大韓藥理學雜誌：第19卷 第2號
Korean J. of Pharmacology
Volume 19, Number 2, 1983

Effects of Cu^{++}-Catalyzed Peroxidation on Collagen Gelation

Myung Hee Chung, Myung Suk Kim and **Chung Soo Lee**

Dept. of Pharmacology, College of Medicine, Seoul National University

=국문초록=

Cu^{++}-Catalyzed Peroxidation이 Collagen Gelation에 미치는 영향

서울대학교 의과대학 약리학교실

정 명 희·김 명 석·이 정 수

reactive oxygen species에 의해 나타나는 reactivity에 있어서 metal ions이 관여함이 시사되고 있다. 이미 알려진 reactive oxygen species와 metal ions의 상호작용 이외에 특히 Cu^{++}과 H_2O_2가 강력한 peroxidative action을 나타낸다는 사실이 알려져 있으며 Cu^{++}-H_2O_2가 biological system에서의 조직파괴에 관여할 가능성이 저자들에 의해서 효소 및 조직의 구조 단백질의 degradation 효과를 관찰함으로써 시사되었다.

본 연구는 H_2O_2 혹은 H_2O_2를 생성하는 효소계(xanthine과 xanthine oxidase 및 glucose과 glucose oxidase)에 Cu^{++}을 첨가하여 Cu^{++}-H_2O_2에 의한 peroxidation의 효과를 collagen gelation을 통하여 확인코저 수행하였으며 다음과 같은 결과를 얻었다.

1) Cu^{++}(20 μM)과 H_2O_2에 의하여 collagen gelation은 현저히 억제되었으며 이같은 억제효과는 양자의 농도에 비례하였다.

2) Cu^{++}-H_2O_2 reactivity를 확인하는 다른 방법으로 glucose oxidase system를 이용하였다. glucose oxidase(2.5μg/ml)와 glucose(0.5 mM)는 collagen gelation에 영향을 미치지 않았으나 이에 Cu^{++}이 존재하면 gelation이 억제되었다. 이때 억제정도는 glucose(0.125~1.25 mM)와 Cu^{++}의 농도에 비례하였다.

3) 여러 reactive oxygen species 가운데 Cu^{++}-H_2O_2 reactivity를 xanthine oxidase system을 이용하여 확인하였다.

(a) collagen gelation은 xanthine oxidase(30 munits/ml)와 xanthine(0.25~2 mM)에 의하여 억제되었다.

(b) 이때 나타나는 collagen gelation의 억제는 superoxide dismutase에 의하여 완전히 회복되었으나 catalase에 의해서는 더욱 촉진되었다. 그러나 catalase에 의한 억제효과의 촉진은 1,4-diazabicyclo(2,2,2)octane에 의하여 완전히 소실되었다. 따라서 이 xanthine oxidase system에서는 O_2^-, H_2O_2, $^1O_2$이 관여함을 알 수 있었다.

(c) 그러나 Cu^{++}(10 μM)이 존재하였을 때 collagen gelation은 superoxie dismutase에 의해 더욱 억제되었고 catalase에 의해서는 완전히 회복되었다.

xanthine oxidase계에서 얻어진 결과는 여러 reactive oxygen species 가운데 H_2O_2가 Cu^{++}에 의하여 peroxidation효과를 나타냄을 알 수 있었다.

이상의 결과로 미루어 볼 때 reactive oxygen species와 metal ions과의 상호작용 가운데 Cu^{++}-H_2O_2는 강한 반응을 나타내는 특이한 구성요소이고 현재 시사되고 있는 reactive oxygen species의 biological effects에 비추어 Cu^{++}-catalyzed peroxidation도 병적상태에서 생체에 유해한 작용을 나타내는 요소임을 시사하며 특히 염증시 조직파괴역할에 관하여 고찰하였다.

* 본 연구는 1983년도 서울대학병원 특진연구비 보조로 이루어진 것임.

INTRODUCTION

Reactive oxygen species are highly reactive and can alter most types of cellular macromolecules. In *in vitro* experiments, they have been shown to oxidize proteins (Venkatasubramanian and Joseph, 1977) and unsaturated fatty acids (Kellogg and Fridovich, 1977), damage nucleic acids (Lavelle et al., 1973) and cleave polysaccharides (McCord, 1974). Among them hydroxyl radicals (OH·) and singlet oxygen ($^1O_2$) are considered to be the most reactive, and the most likely immediate oxidants of the destructive processes. Other less reactive species, such as superoxide radical (O^-_2·) and hydrogen peroxide (H_2O_2) have been postulated to be the precursors of either OH· (Fong et al., 1976) or $'O_2$ (Kellogg and Fridovich, 1977) or both (Lynch and Fridovich, 1978). Metal ions have been suggested as catalysts for the eventual production of either OH· or $'O_2$ (Fong et al., 1976; Fee, 1980; Fridovich, 1978). Alternatively, metal ion-oxygen complexes have also been proposed as proximate reactive species for oxidation of lipid, protein and nucleic acids (Brawn and Fridovich, 1981; Ingraham, 1959; Chung et al., 1981; Chan and Kesner, 1980).

Among the various metal ions, Cu^{++} has been shown to be particularly effective in catalyzing peroxidation by H_2O_2. This include NADH (Chan and Kesner, 1980), lysozyme (Chung et al., 1981) and other proteins (Phelps et al., 1961; Chung et al., 1983) and phospholipids (Chan et al., 1982). Complexes of Cu^{++} and H_2O_2 have been suggested to be the immediate oxidizing species for NADH (Chan and Kesner, 1980), phospholipids (Chan et al., 1982) and proteins (Chung et al., 1981 & 1983) without involving either OH· or $^1O_2$ as an intermediate.

In this study, Cu^{++}-catalyzed peroxidation on collagen was confirmed in the presence of H_2O_2 of various sources, added directly or generated enzymatically using glucose oxidase or xanthine oxidase by observing the inhibition of gelation of this structural protein.

MATERIALS AND METHODS

Xanthine oxidase, superoxide dismutase and catalase were obtained from Sigma Chemical Co.; glucose oxidase from Worthington; 1,4-diazabicyclo (2, 2, 2) octane from Aldrich Chem. CO.; allopurinol from Samil Pharma. Co.; H_2O_2 from Shinyo Pure Chemical Co.; $CuSO_4$ from Fisher Chemical Co. Other chemicals were of analytical reagent grade. Xanthine oxidase and catalase were dialyzed before using 3 times for 12 h at 4°C against 50 mM Tris-HCl, pH 7.6.

1) Preparation of skin collagen

Collagen was prepared from the skin obtained from male rats weighing about 100 g according to the procedures of Oegema et al. (1975). Collagen solution in 0.1 M acetic acid was lyophilized and stored at -20°C. Collagen when used, was dissolved in 0.5 M acetic acid to approximately 2 mg/ml, dialyzed extensively against 0.005 M acetic acid at 4°C and the resulting collagen in 0.005 M acetic acid was used in the experiments.

2) Collagen gelation

Collagen was mixed in 4 ml of reaction medium containing 0.14 M NaCl, 10 mM NaH_2FO_4, pH 7.4 with or without other components as decribed below and incubated for 12 h at 4°C. The final concentration of collagen was 0.25 mg/ml. After dissolved air was removed by vacuum, 3 ml of aliquot was

removed to a cuvette. The cuvette containing collagen was then transfered to the temperature-controlled chamber of a Unicam SP 1750 recording spectrophotometer and it was maintained at 37°C. Gelation was monitored by continuous absorbance recording at 400 nm.

3) Effects of Cu^{++}-catalyzed peroxidation of collagen gelation

Collagen in 4 ml of the reaction medium decribed above was incubated with varying concentrations of $CuSO_4$ in the presence of eitier H_2O_2 added directly or H_2O_2 generating systems i. e., xanthine oxidase plus xanthine or glucose oxidase plus glucose for 12 hr at 4°C. Reaction was stopped by addition of 0.1 ml of 1 mM EDTA and 20 μl of 1 mg/ml catalase, but in case that xanthine oxidase and xanthine were used, 0.4ml of 80 mM allopurinol was used together with 0.1 ml of 1 mM EDTA. The concentrations of xanthine oxidase and glucose oxidase were 30 munits/ml and 2.5 μg/ml, respectively and those of xanthine and glucose, 0.25~2 mM and 0.125~1.25 mM, respectively. Three ml of aliquot was used to observe gelation according to the procedure described above. Xanthine oxidase was assayed according to the method of Greenwald and Moy(1979) and protein concentration was determined by the method of Lowery et al. (1951).

RESULTS AND DISCUSSION

1. Inhibition of collagen gelation by Cu^{++} and H_2O_2

When collagen was incubated with Cu^{++} and H_2O_2 at 4°C, its gelation initiated by warming to 37°C was markedly inhibited as shown in Fig.1. The inhibition was observed only in the presence of both Cu^{++} and H_2O_2. Either

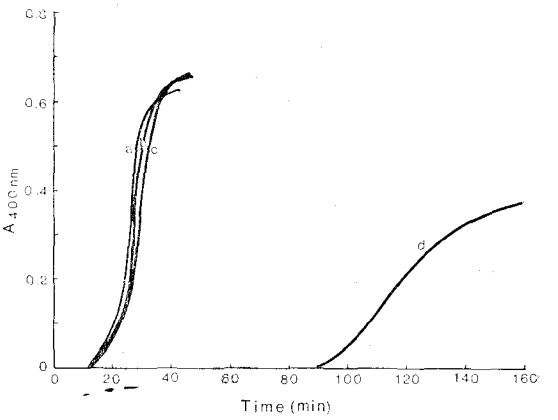

Fig. 1. Effects of Cu^{++} and H^2O^2 on collagen gelation. Collagen(0.25 mg/ml) was incubated in 4 ml of reaction medium containing 0.14 M NaCl, 10 mM sodium phosphate, and varying concentrations of $CuSO_4$ and H_2O_2 for 12 h at 4°C. Termination of the reaction, and gelation were done as described in Materials and Methods. a; no $CuSO_4$ and H_2O_2, b; 20 μM $CuSO_4$ only, c; 0.5 mM H_2O_2 only and d; 20 μM $CuSO_4$ and 0.5 mM H_2O_2.

Cu^{++} or H_2O_2 alone showed essentially no effect on the gelation. Furthermore, when collagen was incubated with Cu^{++} and H_2O_2 in the presence of either 5 μg/ml of catalase or 0.1 mM EDTA, almost no detectable change in gelation was observed(data not shown). The results suggest that both Cu^{++} and H_2O_2 were needed for peroxidative attack to collagen resulting in inhibition of its gelation reflecting alteration of physico-chemical properties of this structural protein.

The dependency of gelation inhibition on both Cu^{++} and H_2O_2 as well as effects of their concentrations was shown in Fig.2 where the inhibitory effects were presented as $T_{1/2max}$, the time required to attain half-maximum turbidity of gelation. In the absence of Cu^{++}, H_2O_2 did not show any significant changes in $T_{1/2max}$. No change in gelation was also observed in the presence of Cu^{++} alone; $T_{1/2max}$ was

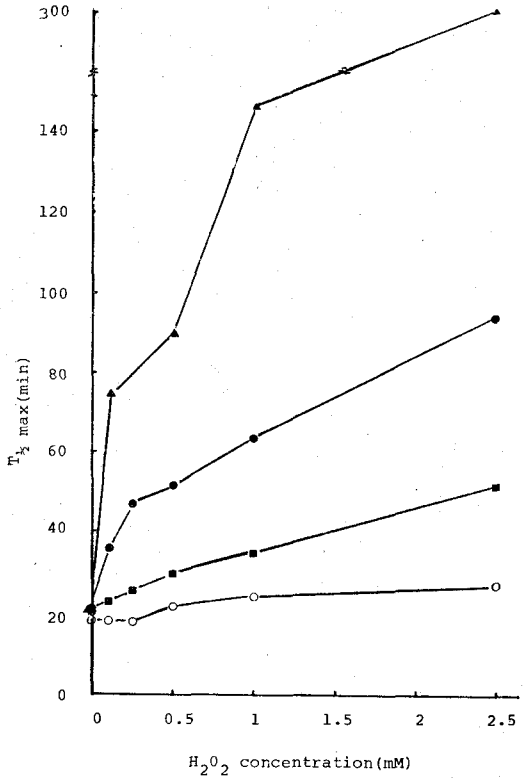

Fig. 2. Effects of concentration of Cu^{++} and H_2O_2 on collagen gelation. All experimental conditions were the same as in Fig. 1. Extent of inhibition of gelation was expressed as $T_{1/2max}$, the time required to attain half-maximum turbidity of gelated collagen. O; no $CuSO_4$ and ■, ● and ▲; 5,10 and 20 μM $CuSO_4$, respectively.

about 20 min up to 20 μM of Cu^{++}. However, $T_{1/2max}$ was increased as a function of H_2O_2 concentration. Moreover, the increment of $T_{1/2max}$ was augmented with increasing concentration of Cu^{++}.

2) Effects of glucose oxidase on collagen gelation in the presence of Cu^{++}

As an alternative to demonstrate Cu^{++}-catalyzed peroxidation, catalytic action of Cu^{++} was tested on collagen gelation with H_2O_2-generating system, glucose plus glucose oxidase. As shown in Fig. 3, Collagen gelation

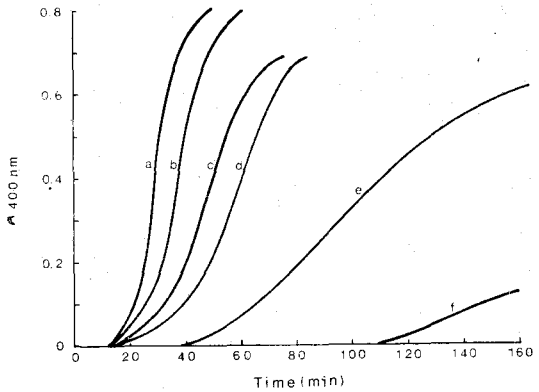

Fig. 3. Effects of glucose oxidase with varying concentration of glucose on collagen gelation in the presence of Cu^{++}. Collagen was treated with glucose oxidase(2.5 μg/ml) and different concentrations of glucose in the presence of 20 μM $CuSO_4$. Other conditions were the same as in Fig. 1. a; no glucose oxidase, glucose and $CuSO_4$, b; glucose oxidase and 5mM glucose and c,d, e and f; 0.125, 0.25, 0.5 and 1.25 mM glucose in the presence of glucose oxidase and $CuSO_4$, respectively.

was inhibited by glucose and glucose oxidase only in the presence of Cu^{++} (Fig. 3, Curve c, d, e and f). The observed inhibition, however, was abolished by 5 μg/ml catalase and 0.1 mM EDTA (data not shown) as in the previous experiment (Fig. 1), The extent of inhibition was proportional to the concentration (0.125~1.25 mM) of glucose used. In the absence of Cu^{++}, slight inhibition was observed even at 5 mM of glucose (Fig. 3, Curve b), whereas in the presence of 20 μM Cu^{++} marked inhibition was observed with much less concentration of glucose, 1.25 mM (Fig. 3, Curve f). Fig. 4 shows effect of Cu^{++} concentration on collagen gelation in the glucose oxidase system. With constant concentration of glucose oxidase (2.5 μg/ml) and glucose (0.5 mM), the degree of inhibition was increased with increasing concentration of Cu^{++}. From the results obtained thus far, the catalytic action of Cu^{++}

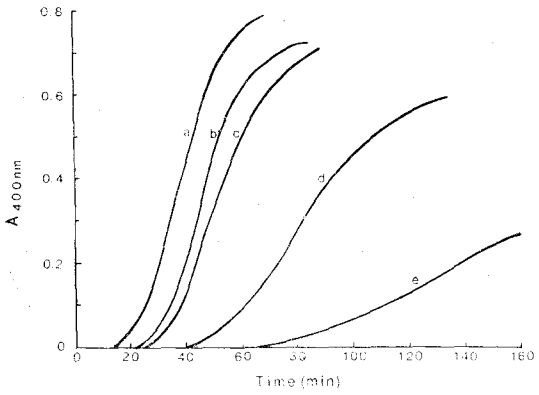

Fig. 4. Effects of glucose and glucose oxidase on collagen gelation in the presence of varying concentrations of Cu^{++}. Collagen was treated with 0.5 mM glucose and 2.5 μg/ml in the presence of different concentions of $CuSO_4$. Other conditions were the same as in Fig. 3. a; no glucose, glucose oxidase and $CuSO_4$ and b, c, d and e; 2, 5, 10 and 20 μM $CuSO_4$ in the presence of glucose and glucose oxidase, respectively.

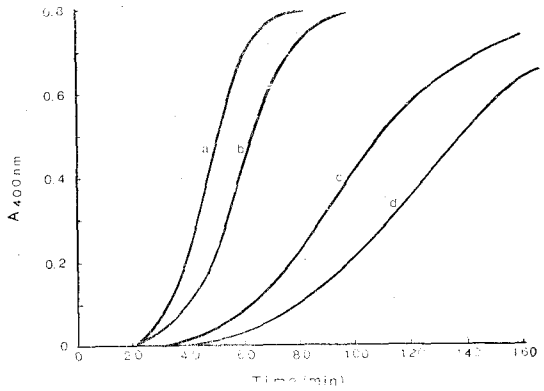

Fig. 5. Effects of xanthine and xanthine oxidase on collagen gelation. Collagen was treated with 30 munits/ml xanthine oxidase in the presence of varying concentrations of xanthine. Reaction was stopped with allopurinol and EDTA as described in Materials and Methods. Other conditions were the same as in Fig. 1. a; no xanthine and xanthine oxidase and b, c and d; 0.25, 1 and 2 mM xanthine in the presence of xanthine oxidase, respectively.

was shown clearly to be identical in two different systems supplying H_2O_2, directly or enzymatically.

3) Effects of xanthine oxidase and xanthine on collagen gelation in the presence of Cu^{++}

In contrast to the findings that glucose oxidase system was able to affect collagen gelation only in the presence of Cu^{++}, xanthine oxidase system (X/XO) by itself caused inhibition of collagen gelation without Cu^{++} added (Fig. 5). The degree of inhibition was dependent on xanthine concentration (0.25~2 mM).

A number of reports have shown that many oxidative processes involving reactive oxygen species generated by X/XO were inhibited by superoxide, catalase and even by quenchers for OH· and $^1O_2$ (Greenwald and Moy, 1979; McCord, 1974; Lynch and Fridovich, 1978; Kellogg and Fridovich, 1977). Based upon these findings O^-_2· and H_2O_2 were suggested to interact to produce more reactive species, OH· and/or $^1O_2$ implicated as final mediators in the oxidative processes as shown in the following equation.

$$O^-_2\cdot + H_2O_2 \rightarrow OH\cdot + OH^- + {}^1O_2 \quad (1)$$

Thus, the observed inhibition of gelation by X/XO may be attributed to the attack to collagen by OH· or $^1O_2$ or both.

As might be expected, superoxide dismutase was effective in limiting the inhibition of collagen gelation in this experiment (Fig. 6, curve c) suggesting O^-_2· was involved in the inhibition of gelaton. Catalase, however, was shown to potentiate the inhibition of collagen gelation by xanthine oxidase system (Fig. 6, Curve e). The observed effect of catalase was contradictory to what have been generally observed with the enzyme in xanthine oxidase system. Moreover, the scavenger for OH· including mannitol, benzoate and formate (Chan and Kesner, 1980) tested in this experiment did not exhibit preventive effect against the

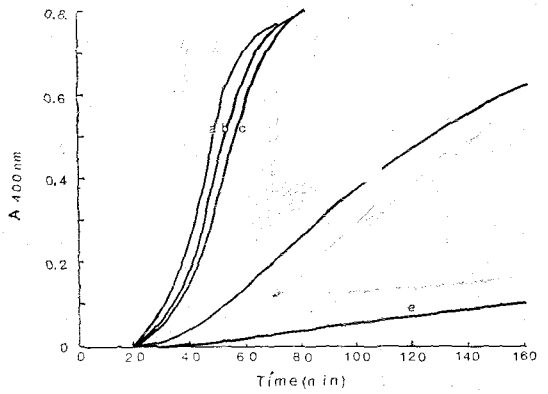

Fig. 6. Effects of superoxide dismutase and catalase on the inhibition of collagen gelation by xanthine and xanthine oxidase. Collagen was treated with 0.5 mM xanthine and 30 munits/ml xanthine oxidase in the presence of either superoxide dismutase or catalase. Other conditions were the same as in Fig. 5. a; 5 μg/ml catalase without xanthine and xanthine oxidase, b; no xanthine and xanthine oxidase, d; xanthine and xanthine oxidase and c and e; 5 μg/ml superoxide dismutase and 5 μg/ml catalase with xanthine and xanthine oxidase, respectively.

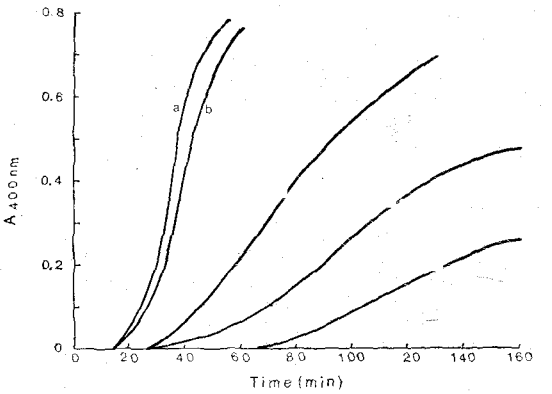

Fig. 7. Effects of 1,4-diazabicyclo(2,2,2)octane on augmented inhibition of collagen gelation by catalase in xanthine oxidase system. Collagen was treated with 0.5 mM xanthine and 30 munits/ml xanthine oxidase in the presence of 2.5 μg/ml catalase with or without 1,4-diazabicyclo(2,2,2) octane. Other conditions were the same as in Fig. 5. a; no xanthine and xanthine oxidase, d and e; xanthine and xanthine oxidase without and with catalase, respectively and b and c; 7.5 and 3.75 mM 1,4-diazabicyclo (2,2,2)octane in the presence of xanthine, xanthine oxidase and catalase, respectively.

inhibition of collagen gelation(data not shown). Interestingly, however, the inhibited collagen gelation by catalase in the xanthine oxidase system was recovered by 1,4-diazabicyclo (2,2,2) octane known as $^1O_2$ quencher in dose-dependent manner(Fig. 7 curve b and c).

Although a statisfactory explanation for the nature of the interaction between reactive oxygen species observed in this system can not be provided, the results obtained from the quencher studies suggest one possible way of interaction,

$$O^-_2\cdot + HO\cdot_2 + H^+ \xrightarrow[\text{dismutation}]{\text{spontaneous}} H_2O_2 + {}^1O_2 \qquad (2)$$

but

$$O^-_2\cdot + HO\cdot_2 + H^+ \xrightarrow[\text{dismutase}]{\text{superoxide}} H_2O_2 + O_2 \qquad (3)$$

In other words, a principal mediator in this system may be $^1O_2$. But this species was produced probably only on spontaneous dismutation not on enzymatic one. The suggested interaction was in agreement with reports showing that $O^-_2\cdot$ spontaneously dismutated to H_2O_2 and $^1O_2$(Kahn, 1970; Mayeda and Bard, 1974).

But two questions to be answered are still remaining. The first is how catalase potentiates inhibition of collagen gelation in the situation where reaction(2) prevails. Possibly, that may be attributable to the shift of reaction(2) to right by removal of H_2O_2 resulting in augmented production of $^1O_2$. The second is why OH· was excluded as a mediator in the quencher studies. In author's works where effects of xanthine oxidase on brain microsomal Na^+-K^+-ATPase(Oh et al., 1982) and calcium binding of cardiac sarcoplasmic reticulum(Kim et al., 1983) the inhibited activi-

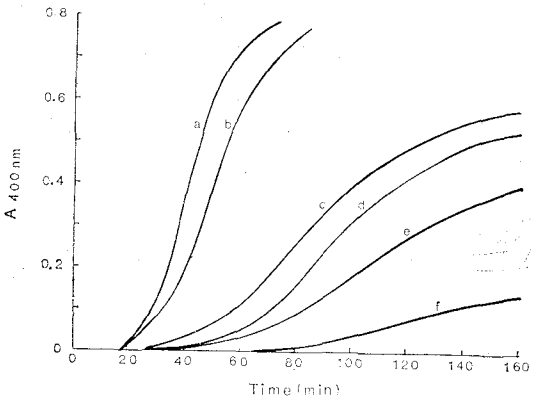

Fig. 8. Effects of Cu^{++} on collagen gelation in the xanthine oxidase system. Collagen was treated with 0.5 mM xanthine and 30 munits/ml xanthine oxidase in the presence of 10 μM $CuSO_4$ and effects of 5 μg/ml superoxide dismutase and 5 μg/ml catalase were observed. Other conditions were the same as in Fig. 5. a; no xanthine oxidase, b; xanthine, xanthine oxidase, $CuSO_4$ and catalase, c; xanthine and xanthine oxidase, d; xanthine, xanthine oxidase and $CuSO_4$, e; xanthine, xanthine oxidase, superoxide dismutase and $CuSO_4$ and f; xanthine, xanthine oxidase and catalase.

ties by xanthine oxidase were not recovered by OH· quenchers, mannitol and others. In spite of frequent observations of exclusion of OH· as a mediator in our xanthine oxidse system, the reason for the phenomena can not be provided. Further study is necessary for elucidating the mechanism related to the observations in this experiment and the two questions above in particular.

At any rate, H_2O_2 was suggested to be produced in the present xanthine oxidase system regardless of its mode of action on the collagen gelation. Under these circumstances the catalytic role of Cu^{++} was also clearly demonstrated in the peroxidative inhibition of collagen gelation. As shown in Fig. 8, Cu^{++} caused marked inhibition of collagen gelation by xanthine and xanthine oxidase even in the presence of superoxide dismutase (Fig. 8, Curve e) which showed complete recovery of the inhibited gelation when Cu^{++} was not present (Fig. 6, Curve c). Furthermore, the observed effect of Cu^{++} in the xanthine oxidase system was prevented by catalase (Fig. 8, Curve b), which, without Cu^{++}, gave rise to potentiation of the inhibition of gelation (Fig. 8, Curve f). The result observed above seems to be consistant of the fact that Cu^{++} interacted with H_2O_2 generated from spontaneous dismutation of O_2^-· produced by xanthine oxidase.

One may have a question why the inhibition of collagen gelation by catalase was not observed in the presence of Cu^{++} (Fig. 8, Curve b) under the condition where reaction (2) prevails as suggested previously. That may be due to superoxide dismutase like activity of Cu^{++} as in reaction (3) showing that O_2 is produced instead of $^1O_2$. This interpretation may be supported by the reports demonstrating that Cu^{++} itself (Rabani et al., 1973) as well as Cu^{++} complexes with amino acids and small peptide (Brigelius et al., 1974) exhibited to dismute O_2^-· to H_2O_2 and O_2. The resulting H_2O_2, however, has no chance to be involved in peroxidation catalyzed by Cu^{++} since it decomposes to H_2O and O_2 in the presence of catalase Gelation was little more inhibited by xanthine and xanthine oxidase in the presence of Cu^{++} alone than in its absence (Fig. 8, Curve d). At this time, the inhibition was not affected by mannitol (10 mM) (data not shown). In this case, therefore, the inhibition of gelation may be attributed to combined oxidative effects of $^1O_2$ and Cu^{++}-H_2O_2. Although contribution of each to the inhibition cannot be quantified, Cu^{++}-H_2O_2 seems to contribute more than $^1O_2$.

Catalytic action of Cu^{++} was confirmed with H_2O_2 of various sources, and more importantly oxidizing activity by Cu^{++}-H_2O_2 was demonstrated in the melieu with various

reactive oxygen species existing. Although specificity of metal ions as catalysts in peroxidatin by H_2O_2 and involvement of OH· or $^1O_2$ in the reaction by Cu^{++}-H_2O_2 were not tested in the present study, studies on peroxidative effects of Cu^{++}-H_2O_2 on proteins (Chung et al., 1981 and 1983), NADH (Chan and Kesner, 1980) and lipids (Chan et al., 1982) showed that among the metal ions, Cu^{++} was uniquely able to catalyze peroxidation by H_2O_2 and OH·and $^1O_2$ were excluded as mediators in this Cu^{++}-catalyzed peroxidation. Thus oxidation exerted by Cu^{++}-H_2O_2 seems to be a different mechanism of interaction from others suggestion involvement of metal ions in the interaction between reactive oxygen species. Further study is still required to explore the mechanism of action of Cu^{++}-catalyzed peroxidation although Cu^+OOH, CuO^+ or CuO_2 was suggested as possible oxidants (Ingraham, 1959).

The significance of peroxidative activities by Cu^{++}-H_2O_2 is not well understood. However, in view of the facts that Cu^{++} is a commonly found metal ion in most biological fluids and tissues and H_2O_2 can be formed as a product of normal oxidative respiration (Fridovich, 1979; Chance and Oshino, 1971; Loshen et al., 1971; Boveris and Chance, 1973), peroxidative activities by Cu^{++}-H_2O_2 to many biological compounds may be a potential mechanism to tissue toxicity in general. But more importantly, the observed effect of Cu^{++}-H_2O_2 on collagen gelation may have pathological implications in inflammatory conditions, paricularly in rheumatoid arthritis. In other study (Chung et al., 1983) even artilcular cartilage was observed to be degraded by Cu^{++}-H_2O_2.

In rheumatoid arthritis, there is a significant increase of copper concentration in the serum (Lorber et al., 1968; Sorenson, 1978) and synovial fluid (White et al., 1978). At the same time, the infiltration of phagocytic leukocytes at the inflamed joints can cause a localized high concentration of H_2O_2 (Root et al., 1975; Nathan et al; 1979). Cu^{++} in this pathological condition has been implicated as a destructive agent (Lorber et al., 1968; Gerber, 1974) and reactive oxygen species having degradative effects on collagen (Greenwald and Moy, 1979) and hyaluronic acid (McCord, 1974) have been suggested to have a role in the damage of joint tissue.

This hypothesis of a deleterious effect Cu^{++} and H_2O_2 is also supported by the observation that catalase was the most anti-inflammatory agent in the rate of all reactive species scavengers tested (Bragt et al., 1980) and EDTA was an effective suppressor of manifestations of rheumatoid arthritis (Leipzig et al., 1970). It has been suggested that lysosomal degradative enzymes play an tissue degradation in the inflamed joint (Ignaro, 1974; Barrett, 1978; Weissmann et al; 1980). However, Cu^{++}-H_2O_2 may also be a contributing factor.

REFERENCES

Barett, A.J.: *The possible role of neutrophil proteinases in damage to articular cartilage. Agents and Actions, 8:11-18, 1978.*

Boveris, A. and Chance, B.: *The mitochondrial generation of hydrogen peroxide: general properties and effect of hyperbaric oxygen. Biochem. J., 134:707-716, 1973.*

Bragt, P.C Bansberg, J.I. and Benta, I.L.: *Anti-inflammatory effects of free radical scavengers and antioxidants. Further support for proinflammatory roles of endogenous hydrogen peroxide and lipid peroxides. Inflammation, 4:289-299, 1980..*

Brawn, and Fridovich, I.: *DNA strand scission by enzymically generated oxygen radicals. Arch.*

Biochem. Biophys., 206:414-419, 1981.

Brigelius, R., Spottl, R., Bois, W., Lengfelder, E., Saran, M and Wesser, U.: *Superoxide dismutase activity of low molecular weight Cu^{++}-chelates studied by pulse radiolysis. FEBS Lett., 47:72-75, 1974.*

Chan, P.C. and Kesner, L.: *Copper(II) complex-catalyzed oxidation of NADH by hydrogen peroxide. Biol. Trace Element Res., 2:159-174, 1980.*

Chan, P.C., Peller, O.G. and Kesner, L.: *Copper (II)-catalyzed lipid peroxidation in liposomes and erythrocyte membranes. Lipids, 17:331-337, 1982.*

Chance, B. and Oshino, N.: *Kinetics and Mechanisms of catalase in peroxisomes of mitochondrial fraction. Biochem. J., 122:225-235, 1971.*

Chang, M.H., Kesner, L. and Chan, P.C.: *Degradation of lysozyme by Cu(II) and H_2O_2. Federation Proceedings, 40:1613, 1981.*

Chung, M.H., Kesner, L. and Chan, P.C.: *Degradation of articular catilage by copper and hydrogen peroxide. Agents and Actions, 1983 in press.*

Fee, J.A.: *Superoxide, superoxide dismutase and oxygen toxicity in Metal Ion Activation of Dioxygen pp. 209-237(ed. T.G. Spiro, John Wiley & Sons, INC.), 1980.*

Fong, K.L., McCay, P.B., Poyer, J.L., Misra, H.P. and Kelle, B.B.: *Evidence for superoxide-dependent reduction of Fe^{+++} and its role in enzyme-generated hydroxyl radical formation. Chem. Biol. Interactions, 15:77-89, 1976.*

Fridovich, I. *The biology of oxygen radicals. Science, 201:875-880, 1978.*

Fridovich, I.: *Hypoxia and oxygen toxicity in Advances in Neurology,pp. 255-259(ed. S. Fahn, Raven Press N.Y.), 1979.*

Gerber, D.A.: *Copper-catalyzed thermal aggregation of human gammaglobulin. Arthritis Rheumat., 17:85-91, 1974.*

Greenwald, R.A. and Moy, W.W.: *Inhibition of collagen gelation by action of the superoxide radical. Arthritis Rheumate., 22:251-259, 1979.*

Ignarro, L.J.: *Regulation of lysosomal enzyme secretion: role in inflammation. Agents and Actions, 4:241-258, 1974.*

Ingraham, L.L.: *Model copper oxidase: the cupric ion-catalyzed hydrogen peroxide oxidation of catechol, Arch. Biochem. Biophys., 81:309-318, 1959.*

Kahn, A.U.: *Singlet molecular oxygen from superoxide anion and sensitized fluorescence of organic molecules. Science, 168:476-477, 1970.*

Kollogg, III, E.W. and Fridovich, I.: *Liposome oxidation and erythrocyte lysis by enzymically generated superoxide and hydrogen peroxide. J. Biol. Chem., 252:6721-6728, 1977.*

Kim, H.W., Park, C.W., Chung, M.H. and Kim, M.S.: *Effects of xanthine and xanthine oxidase on calcium binding of cardiac sarcoplasmic reticulum. Unpublished.*

Lavelle, F., Michelson, A.M. and Dimitrijevic. L.: *Biologic protection by superoxide dismutase. Biochem. Biophys. Res. Commun., 55:350-357, 1973.*

Leipzig, L.J., Boyle, A.J. and McCan, D.S.: *Case histories of Rheumatoid arthritis treated with sodium or magnesium EDTA. J. Chron. Dis., 22:553-563. 1970.*

Lorber, A., Culter, L.S. and Chang, C.C.: *Serum copper levels in rheumatoid arthritis: Relation ship of elevated copper to protein alteration. Arthritis Rheumat., 11:65-71, 1968.*

Loshen, G., Flohe, L. and Chance, B.: *Respiratory Chain linked H_2O_2 production in pigeon heart mitochondria. FEBS Lett., 18:261-264.1971.*

Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Raudall, R.J.: *Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193:265-275, 1951.*

Lynch, R.E. and Fridovich, I.: *Effects of superoxide on erythrocyte membrane. J. Biol. Chem., 253:1838-1845, 1978.*

McCord, J.M.: *Free radical and inflammation: protection of synovial fluid by superoxide dis-*

mutase. Science, 185:529-531, 1974.

Mayeda, E.A. and Bard, A.J.: *Singlet oxygen. The suppression of its produclion in dismutation of superoxide ion by superoxide dismutase. J. Am. Chem. Soc., 96:4023-4024, 1974.*

Nathan, C.F., Brukner, L.H., Silverstein, S.C. and Cohn, Z.A.: *Extracellular cytolysis by activated macrophages and granulocytes. I. Pharmacologic triggering of effector cells and the release of hydrogen peroxide. J. Exp. Med., 149:84-99, 1979.*

Oegema, T.R., Laidlaw, J., Hascall, V.C. and Dziewiatkowski, D.D.: *The effect of proteoglycans on the formation of fibrils from collagen solutions. Arch. Biochem. Biophys., 190:698-709, 1975.*

Oh, S.M., Son, Y.S., Choi, K.S., Lim, J.K. and Chung, M.H.: *Effect of oxygen derived free redicals on brain microsomal Na^+-K^+-ATPase activity. Korean J. Pharmacol., 18: 1-14, 1982.*

Phelps, A.A., Neet, K.E., Lynn, L.T. and Putnam, F.W.: *The cupric ion catalysis of the cleavage of γ-globulin and other proteins by hydrogen peroxide. J. Biol. Chem., 236:96-105, 1961.*

Rabani, J., Klug, R.D. and Lilie, J.: *Pulse radiolytic investigation of the catalyzed disproportionation of peroxy radicals. Aqueous cupric ions. J. Phys. Chem., 77:1169-1175, 1973.*

Root, R.K., Metcalf, J., Oshino, N. and Chance, B.: *H_2O_2 release from human granulocytes during phagocytosis. I. Documentation, quantitation, and some regulating factors. J. Clin. Invest., 55:945-955, 1975.*

Sorenson, J.R.J.: *Copper complexes - A unique class of anti-arthritic drugs. Prog. Med. Chem., 15 :211-260, 1978.*

Weissmann, G. Smolen, J.E. and Korchak, H.M.: *Release of inflammatory mediators from stimulated neutrophils. New Engl. J. Med., 303: 27-34, 1980.*

Venkatasubramanian, K. and Joseph, K.T.: *Action of singlet oxygen on collagen. Indian J. Biochem. Biophys. 14:217-220, 1977.*

White, A.G., Scudder, P.R., Dormandy, T.L. and Martin, V.M.: *Copper - An index of errosive activity. Rheum. Rehabilitation, 17:3-5, 1978.*