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COPPER(II)ETHYLENEDIAMINETETRAACETATE DOES DISPROPORTIONATE SUPEROXIDE

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SUMMARY. The superoxide dismutase (SOD) mimetic reactivity of Cu(II)EDTA was studied in the pH range of 6.0 to 8.0. Cu(II)EDTA disproportionated superoxide without inhibiting superoxide production by xanthine oxidase, as a result of bonding sites becoming available on the copper complex with increasing acidity. This disproportionation by Cu(II)EDTA is offered as evidence that the addition of EDTA to biological preparations for the purpose of complexing copper and thereby inhibiting copper-dependent superoxide disproportionation and promoting superoxide-dependent reactions is not a valid practice.

Interest in copper containing cupreins as protective enzymes arose after the report of McCord and Fridovich (1) that a cuprein had superoxide dismutase (SOD) reactivity. Mann and Keilin (2), Markowitz et al. (3), Kimmel et al. (4), and Porter and Folch (5) had isolated cupreins but McCord and Fridovich were the first to show that erythrocuprein disproportionated superoxide according to the following equation:

$$O_2^- + O_2^- + 2H^+ \longrightarrow O_2 + H_2O_2$$

It has also been shown that many small molecular weight copper complexes (6,7,8,9) as well as inorganic copper(II) compounds (10,11) disproportionate superoxide in a manner similar to superoxide dismutase. The suggestion that

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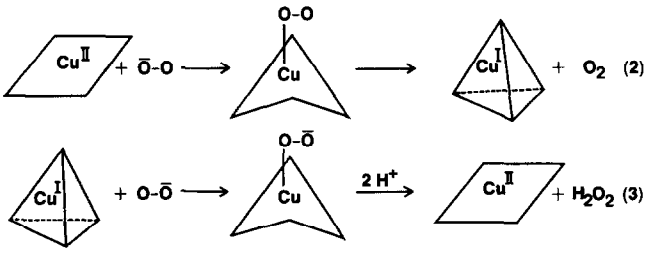
Cu(II)EDTA did not disproportionate superoxide was originally offered by McCord and Fridovich (1) and subsequently promulgated by Fridovich (12). Consequently, EDTA is commonly added to biological systems to scavenge inorganic copper or loosely bonded copper in order to prevent its disproportionation of superoxide (11,14).

The notion that Cu(II)EDTA does not disproportionate superoxide appeared somewhat unusual since many copper complexes show this reactivity. It is known that superoxide disproportionating complexes must have available sites for superoxide bonding as illustrated in the following reaction sequences:



Such sites would become available on Cu(II)EDTA under acidic conditions as depicted in Figure 1; therefore, this complex was investigated for Cu-ZnSOD-mimetic reactivity in the 8 to 6 pH range.

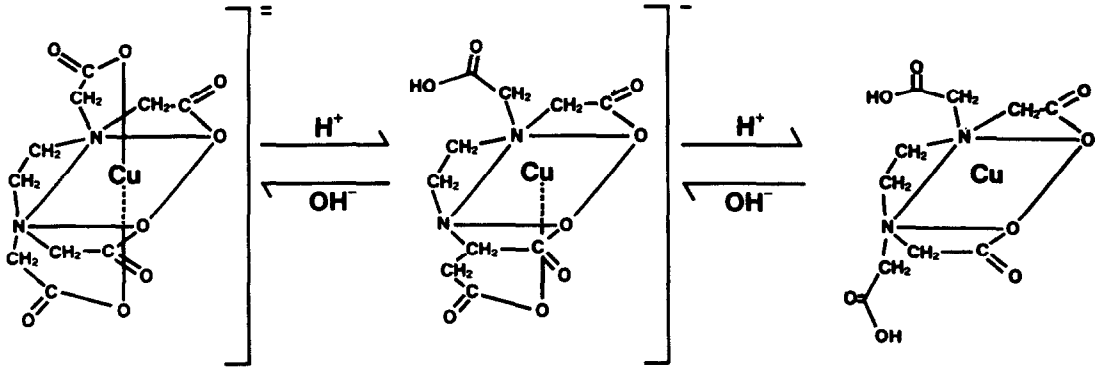


Figure 1. Influence of hydrogen ion concentration on the structure of Cu(II)EDTA.

To examine the possibility that the observed pH related change in Cu-ZnSOD-mimetic reactivity of Cu(II)EDTA might be due to inhibition of the reduction of oxygen to superoxide and the concomitant conversion of xanthine to uric acid by xanthine oxidase, uric acid synthesis was measured under the same conditions used to determine Cu-ZnSOD-mimetic reactivity.

MATERIALS AND METHODS

Spectrophotometric assays were done with a Shimadzu Spectronic 200 uv-visible spectrophotometer. Beef liver catalase was purchased from ICN Pharmaceuticals Inc., NBT was purchased from Aldrich Chemical Co., xanthine oxidase (0.52 U/10 g protein) and xanthine (Grade V) was purchased from the Sigma Chemical Co. $CuSO_4$ was purchased from the Baker Chemical Co., Na_2H_2EDTA was purchased from Alpha, and Cu-ZnSOD was purchased from Diagnostic Data Inc.

The Cu(II)EDTA was synthesized according to the method of Kirschner (13) with minor modifications. The Na_2H_2EDTA (16.8 g) was added slowly to a $CuSO_4$ solution (12.6 g in 100 ml of H_2O) with constant stirring and heating at 85°C. The hot solution was filtered and allowed to cool over a four hour period. The blue solid that separated on cooling was collected by filtration and partially dried at 15 mm Hg in a heated, 50°C, desiccator overnight. It decomposed on heating from 245 to 265°C, and was identified as $[Cu(C_{10}H_{14}N_2O_8)].H_2O$ Analysis calculated: C, 32.30; H, 4.34; N, 7.54. Found: C, 32.69; H, 4.28; N, 7.46.

Inhibition of NBT reduction by superoxide produced with the xanthine oxidase superoxide generating system at various pH values was determined by measuring absorbance at 540 nm 5 minutes after initiating the reaction with the addition of xanthine. The reaction mixture consisted of 52 ml 0.05 M sodium phosphate buffer (pH 7.8), 20 ml of 1% gelatin solution, 8 ml of phosphate buffer solution containing 4 units of xanthine oxidase and 1 ul of catalase solution containing 316,000 units of enzyme per ml. A 1,000 uM stock solution of Cu(II)EDTA was prepared by dissolving 3.716 mg in 5 ml of distilled deionized water at pH 7.0 and diluting to 10 ml. pH adjustments were made using either 1 percent HCl or 1 percent NaOH. Appropriate aliquots of stock solution were used to obtain the desired concentrations.

Individual cuvettes contained 1.3 ml of buffer, 0.5 ml of the gelatin solution, 0.2 ml of NBT solution, 0.2 ml of deionized distilled H_2O containing Cu(II)EDTA, 0.2 ml of the xanthine oxidase solution and 0.5 ml of xanthine solution containing 5 mg of xanthine per 50 ml of solution. The xanthine addition resulted in a final liquid volume in each cuvette of 2.9 ml. This addition was also used to start the reaction. The

absorbance observed with no Cu(II)EDTA added was taken as 100 percent reduction of NBT or no SOD-mimetic reactivity.

The reaction mixture and the method of initiating uric acid synthesis with xanthine were the same as that for SOD-mimetic reactivity except the NBT was omitted. Uric acid synthesis was monitored by observing the change in absorbance at 292 nm, the λ_{max} for uric acid.

RESULTS AND DISCUSSION

Cu(II)EDTA had no significant superoxide dismutase mimetic reactivity at pH 8; however, significant mimetic reactivity was observed in a pH-related manner within the range studied as evidenced by data in Figure 2. As can be observed, the complex has sufficient SOD-mimetic reactivity at pH 7 to be classified as active according to criteria suggested by Roberts and Robinson (14).

As shown in Table 1, uric acid synthesis did not decrease under conditions of superoxide disproportionation measurement and, therefore, there was no inhibition of xanthine oxidase. Also, as shown in Table I, the rate of uric acid synthesis was constant for given pH values over the

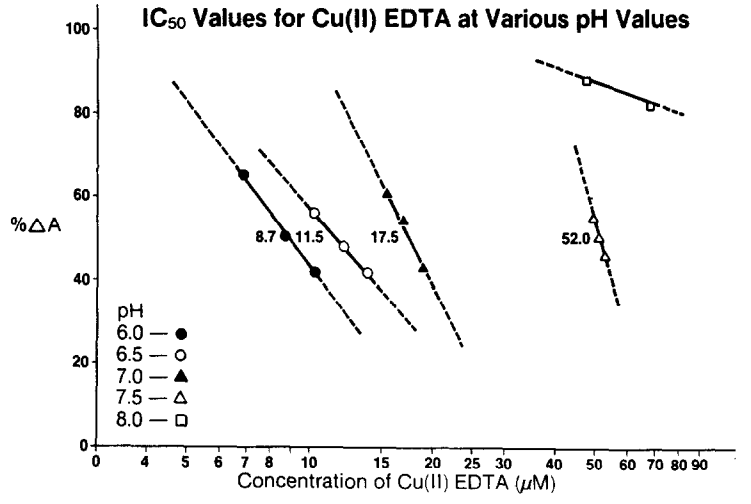


Figure 2. Influence of hydrogen concentration on superoxide disproportionation by Cu(II)EDTA.

Table 1. Absence of an effect of Cu(II)EDTA on uric acid synthesis by xanthine oxidase in the pH range of 8 to 6

Concentration of Complex in Cuvette (uM)	Absorbance	pH	△A/Min
		8.0	
0.0	0.243		0.24
55.2	0.266		0.23
62.2	0.383		0.25
69.0	0.395		0.25
		7.5	
0.0	0.466		0.23
50.0	0.461		0.21
51.7	0.472		0.21
53.5	0.498		0.21
		7.0	
0.0	0.240		0.15
13.8	0.243		0.16
15.5	0.268		0.16
17.2	0.292		0.16
19.0	0.336		0.16
		6.5	
0.0	0.442		0.14
10.4	0.453		0.14
12.1	0.482		0.14
13.8	0.486		0.14
		6.0	
0.0	0.333		0.12
6.8	0.376		0.12
8.6	0.457		0.10
10.4	0.462		0.11
12.1	0.480		0.10

range of Cu(II)EDTA concentrations employed which is further evidence that xanthine oxidase was not inhibited by Cu(II)EDTA. These data are consistent with data reported by Dollwet et al.(15) that showed certain copper complexes, Cu(II)(3,5-Diisopropylsalicylate)$_2$ and Cu(II)$_2$(Asprinate)$_4$, did not decrease uric acid synthesis.

These data support the superoxide disproportionation mechanism proposed by Fridovich for Cu-ZnSOD (12) and deAlvare et al. for Cu(II)(salicylate)$_2$ (16), a small

molecular weight copper complex. Moreover, these results are consistent with the suggestion that superoxide bonding sites become available on Cu(II)EDTA under acidic conditions and that the complex, like many other copper(II) complexes, disproportionates superoxide.

In light of these findings, it is suggested that care be exercised by investigators when assessing superoxide obligate reactions in the presence of Cu(II)EDTA. The use of EDTA to inhibit supposed copper-dependent superoxide disproportionation and those published reports wherein EDTA was added to rule out the possibility that observed disproportionation was copper-dependent are also questioned.

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