

## PULSE RADIOLYTICALLY GENERATED SUPEROXIDE AND Cu(II)-SALICYLATES

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**SUMMARY:** The rate constants of the reactions between pulse radiolytically produced superoxide anions and the Cu(II) chelates of salicylate, acetylsalicylate, p-aminosalicylate and diisopropylsalicylate were determined at pH 7.5 and found to range from  $0.8$  to  $2.4 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$ . It was intriguing to note that they had a superoxide dismutase activity identical with that of native cuprein-copper ( $k_{245} = 1.3 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$  per g-atom of Cu). These measurements confirm our earlier observations using indirect assays that all copper salicylates act as perfect model superoxide dismutases and favour the proposal that the activity of anti-inflammatory agents might be assigned to their in vivo formed Cu complexes.

Inflammatory response is in part attributed to excited oxygen species, such as  $\cdot\text{O}_2^-$  and  $\cdot\text{OH}$  (1,2). Based on the observation that the serum copper is increased during arthritic diseases (3) Sorenson could show that the anti-inflammatory activity of the Cu(II) chelates of many anti-arthritic drugs is much higher than that of the ligands (4,5). He proposed that anti-inflammatory agents form copper complexes in vivo which are responsible for the therapeutic action.

As our previous work revealed that low molecular weight Cu(II) chelates had nearly the same ability to catalyze the spontaneous dismutation of superoxide as native bovine cuprein (2Cu,2Zn-superoxide dismutase) (6-9) and that  $\text{Cu}(\text{tyr})_2$  suppressed microsomal dealkylation reactions (10-12), we could recently show that Cu(II) complexes with four different acetylsalicylate-like drugs were able to suppress the xanthine-xanthine oxidase mediated reduction of both cytochrome c and nitroblue tetrazolium as well as the formazan formation by  $\text{KO}_2$ . Furthermore, these complexes were able to inhibit the hydroxylation of benzo-[a]-pyrene as well as the dealkylation of 7-ethoxycoumarin by induced hepatic rat microsomes (13).

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The Cu-complex of penicillamine was also shown to possess a superoxide dismutase activity (14).

Owing to the fact that all the assays employed above were of an indirect nature, it was of interest to study the direct reaction between the anti-inflammatory active copper chelates with pulse radiolytically produced superoxide and to determine the rate constants of these reactions.

### EXPERIMENTAL

The copper chelates were prepared as described in (3,13). Elemental analyses revealed a stoichiometry of 1 Cu per 2 moles of ligand. X-ray photoelectron spectrometric measurements confirmed that copper is present as Cu(II) throughout (13). All chemicals employed were of analytical grade purity. In pulse radiolysis experiments triply distilled and pyrolyzed water was used.

For the assays of the superoxide dismutase activity a Febetron pulse radiolysis unit was employed. A xenon lamp, Osram XBO 450 W4, served as light source and the optical detection system was composed of a Zeiss M4 Q III monochromator, an EMI 9659 QB photomultiplier and a Tectronix 7704 oscilloscope. Oscilloscope traces showing the decay of  $\cdot\text{O}_2^-$  (at 250 nm) in the absence and in the presence of the Cu-chelates were photographed with a Polaroid camera. 40 nsec pulses of high-energy electrons (1.81 MeV) produced  $\cdot\text{O}_2^-$  concentrations ranging from 4 to 35  $\mu\text{M}$ . The respective superoxide concentration was evaluated using thiocyanate dosimetry (15). The rate constants were calculated on a Wang 2200 computer by the method described in (16) and were expressed per equivalent of copper. The Cu(II)-chelates were dissolved in pyrolyzed water containing 1 mM formate; the pH was adjusted to 7.5 with 0.1 N NaOH.  $\text{O}_2$  was bubbled at least 15 minutes prior to the start and during the pulse radiolysis experiments to achieve oxygen saturation. The quartz cuvettes were filled with the solutions investigated employing a remotely controlled flow system.

### RESULTS

The ability of the Cu(II)-chelates of four acetylsalicylate-like drugs (salicylate, acetylsalicylate, p-aminosalicylate and diisopropylsalicylate) to catalyze the spontaneous dismutation of superoxide anions at pH 7.5 was assayed directly using pulse radiolytically produced  $\cdot\text{O}_2^-$ . This was achieved by a 40 nsec pulse in oxygen saturated solutions containing 0.1 M formate and the respective complex. Formate was taken to convert  $\cdot\text{OH}$  radicals to superoxide (17,18). In such solutions the following series of reactions occur:

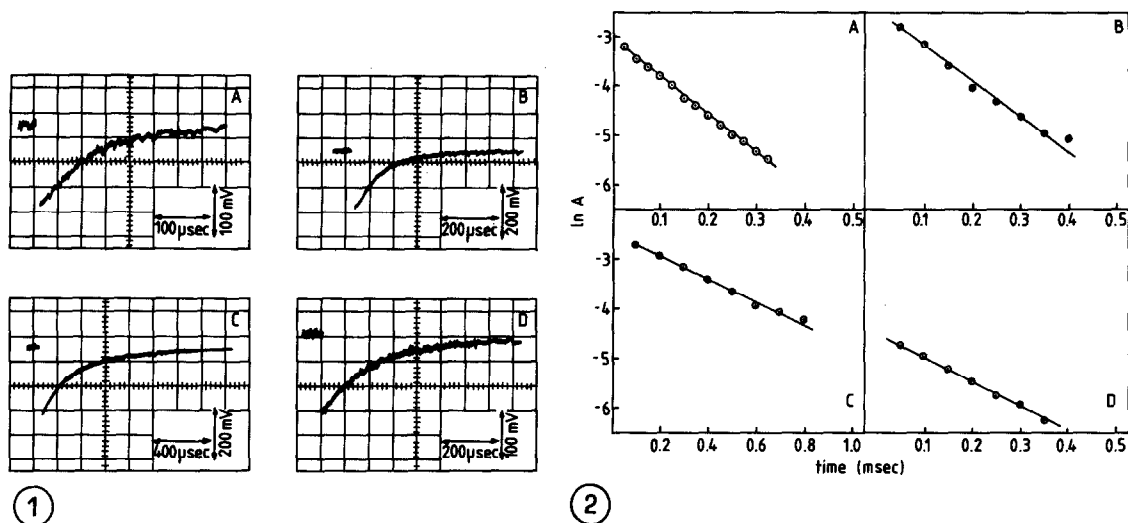
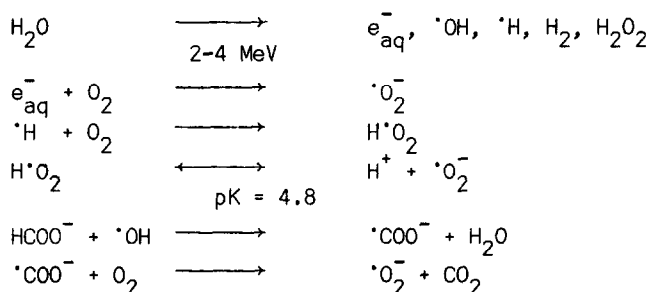


Figure 1: The decay of pulse radiolytically produced superoxide in the presence of A  $\text{Cu}(\text{salicylate})_2$ , 5.0  $\mu\text{M}$ , B  $\text{Cu}(\text{acetylsalicylate})_2$ , 8.4  $\mu\text{M}$ , C  $\text{Cu}(\text{p-aminosalicylate})_2$ , 5.0  $\mu\text{M}$ , and D  $\text{Cu}(\text{diisopropylsalicylate})_2$ , 2.2  $\mu\text{M}$  as observed at 250 nm employing a Tectronix 7704 oscilloscope equipped with a Polaroid camera. The measurements were carried out in oxygen saturated solutions containing 1 mM formate, the pH of which was adjusted to pH 7.5. 40 nsec electron pulses of 1.81 MeV were generated using a Febetron pulse radiolysis unit. A xenon lamp (Osram XBO 450 W4) served as light source. The light path was 2cm.

Figure 2: Pseudo first order plots for the reactions of the different  $\text{Cu}(\text{II})$ -salicylates with  $\cdot\text{O}_2^-$ . The experimental conditions were described in the legend to fig. 1. A  $\text{Cu}(\text{salicylate})_2$ , 5.0  $\mu\text{M}$ ; B  $\text{Cu}(\text{acetylsalicylate})_2$ ; 8.4  $\mu\text{M}$ , C  $\text{Cu}(\text{p-aminosalicylate})_2$ , 2.0  $\mu\text{M}$ ; D  $\text{Cu}(\text{diisopropylsalicylate})_2$ , 2.2  $\mu\text{M}$ .



The G-value of superoxide (yield of  $\cdot\text{O}_2^-$  molecules per 100 eV of absorbed energy) was thus raised to 5.8 (17).

The oscilloscope traces showing the decay of superoxide in the absence and in the presence of different concentrations of each of the copper chelates investigated were photographed. The catalyzed dismutation re-

Table 1: Calculated second order rate constants of the reactions between superoxide and four anti-inflammatory active copper complexes.

Cu(II)-chelate	Copper concentration ( $\mu\text{M}$ )	$k_{250} \times 10^{-9} \text{ M}^{-1} \text{ sec}^{-1}$ (mean value for all $\cdot\text{O}_2^-$ concentrations)	Mean value ( $\times 10^{-9} \text{ M}^{-1} \text{ sec}^{-1}$ )
$\text{Cu}(\text{salicylate})_2$	1.0	1.50	
	5.0	1.79	$1.64 \pm 0.15$
$\text{Cu}(\text{acetylsalicylate})_2$	1.1	1.21	
	2.2	0.61	$0.96 \pm 0.20$
	8.4	1.07	
$\text{Cu}(\text{p-amino-salicylate})_2$	2.0	1.03	
	5.0	0.65	$0.79 \pm 0.16$
	10.0	0.69	
$\text{Cu}(\text{diisopropyl-salicylate})_2$	2.2	2.28	
	5.4	2.52	$2.40 \pm 0.12$

Mean values at different  $\cdot\text{O}_2^-$  concentrations are given ( $4\text{--}36 \mu\text{M}$ ). For experimental details see legend to fig. 1.

actions showed pseudo first order characteristics (fig. 1). When  $\ln A$  (the natural logarithm of the absorption of  $\cdot\text{O}_2^-$  at 250 nm) was plotted against time linear plots were obtained which allowed the graphic determination of the pseudo first order rate constants (fig. 2).

The second order rate constants were obtained by dividing the pseudo first order rate constants by the respective concentration of chelated Cu(II). Values ranging between  $0.79$  and  $2.40 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$  were obtained for the four copper chelates of the salicylate derivatives (table 1) showing that these complexes react with  $\cdot\text{O}_2^-$  as rapidly and effectively as native bovine cuprein (2Cu,2Zn-superoxide dismutase) and other low molecular weight copper chelates (6,7,19,20). The second order rate constant for the spontaneous dismutation of superoxide was found to be  $1.06 \times 10^7 \text{ M}^{-1} \text{ sec}^{-1}$  at pH 7.5.

#### DISCUSSION

The ability of Cu(II) complexes of four salicylate derivatives to exhibit superoxide dismutase activity has been previously demonstrated using an

enzymical and a strictly inorganic source of superoxide (and perhaps other excited oxygen species) (13). We could also show that these compounds can express their activities to scavenge  $\cdot\text{O}_2^-$  in more complex systems, i.e. during hepatic microsomal dealkylations (13) which proved to implicate the presence of superoxide anion radicals (10-12).

In this study, using a pure source of  $\cdot\text{O}_2^-$ , it was demonstrated that these chelates react with superoxide in a fast and effective manner. The suggestion that Cu(II) chelates are the active forms of anti-inflammatory drugs (4,5,13) is favoured by these results. The efficacy of these chelates as anti-inflammatory agents may be, in part, due to the dismutation of superoxide anions.

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