irreversibility was demonstrated by dialysis of the enzyme after its inactivation by diazo-ICA in the presence of substrate, i.e., the percentage inhibition did not change on dialysis for 6 hr at 4° against 300 vol. of 0·1 M Tris–HCl buffer (pH 7·5).

Site of the inhibitory action of diazo-ICA. Many reports[16–22] have stated that various sulfhydryl reagents inhibit milk xanthine-oxidase and that thiol groups are important in the enzyme reaction. Yamamoto[5] and Iwata *et al.*[23–25] reported that sulfhydryl groups were involved in the mechanisms of some biological activities of diazo-ICA. We therefore, tested to see whether these groups of the enzyme took part in the inhibitory action of diazo-ICA. When a sulfhydryl compound, such as cysteine, cysteamine or reduced glutathione, was added to the assay mixture simultaneously with diazo-ICA, the inhibitory activity of diazo-ICA was reduced (Fig. 6). Other

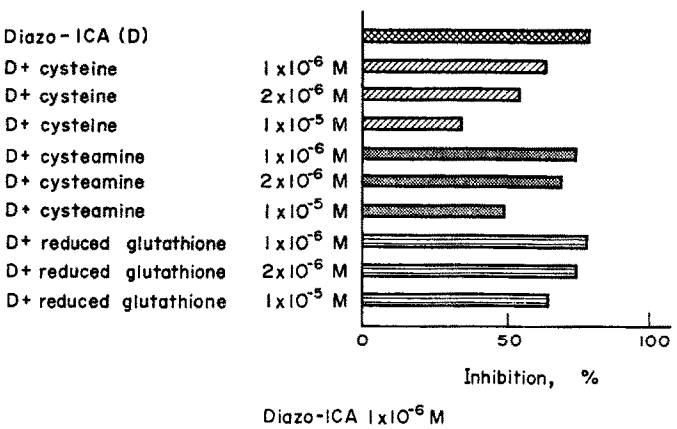

FIG. 6. Protection of xanthine oxidase by sulfhydryl compounds from inhibition by diazo-ICA. Sulfhydryl compounds were added to the reaction mixture simultaneously with diazo-ICA. Percentage inhibition was determined relative to the activity of the enzyme in the absence of diazo-ICA and sulfhydryl compounds. Sulfhydryl compounds at these concentrations had no effect on milk xanthine oxidase activity.

amino acids had no effect on the inhibition by diazo-ICA at the same concentrations. Sulfhydryl compounds also protected xanthine oxidase against inactivation by thioazo-ICAs. Inhibition by diazo-ICA could not be reversed by subsequent addition of excess cysteine.

Comparison of the inhibitory actions of diazo-ICA and other sulfhydryl reagents. The inhibitory action of diazo-ICA was compared with those of other sulfhydryl reagents. At a concentration of 1×10^{-6} M, diazo-ICA caused 79 per cent inhibition, while *p*-chloromercuribenzoate (PCMB) and *o*-iodosobenzoate caused 19 and 16 per cent inhibition, respectively, and *N*-ethylmaleimide (NEM) and iodoactamide caused little or no inhibition (Table 1). Therefore, of these sulfhydryl reagents, diazo-ICA seems to be the strongest inhibitor of xanthine oxidase.

Figure 7 shows that inhibitions by NEM, *o*-iodosobenzoate and PCMB increased with time, but that by Diazo-ICA was maximal immediately after its addition, so diazo-ICA probably inactivates xanthine oxidase very rapidly.

TABLE 1. INHIBITION OF XANTHINE OXIDASE BY DIAZO-ICA AND SULFHYDRYL REAGENTS

Inhibitor	Inhibition (%) conc. (M)					
	1×10^{-6}	1×10^{-5}	5×10^{-5}	1×10^{-4}	5×10^{-4}	1×10^{-3}
Diazo-ICA	79	96				
o-Iodosobenzoate	16	78	94	96		
PCMB	19	69	91	94		
NEM				34	76	93
Iodoacetamide				4	13	25

Effect of metal ions on the inhibition of xanthine oxidase by diazo-ICA. Xanthine oxidase is known to contain three cofactors, FAD, molybdenum and iron[26] and besides sulfhydryl groups these cofactors may be involved in the mechanism of inhibition by diazo-ICA. FAD, when added to the assay mixture at a concentration of 5×10^{-6} M simultaneously with diazo-ICA, had no effect on the inhibitory activity of the latter. Other nucleotides, such as FMN, NAD and ATP, also had no effect at the same concentration.

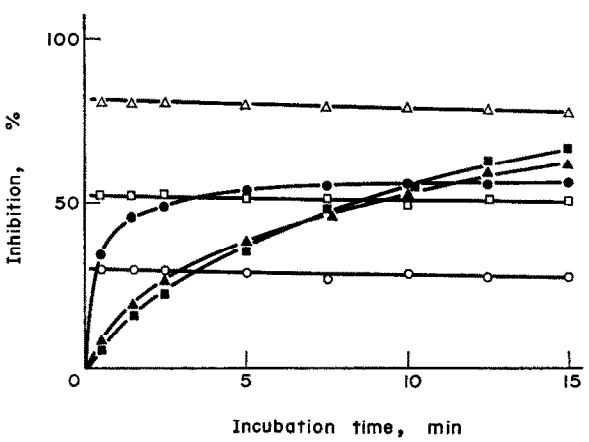

FIG. 7. Comparison of inhibitions of xanthine oxidase by diazo-ICA and sulfhydryl reagents. ○, 5×10^{-7} M diazo-ICA; □, 8×10^{-7} M diazo-ICA; △, 1×10^{-6} M diazo-ICA; ●, 5×10^{-6} M PCMB; ■, 4×10^{-6} M *o*-iodosobenzoate; ▲, $2{\cdot}5 \times 10^{-4}$ M NEM.

Unexpectedly, Fe^{2+} greatly potentiated the inhibitory activity of this drug (Fig. 8). The dose–response curve of this potentiation is shown in Fig. 9. Fe^{3+} had scarcely any effect and Mo^{5+} caused slight potentiation. Mn^{2+}, Co^{2+} and Cu^{2+} potentiated the inhibitory activity of diazo-ICA to the same extent as Fe^{2+}. Ni^{2+}, Cd^{2+}, Mg^{2+} and Ca^{2+} and other metal ions, such as Zn^{2+}, Ba^{2+}, Sn^{2+}, Sr^{2+}, Cr^{3+} and Sn^{4+}, showed no effect at a tested concentration. The inhibitory activities of thioazo-ICAs were also greatly potentiated by Fe^{2+}, but not by Fe^{3+} (unpublished data). These observations provide further evidence that the inhibitory activities of thioazo-ICAs

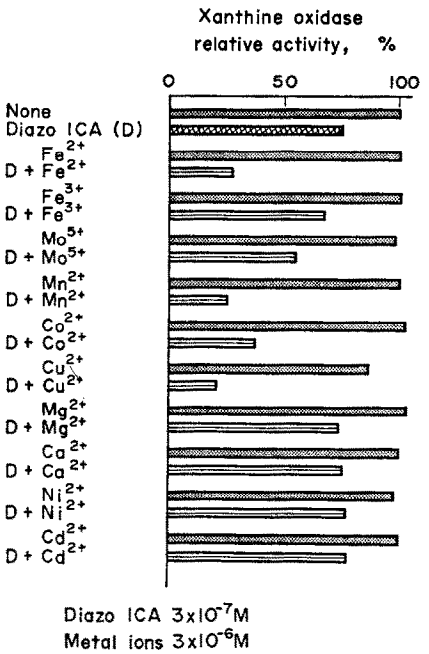

FIG. 8. Effect of metal ions on inhibition of xanthine oxidase by diazo-ICA. Metal ions were added to the reaction mixture simultaneously with diazo-ICA. EDTA was not added to the reaction mixture. The enzyme activity is expressed relative to the activity in the absence of diazo-ICA and metal ions.

are due to formation of diazo-ICA. Fe^{2+} and Fe^{3+} (1×10^{-5} M or 1×10^{-4} M) had no effect on the inhibition of xanthine oxidase by allopurinol, 2-azahypoxanthine, PCMB, NEM or iodoacetamide. In the experiments on the effects of metal ions EDTA was omitted. The percentage inhibition of xanthine oxidase activity by diazo-ICA was 10 to 15 per cent more in the absence of EDTA than in its presence. In the absence of diazo-ICA, enzyme activity was the same in the presence and absence of EDTA.

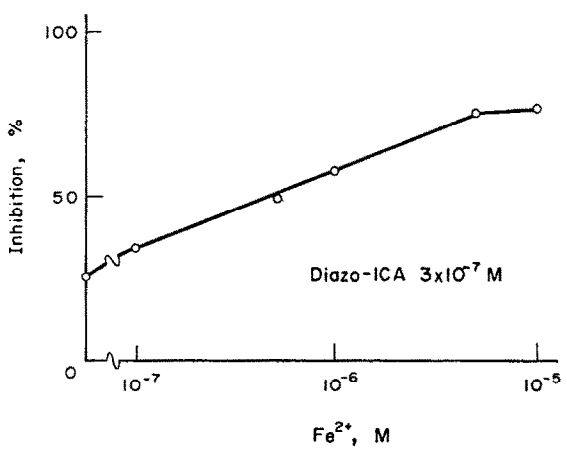

FIG. 9. Potentiation of diazo-ICA inhibition of xanthine oxidase by Fe^{2+}. Conditions were as for Fig. 8. Percentage inhibition was determined relative to the activity of the enzyme in the absence of diazo-ICA and Fe^{2+}. Fe^{2+} at these concentrations had no effect on enzyme activity.

Alteration in the visible absorption spectrum of milk xanthine oxidase on treatment with diazo-ICA. Some inhibitors and substrates of xanthine oxidase are known to alter the absorption spectrum of the enzyme.[26–31] Figure 10 shows that treatment of

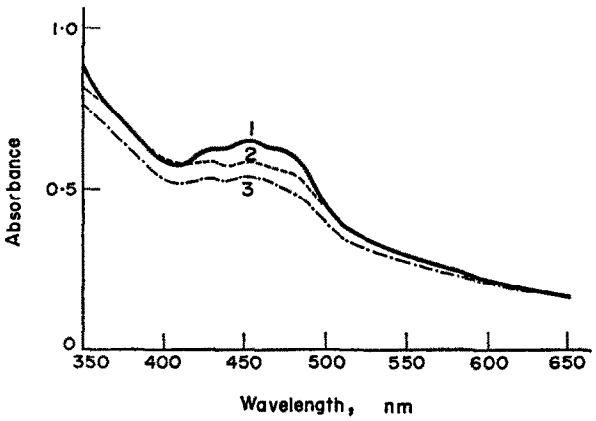

FIG. 10. Effect of diazo-ICA on the absorption spectrum of milk xanthine oxidase. The enzyme (14·2 mg protein) was allowed to react with 2×10^{-4} M diazo-ICA or 1×10^{-3} M hypoxanthine in 0·1 M phosphate buffer (pH 7·0) at 25° in a final volume of 4·0 ml and the absorption spectrum was recorded against buffer. Diazo-ICA has slight absorption near 350 nm, but 30 min after dissolving it in buffer this absorption became negligible due to its cyclization to 2-azahypoxanthine. The oxidation product of 2-azahypoxanthine by xanthine oxidase has slight absorption near 350 nm. Hypoxanthine, xanthine and uric acid have no absorption at these wavelengths. Curve 1, unreacted enzyme; curve 2, 30 min after addition of 2×10^{-4} M diazo-ICA; curve 3, immediately after addition of 1×10^{-3} M hypoxanthine.

milk xanthine oxidase with diazo-ICA caused a change in the absorption spectrum of the enzyme: the absorbance between 410 and 515 nm decreased. This spectral alteration resulting from treatment with diazo-ICA did not change on standing milk xanthine oxidase for 5 hr, suggesting that it was irreversible, while a spectral change of the enzyme caused by reduction with hypoxanthine was reversible. 2-Azahypoxanthine, allopurinol and PCMB (2×10^{-4} M) did not change the absorption spectrum of milk xanthine oxidase under the same conditions.

DISCUSSION

Diazo-ICA is unstable in an aqueous solution and cyclizes rapidly with ring-fusion to the isomer, 2-azahypoxanthine,[1] which is an analog of hypoxanthine. Therefore, Iwata *et al.*[8,9] examined the effect of diazo-ICA on purine catabolism and found that it strongly inhibited xanthine oxidase and uricase. 2-Azahypoxanthine was found to inhibit uricase strongly,[9] and xanthine oxidase weakly.[8,9,13] This suggests that diazo-ICA itself inhibits xanthine oxidase activity.

On preincubation in the absence of xanthine oxidase and substrate the inhibitory activity of diazo-ICA decreased with time (unpublished data). Figure 1 shows that the inhibitory activity of aminocarboxyethylthioazo-ICA also decreased on preincubation before addition of the substrate and enzyme. We found that thioazo-ICAs decomposed to yield diazo-ICA and were ultimately converted to 2-azahypoxanthine and that the inhibitory activities of the thioazo compounds did not exceed that of diazo-ICA.

These findings suggest that the inhibitory actions of thioazo-ICAs on xanthine oxidase may be attributable to that of diazo-ICA.

The possibility of the reaction of diazo-ICA with sulfhydryl groups was suggested by the fact that various biological activities of diazo-ICA were inactivated by addition of mercapto compounds.[5,23–25] In the present work we found that sulfhydryl compounds, such as cysteine, cysteamine and reduced glutathione, reduced the inhibition of xanthine oxidase by diazo-ICA, but other amino acids did not. These results and the finding that various sulfhydryl reagents inhibit the enzyme[16–22] suggest that the inhibitory effect of diazo-ICA on xanthine oxidase may be related to its reactivity with thiol groups. Bray and Watts[21] showed that the initial reaction of iodoacetamide with milk xanthine oxidase probably involved the reaction of 1 thiol group/mole of enzyme and they discussed possible mechanisms for the apparent exposure of this essential thiol group when xanthine oxidase was reduced by xanthine, salicylaldehyde or dithionite. They reported that the initial rate of the reaction with iodoacetamide was followed by a slower phase, during which about 12 thiol groups/mole of enzyme reacted. Fridovich and Handler[18] found that, in the presence of substrate, the enzyme was rapdily inactivated by PCMB. Green and O'Brien[22] reported that the presence of xanthine was required for the inhibition of bovine milk xanthine oxidase by *o*-iodosobenzoate, iodoacetamide, hydrogen peroxide or PCMB. We found that the inhibition of xanthine oxidase by diazo-ICA was uncompetitive, that is, that diazo-ICA inhibited this enzyme only in the presence of the substrate at concentrations used in this inhibition experiment. Diazo-ICA caused stronger and more rapid inhibition of xanthine oxidase than other sulfhydryl reagents. This inhibition was irreversible, even by excess cysteine. However, simultaneous addition of cysteine partially blocked the inhibition.

A metal ion is important in the biological activities of diazo-ICA. Yamamoto and Iwata[32] showed that Ca^{2+} was required for release of 5-hydroxytryptamine from rabbit platelets by diazo-ICA and Yamamoto *et al.*[33] found that activation of monoamine oxidase by diazo-ICA was greater in the presence of Ca^{2+} than in its absence. In this work some metal ions, such as Fe^{2+}, Mn^{2+}, Co^{2+} and Cu^{2+}, markedly potentiated the inhibitory activity of diazo-ICA on xanthine oxidase. The mechanisms of these potentiations are unknown. Fe^{2+}, and possibly other metal ions, did not seem to stabilize the diazomidazole in an aqueous solution, because the inhibitory effect of diazo-ICA decreased to the same extent on preincubation in the presence and absence of Fe^{2+}.

Treatment of milk xanthine oxidase with diazo-ICA produced a spectral alteration of the enzyme. 2-Azahypoxanthine is known to serve as a substrate of this enzyme,[13] but it did not alter the absorption spectrum of the enzyme under the same conditions. Therefore, this action of diazo-ICA is not due to 2-azahypoxanthine formed from it, but it is uncertain whether the presence of 2-azahypoxanthine is required in this action of diazo-ICA. Diazo-ICA inhibited xanthine oxidase uncompetitively, at least at low concentrations (below 1×10^{-6} M) used in this experiment. However, higher concentrations of diazo-ICA (above 1×10^{-6} M) caused inhibition after preincubation with the enzyme in the absence of xanthine, but not after preincubation in the absence of both enzyme and xanthine. Further studies are required on whether higher concentrations of diazo-ICA inactivate xanthine oxidase even in the absence of substrate, because 2-azahypoxanthine serves as a substrate of the enzyme. These findings suggest that there is some relationship between the spectral alteration and the inhibition of xanthine oxidase by diazo-ICA in the absence of xanthine.

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