# EFFECT OF METAL-COMPLEXING AGENTS ON THE OXYGENASE ACTIVITY OF SHEEP VESICULAR GLANDS

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## ABSTRACT

A variety of agents that are known to complex copper can reversibly inhibit the oxygenation of unsaturated fatty acids by particulate preparations from sheep vesicular glands. The time-dependent activation of the oxygenase preparation by phenol, on the other hand, was not affected by the copper chelator, diethyldithiocarbamate. The results suggest that protein-bound copper could play a role in the interaction of the oxygenase enzyme with oxygen and the fatty acid substrates. Other findings indicate that phenanthroline type compounds may inhibit both the oxygenation reaction and phenol activation by binding to an hydrophobic site on the oxygenase.

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## INTRODUCTION

The mechanism of prostaglandin biosynthesis has been studied (1-3), and the initial oxygenation resembles that catalyzed by plant lipoxygenases (4-6). Soybean lipoxygenase has recently been shown to contain ferric iron (7). However, the requirement of a metal ion for mammalian dioxygenase activity leading to prostaglandins has not yet been resolved. It has been reported that 5 x $10^{-3}$ M o-phenanthroline and diethyldithiocarbamate (DDC) gave a somewhat lower yield of $PGE_1$ in a system consisting of a lyophilized particulate fraction of sheep vesicular glands (2), although $10^{-3}$ M EDTA, 2,2'-dipyridyl, o-phenanthroline, 8-hydroxyquinoline, DDC and KCN were reported to have no effect on prostaglandin synthesis. These same chelating agents were also reported to be ineffective as inhibitors of a purified microsomal enzyme preparation (8,9). On the other hand, mercaptoethanol, dithiothreitol and dihydrolipoate inhibited the prostaglandin synthetase system isolated from bovine seminal vesicles (10), and various dithiols decreased the amount of oxidized products obtained from sheep vesicular gland preparations (11). Furthermore, the release of a rabbit aorta contracting substance (RCS) in guinea pig lungs (12), a component considered to be an intermediate of prostaglandin formation (13), has been reported to be suppressed by sulfhydryl and antioxidant compounds.

## METHODS

For the experiments in this report, sheep vesicular gland oxygenase activity was followed using an oxygen monitor as described by Smith and Lands (5). Acetone powder preparations (14) of sheep vesicular gland were homogenized (50 mg/ml) at 0°C in 0.1 M Tris-HCl (pH 8.5) containing 0.7 mM phenol and activation (5) was carried out for 30 minutes at room temperature after which the homogenate was placed in an ice bath. Studies on the unactivated oxygenase from acetone powder were carried out with powder homogenized as stated above, but without phenol.

The inhibitors listed in Table I were studied as follows. For lipoxygenase and unactivated oxygenase from acetone powder, the enzyme (4 mg of unactivated vesicular gland acetone powder or 0.02 mg of lipoxygenase) was added to an assay chamber containing 2.9 cc of 0.1 M Tris-HCl (pH 8.5). After standing for 15 sec, inhibitor was added and 15 sec later the oxygenation reaction was initiated with 23 μM 5,8,11,14-eicosatetraenoic acid (20:4).

In the case of phenol-activated vesicular gland oxygenase, 2.5 mg of the activated enzyme preparation was added to an assay chamber containing 0.66 mM phenol in 2.9 cc of 0.1 M Tris-HCl (pH 8.5). After standing for 1 min, inhibitor was added and the reaction was initiated 15 sec later by the addition of 23 μM 20:4.

Activity measurements were conducted in the above manner with all inhibitors except o-phenanthroline, 2,2'-bipyridine, neocuproine and bathocuproine sulfonate. For these three, the inhibitor was present in

the chamber before the addition of enzyme, and fifteen seconds after the addition of enzyme, 23 μM 20:4 was added to the assay chamber.

## RESULTS AND DISCUSSION

Inhibition studies were carried out with a variety of agents which are known to complex metal ions and one non-chelating agent. These are listed in Table I along with $[I]_{50}$ values* obtained from experiments on unactivated and phenol-activated oxygenase from vesicular gland and also soybean lipoxygenase. All of the compounds in Table I complex copper (15-17) except for Tiron, a $Fe^{+++}$ chelator (18) and m-phenanthroline, a non-chelator. Bathophenanthroline sulfonate, o-phenanthroline, 2,2'-bipyridine (15) and NaCN (19) also complex ferrous ions in addition to copper. The phenol-activated oxygenase activity was inhibited by all of these metal complexing agents, except Tiron, and the unactivated oxygenase activity was also inhibited by all of those tested (Table I). Therefore, copper (or perhaps ferrous iron) may be associated with normal oxygenase activity in both the activated and untreated enzyme preparations. The $[I]_{50}$ values for the activated enzyme were generally slightly lower than those for the unactivated acetone powder (Table I). Two of the complexing agents studied strongly inhibited the soybean lipoxygenase, but the mechanism for this effect is not yet apparent.

The inhibition of the activated vesicular gland enzyme by DDC was found to be reversible. Reversibility was demonstrated by observing no loss in enzyme activity (taking into consideration the percent inhibition caused by the small amount of DDC transferred to the assay chamber) when a concentrated solution of enzyme (50 mg/ml) and inhibitor (10 mM) were incubated at room temperature for various times and then diluted (60-fold) by transfer to the assay chamber for activity measurements. The inhibition of the activated oxygenase by DDC and NaCN was also reversed by adding $CuSO_4$. In addition, an unactivated enzyme preparation that was inhibited by NaCN yielded 60-80% of its initial activity after centrifugation at $9 \times 10^6$ g-min and resuspension of the pellet in 0.1 M Tris-HCl (pH 9). The results were the same when a similar experiment was done with activated enzyme. Therefore, in both cases, the cyanide complex could not remove the presumed metal ion from the protein as in the case of tyrosinase (19). This result makes it likely that the recovery of activity upon adding $Cu^{+2}$ noted above was due to complexing the inhibitor rather than replacing $Cu^{+2}$.

Tiron, a chelating agent specific for ferric ion (8), did not inhibit either the activated oxygenase or the soybean lipoxygenase. On the other hand, o-phenanthroline, which can complex $Cu^{+}$ or $Fe^{+2}$, inhibited both the activated and untreated oxygenase and 2,2'-bipyridine also inhibited the activated enzyme. We reported earlier that o-phenanthroline was a reversible inhibitor of the activated enzyme (10); however, it is still not known whether the inhibition caused by this type of

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* $[I]_{50}$ = concentration of inhibitor that gives 50% inhibition.

Table I. Effect of Metal-Complexing Agents on Oxygenase Activities

| Complexing Agent | $[I]_{50}$ (mM) | | |
|---|---|---|---|
| | Vesicular Gland (Unactivated) | Vesicular Gland (Phenol Activated) | Soybean Lipoxygenase |
| Diethyldithio-carbamate | N | 2 (12) | >50 (8) |
| Ethylxanthate | N | 25 (4) | >66 (4) |
| Bathocuproine Sulfonate | 20 (6) | 7 (5) | >30 (2) |
| Toluene-3 4-Dithiol | .1 (4) | .1 (5) | 1 (4) |
| Dithizone | N | .8 (3) | 1 (4) |
| NaCN | 2. (4) | .5 (5) | >50 (2) |
| Neocuproine | .5 (4) | 1. (5) | >10 (4) |
| Cuprizone | 6 (4) | 6 (4) | > 6 (2) |
| o-Phenanthroline | 4 (5) | 2 (4) | >10 (2) |
| 2,2'-Bipyridine | N | 8 (4) | >10 (2) |
| Tiron | N (3) | >50 (2) | >50 (2) |
| m-Phenanthroline | N | 1. (4) | >10 (2) |

( ) - number of experiments

N - could not be determined

compound is due to the binding of iron or copper or is of a hydrophobic nature as seen with alcohol dehydrogenase (21). The inhibition of the activated oxygenase by m-phenanthroline, an isomer of o-phenanthroline which cannot chelate metals, indicates that these compounds may be inactivating reversibly by binding to a hydrophobic site on the enzyme.

In addition to the reversible inhibition by o-phenanthroline, we have reported that o-phenanthroline prevented the phenol stimulated activation of the oxygenase in the acetone powder (20). Since DDC inhibited the activated oxygenase action in a reversible manner, as did o-phenanthroline, we examined its effect on the process of phenol

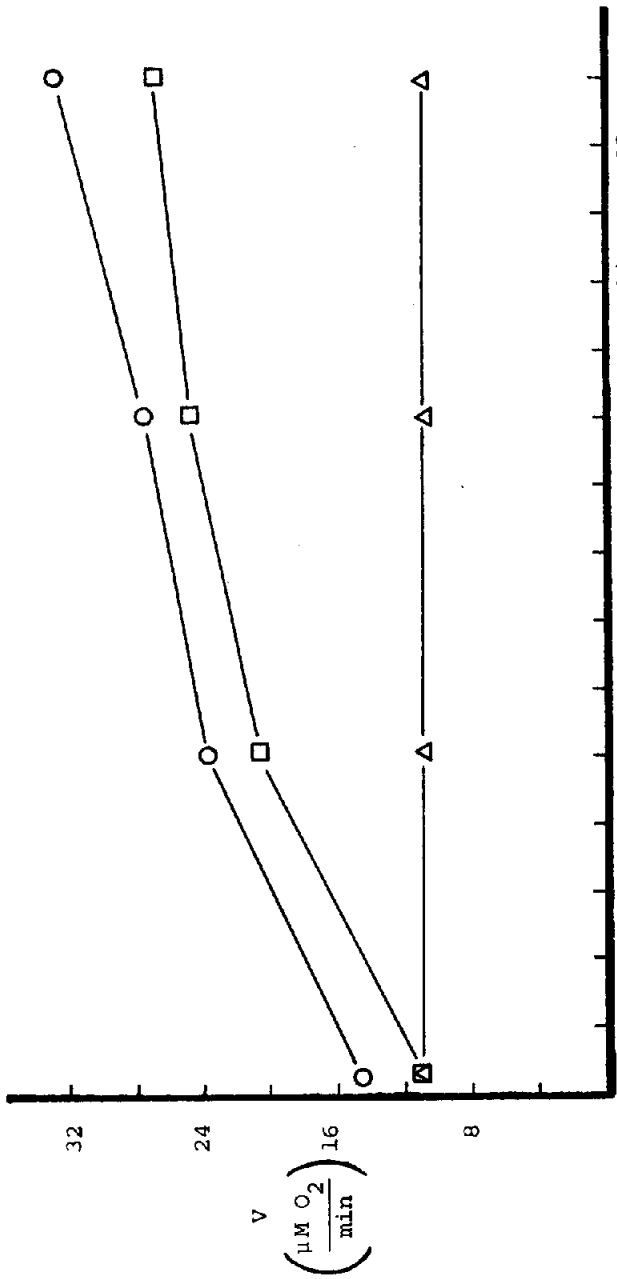


Figure 1. Phenol activation of vesicular gland oxygenase in the presence of diethyldithiocarbamate and o-phenanthroline. The acetone powder preparation of vesicular gland was homogenized (50 mg/ml) at 0°C in 0.1 M Tris-HCl (pH 8.5) and diluted to 12.5 mg/ml. The diluted protein solution was allowed to warm to room temperature for two minutes and then treated with 0.7 mM phenol (o-o); 0.7 mM phenol and 10 mM DDC (□-□); 0.7 mM phenol and 10 mM o-phenanthroline (Δ-Δ). The period of incubation was initiated when the additions were made. After the indicated times, 200 μl aliquots (2.5 mg of protein) of these solutions were transferred to assay chambers containing 0.66 mM phenol and 61 μM 5,8,11,14-eicosatetraenoic acid in 2.9 ml of 0.1 M Tris-HCl (pH 8.5) and the oxygenase activity was determined.

activation. Figure 1 shows that o-phenanthroline definitely behaves differently from DDC in that it did prevent phenol activation, whereas DDC had no effect. The difference between the initial value for the curve (o-o) and (□-□)-(Δ-Δ) indicates an inhibition of the enzyme by the low level (0.66 mM) of inhibitors that are transferred to the assay chamber from the incubated solution of enzyme. These differences would be expected from the degree of inhibition described in Table I. Moreover, m-phenanthroline also prevents phenol activation. These results suggest that o-phenanthroline and DDC can affect two different types of processes that may influence the oxygenation of fatty acids which is necessary for prostaglandin biosynthesis. The strong inhibition by DDC type agents and cuprizone noted here may be due to metal ion complexing whereas that by phenanthroline and other aromatic hydrocarbons (22,23) may be at a hydrophobic site.

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