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# An Fe (III) - initiated oxidase reaction with ferroxidase-II

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# AN FE(III)-INITIATED OXIDASE REACTION WITH FERROXIDASE-II

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# BIOGRAPHICAL SKETCH

Glenn Alexander Hegamyer was born on June 14, 1950 in Roanoke Rapids, North Carolina to Leila Murray and Oscar William Hegamyer. He completed his primary and secondary education in Roanoke Rapids.

After high school, he attended the University of Richmond where he majored in Chemistry and from which he received the Bachelor of Arts degree in June of 1972.

In September of 1972, he re-entered the University of Richmond as a special student in the Department of Chemistry. In September of 1973 he became a degree candidate under the direction of Dr. Richard W. Topham. The author was awarded a Puryear Fellowship during the course of this study. This work was partially supported by a Grant-in-aid from Research Corporation and a faculty research grant from the University of Richmond.

In September of 1974, the author began work as a chemist for the Viral Oncology branch of the National Cancer Institute.

On December 21, 1974 he married Alison Anne Wagner in Annandale, Virginia.

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A special thanks is extended to Mrs. Topham for her hospitality during the authors many trips to Richmond. Deep appreciation is also expressed to his loving wife and parents for their encouragement throughout the course of this study.

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## ABSTRACT

A reaction involving iron as an intermediary electron carrier for the coupling of electron transfer from an endogenous substrate to ferroxidase-I has been proposed.

The enzymic nature of the reaction has been established and studies with iron chelating agents have demonstrated the involvement of Fe(III). The kinetic parameters of ferroxidase-II with Fe(III) were found to be quite similar to those previously established for Fe(II). The participation by the copper associated with ferroxidase-II was ascertained by use of the copper chelating agent, diethyl-dithio-carbamate.

A variety of substances normally found in blood were surveyed as possible candidates for the endogenous substrate but none were oxidized by ferroxidase-II in a coupled reaction with Fe(III). Compiete loss of the Fe(III)-initiated oxidase activity was observed when ferroxidase-II was treated with phospholipase C and passed over a column of Agarose A-5m. This procedure removes the bound lipid from ferroxidase-II and the loss of activity observed suggested that the endogenous substrate was a lipid or was dissolved in the lipid matrix of the enzyme. Attempts to reconstitute this activity with lipid extracts of ferroxidase-II were unsuccessful. The endogenous substrate responsible for the Fe(III)-initiated reaction was not definitely identified; however, significant progress towards its characterization was made.

# LITERATURE REVIEW

The element copper is fundamental to the life processes of both plants and animals. Copper has long been a familiar element but its presence in biological systems has been known for only 150 years. One of the reasons its presence has gone undetected until recent times is that copper deficiency is rare in plants and has never been observed in animals. This is due to the natural abundance of copper in the diet. An excess of copper could be toxic but the protective mechanisms in the body restrict the absorption of copper and other metals.

Copper's importance arises from the fact that it is a required constituent of numerous essential proteins and enzymes. Although the amount of copper required by organisms is quite small, it is indispensable to the life processes. The adult human body contains only about 100 milligrams of copper, a small amount, but one which is vital to our existence.

In living systems it is very difficult to determine precise forms and locations of copper. This is true because copper is seldom found in any biological compound less complicated than a protein. Nearly all of the 100 milligrams of copper in the human body is incorporated into proteins. A few of the copper proteins are given in Table I.

There are primarily three properties which make copper so ideally suited for metabolic processes(1):

1. copper ions react with amino acids or proteins more strongly than other metal ions do
2. copper is an exceptionally effective catalytic agent
3. copper's ionization states have flexible properties that qualify it uniquely for metabolic functions

The element copper is a member of the first transition series. Several of the elements of this series share these three important properties

TABLE I

IMPORTANT COPPER PROTEINS AND ENZYMES

| Name | Where Found | mol.wt $10^3$ gm | Biochemical Function |
|---|---|---|---|
| Cytochrome oxidase | Most plants and animals | 70 | Terminal oxidase |
| Hemocyanin | Mollusk and arthropod plasma | 450-6,680 | Oxygen carrier |
| Ceruloplasmin | Plasma of most animals | 151 | Fe(III)-transferrin formation, Copper transport |
| Amine oxidase | Most animals, etc. | 225 | Elastin, collagen formation |
| Tyrosinase | Animal skin, melanoma, insects, plants | 35<br>100 | Tyrosine oxidation, skin pigment (melanin) formation |
| Dopamine-β-hydroxylase | Adrenals | 290 | Epinephrine biosynthesis |
| Galactose oxidase | Molds | 75 | Galactose oxidation |
| Erythrocuprein | Red blood cells of most animals | 34 | Superoxide dismutase |
| Ascorbic acid oxidase | Many plants | 150 | Terminal oxidase |
| Plastocyanin | Algae, green leaves and other plants | 21 | Photosynthesis |

and also serve as biological trace elements. Although they do serve as trace elements, each is very specific in its function.

Research concerning the biological role of copper has greatly expanded since the recognition of its involvement in a number of important physiological processes(2,3,4). The best known of these is copper's involvement in hemoglobin formation(5,6,7).

The principal metabolic disturbances arising as a result of copper deficiency are as follows(8):

1. reduction in the absorption and utilization of iron leading to hypochromic and microcytic anemia and a deficiency of heme enzymes including cytochrome oxidase
2. a defect in phospholipid biosynthesis resulting in demyelination and "swayback" disease in lambs
3. impairment of osteoblastic activity leading in some animals to skeletal changes similar to those reported in scurvy
4. abnormalities in keratin and pigment formation resulting in wool defects in lambs and achromotrichia in rats

In 1928, Hart et al(5) were the first to report anemia in copper-deficient animals. Since the early 1950's, Cartwright, Wintrobe, and their associates at the University of Utah School of Medicine have done extensive research on this defect in iron metabolism. Their studies led them to propose a role for copper in the biosynthesis of hemoglobin(7,9). They theorized that copper could act at any of the three main lines of hemoglobin biosynthesis: the biosynthesis of protoporphyrin of heme, the utilization of iron, or the biosynthesis of globin. Earlier work by Lee et al(10) showed that as anemia developed there was an increase in the activity of heme biosynthetic enzymes. Thus, it did not appear that copper acted at this point of hemoglobin biosynthesis. Since there was no evidence for impairment of the biosynthesis of globin, they concluded that copper was important in the proper utilization of iron(10). In 1966, Osaki

et al reported on the possible significance of the ferroxidase activity of a copper protein, ceruloplasmin. They proposed that its catalytic activity involved promoting the rate of iron saturation of transferrin and iron utilization in the plasma(11).

Ceruloplasmin was first isolated and characterized in 1947 by the Swedish biochemists Holmberg and Laurell(12). They found the protein to have a molecular weight of approximately 151,000 and a copper content of eight atoms per molecule. Ceruloplasmin is found in the plasma of most vertebrates and normally accounts for over 98% of the serum copper.

Since ceruloplasmin had greater than 98% of the serum copper bound to it, many believed that ceruloplasmin was directly involved in the transfer of copper. To test this hypothesis, a radioactive copper salt was injected into a human body. The copper first appeared in combination with the serum albumin in the bloodstream. From there it was rapidly absorbed by the liver and finally reappeared in the serum in the form of ceruloplasmin(1). Thus, ceruloplasmin could be a convenient storage form of copper ion, thereby preventing its interaction with other proteins.

In later studies of ceruloplasmin, Holmberg and Laurell found oxidase activity in vitro towards many substrates. They observed maximum substrate activity with p-phenylenediamine(PPD). They also found the activity of ceruloplasmin to be markedly affected by anions(14). Walaas and coworkers (15,16) have reported that catecholamines serve as substrates for ceruloplasmin although their reaction rates are slower than those observed with PPD. In 1958 Broman reported inhibition of crude ceruloplasmin by dicarboxylic acids and ethylenediaminetetraacetic acid(17). In 1960, Curzon reported the results of his studies on the effects of some anions, cations and chelating agents on the oxidase activity of purified ceruloplasmin.

He observed that low concentrations of metal ions, in particular Fe(II), increased the activity of ceruloplasmin. In similar studies Curzon observed inhibition of activity at higher metal ion concentrations(18). In 1960, Curzon and O'Reilly(19) noted the catalytic oxidation of Fe(II) by ceruloplasmin. Their data indicated a coupled iron-ceruloplasmin oxidation system to be involved.

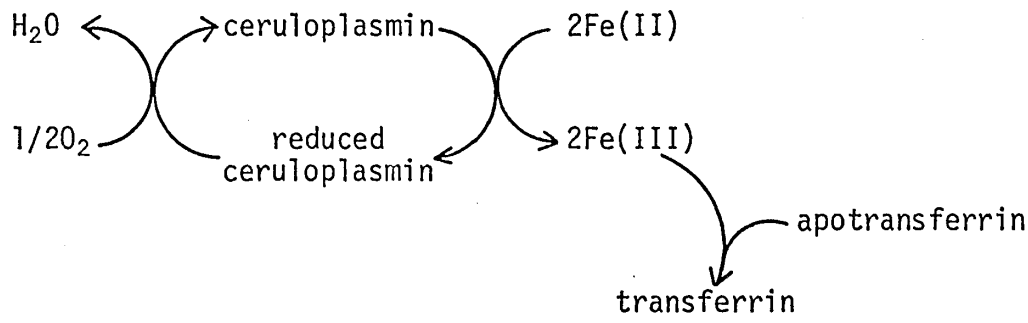
S. Osaki(20) studied the kinetics of the catalysis of Fe(II) oxidation by crystalline human ferroxidase. At the pH optimum of 6.5 Osaki obtained two $K_m$ values with respect to Fe(II). Competition between the two substrates PPD and Fe(II) was also observed(20).

Huber and Frieden(21) found that the ceruloplasmin catalyzed oxidation of Fe(II) and aromatic diamines exhibited nonlinear kinetics in Eadie-Hofstee plots. They accounted for the kinetics of Fe(II) oxidation by a mechanism based on substrate activation.

In a subsequent study, Huber and Frieden studied the inhibition of ferroxidase by trivalent and other metal ions. They observed inhibition with all trivalent ions tested but strongest inhibition was from those ions which had an ionic radius of 0.81A or less(22).

As reported earlier, Osaki et al(11) were interested in the role of ceruloplasmin in hemoglobin biosynthesis. In 1966, they reinvestigated the problem of serum Fe(II) oxidation under conditions approaching the state in vivo. They estimated the nonenzymic oxidation of Fe(II) to be insufficient to account for a rate of Fe(III)-transferrin formation necessary to provide adequate iron supply for hemoglobin and other biosyntheses requiring Fe(II). These results suggested an appreciable catalytic activity was involved in Fe(II) oxidation in serum. From their work they proposed a biological role for ceruloplasmin in converting Fe(II) to Fe(III),

thereby promoting the rate of incorporation of Fe(III) into apotransferrin. This leads directly to the biosynthesis of hemoglobin and other iron-utilizing systems(23). The possible role of ceruloplasmin is illustrated below(11):




The ferroxidase activity of ceruloplasmin explains its broad capacity to catalyze the oxidation of a variety of reducing agents, all of which react with ferric ion. Thus it was proposed(11) that when describing the enzymic activity of ceruloplasmin, it be designated as a ferro-$O_2$-oxidoreductase or simply ferroxidase. Thus, the term ferroxidase is often used to describe ceruloplasmin's catalytic activity and its specific role in iron mobilization.

Johnson, Osaki and Frieden reported a very strong positive linear correlation between PPD oxidase activity and ferroxidase activity(24). With the exception of Wilson's disease sera, this correlation was observed in normal as well as in pathological sera.

There is now considerable evidence supporting the proposal that ceruloplasmin is the key link between iron and copper metabolism. The best evidence comes from Ragan et al(25) at the University of Utah School of Medicine. They observed a rapid rise in plasma iron upon injection of ceruloplasmin in in vivo studies with copper-deficient pigs.

Subsequent studies by Cartwright, Lee and coworkers have demonstrated that a rise in serum ferroxidase activity precedes a rise in serum iron

following copper injection. This rise in plasma iron concentration was followed by a gradual rise in the hemoglobin concentration of the blood stream(26). These experiments at the University of Utah indicated that the defect in the release of iron in the copper-deficient animal is quickly reversed by intravenous ceruloplasmin(13). Osaki et al confirmed these results in their studies of iron mobilization from perfused mammalian livers(27,28).

Although the evidence for the role of ceruloplasmin in iron mobilization was quite strong(11,13,25,26,27,28), there was no explanation for the normal iron mobilization found in Wilson's disease (Hepatolenticular Degeneration).

In Wilson's disease, the serum is largely depleted of ceruloplasmin and copper diffuses into the tissues. The copper may then accumulate to high levels, especially in the liver and brain. If ceruloplasmin were the only ferroxidase in the sera, the Fe(II) oxidase activity would be expected to be reduced and a defect in iron metabolism should be observed. Johnson et al observed reduced PPD oxidase activity but there was still an appreciable Fe(II) oxidase activity(24). Thus, despite the reduction in ceruloplasmin, sufficient ferroxidase activity remained to prevent anemia(29). This find- indicated the possibility of the presence of a modified ferroxidase or an additional unknown catalyst(29).

In 1970, Topham and Frieden announced the identification and purification of a second serum ferroxidase which they designated ferroxidase-II. They isolated this non-ceruloplasmin ferroxidase protein from normal as well as Wilson's disease sera(30).

Some of the properties distinguishing ferroxidase-II from ceruloplasmin are given in Table II(30). Three of the primary differences are that

TABLE II

PROPERTIES DISTINGUISHING FERROXIDASE-II FROM CERULOPLASMIN

| PROPERTY | CERULOPLASMIN | FERROXIDASE-II |
|---|---|---|
| Inhibition with 1mM Azide | > 98% | None |
| Apparent $K_m$ values for Fe(II) | 2 | 1 |
| Specific activity ($A_{460}$ per 10min per mg of protein) | 30 | 4.9 |
| p-Phenylenediamine oxidase activity | Yes | None |
| Elution after chromatography[a] | .2 to .3M NaCl required | No NaCl required |
| Color of purified preparation | Blue | Yellow |
| Molecular Weight | 151,000 | > 800,000 |
| Copper content(millimicromoles of copper per mg of protein) | 44 | 12 |
| Immunoelectrophoretic migration from origin | Large | Small |
| Effect of dialysis[b] | ∿ 35% remaining activity | 100% activity |

[a]Adsorption on DEAE-Sephadex; eluted with .05M Acetate buffer, pH 5.5, with or without .2 to .3M NaCl

[b]Dialysis for 2 days at 4°C against .05M Acetate buffer, pH 5.5

ferroxidase-II:

1. is yellow rather than blue as ceruloplasmin
2. is not inhibited by azide
3. exhibits no PPD oxidase activity

Topham observed that oxygen consumption with ferroxidase-II occurred simultaneously with iron oxidation.

Topham and Frieden found that although ferroxidase-II was decreased in Wilson's disease serum it was reduced to a lesser extent than ferroxidase-I. They observed the total ferroxidase activity of Wilson's disease serum to be approximately 5 to 10% of that of normal serum(30). Osaki et al(28) had previously reported that only about 10% of the total ferroxidase activity of normal serum was necessary for maximum iron mobilization response from the liver. Thus, the total ferroxidase activity of Wilson's disease serum accounts for the maintenance of near normal iron metabolism(30).

Gel filtration data and disc gel electrophoresis indicated that ferroxidase-II had a molecular weight greater than 800,000. Immunoelectrophoretic mobility, the oil red-staining property, and the large molecular weight suggested that it might be a serum lipoprotein(30).

Sung and Topham(31,32) have recently reported the identification and quantitative analyses of the lipid components bound to ferroxidase-II. They found ferroxidase-II to contain lipids both in the form of cholesterol and phospholipid. Thin-layer chromatographic analyses were carried out to determine which individual phospholipids were bound to ferroxidase-II. Their findings indicated that phosphatidyl choline accounted for most of the bound phospholipid with small amounts of sphingomyelin and lysophosphatidyl choline accounting for most of the remaining phospholipid(31,32,33).

They also found that intact phospholipids appear essential for the enzymic activity of ferroxidase-II to be maintained.

Topham et al(34,35) have reported a detailed study of the kinetics of Fe(II) oxidation by ferroxidase-II. Their findings are presented in Table III. Their results suggest that ferroxidase-II would be capable of functioning as an alternative for ferroxidase-I in human sera and as the major ferroxidase in the sera of species having low ferroxidase-I levels.

TABLE III

COMPARISON OF THE KINETIC PARAMETERS OF FERROXIDASE-I AND FERROXIDASE-II

| PROPERTY | FERROXIDASE-I | FERROXIDASE-II |
|---|---|---|
| pH optimum | 6.5 | 7.2 |
| $K_m$(s) for Fe(II) (μM) | 0.6, 50 | 14 |
| $K_m$ for $O_2$ (μM) | 9.0 | 4.1 |
| Molar activity (μM Fe (III)/min)/(μM enzyme) | 550 | 525[a] |
| Activity per Cu atom (μM Fe(III)/min)/(Cu atom) | 69 | 53 |

[a]Calculated assuming a molecular weight of $8 \times 10^5$ which was estimated from gel filtration and disc-gel polyacrylamide electrophoresis(32).

# INTRODUCTION

Huber and Frieden(22) previously reported that certain metal ions markedly influence the ferroxidase reaction catalyzed by ferroxidase-I. Substrate activation was observed with iron, whereas inhibition was observed with certain trivalent and other metal ions. These studies provided considerable insight into the molecular mechanism of the ferroxidase reaction catalyzed by ferroxidase-I.

This thesis proposed to undertake similar experiments with ferroxidase-II in the hopes of 1) further differentiating ferroxidase-II from ferroxidase-I and 2) providing information concerning the molecular mechanism of the ferroxidase reaction catalyzed by ferroxidase-II.

# EXPERIMENTAL METHODS

## Isolation and Purification of Ferroxidase-II from Human Plasma

Pure ferroxidase-II was prepared from the Cohn IV-1 fraction of human plasma. The method was an adaptation of the procedure used previously by Topham and Frieden (30). This plasma fraction contains ceruloplasmin (ferroxidase-I) and ferroxidase-II as well as other contaminating proteins but contains only 5% of the total protein of blood plasma. Thus, Cohn IV-1 is an excellent starting material for the purification of ferroxidase-II. Five grams of lyophilized Cohn IV-1 (obtained from the E. R. Squibb and Sons, Inc., New York, New York) was extracted with 100 ml of 0.05 M acetate buffer, pH 5.5, overnight at 4° with gentle continuous stirring. In order to remove any undissolved material, the resulting extract was centrifuged at 25,000 x gravity for 30 minutes at 4°. The supernatant was then decanted through a thin layer of fine glass wool to remove the floating lipid layer.

At pH 5.5 ferroxidase-I and ferroxidase-II differ greatly in charge. At this pH, ceruloplasmin is highly negatively charged; however, ferroxidase-II bears little charge. Thus, these two proteins may be completely separated by chromatography on an anion exchange resin, DEAE-Sephadex A-50. The entire extract of Cohn IV-1 was chromatographed on a DEAE-Sephadex A-50 column (4 cm x 10 cm). The column was washed with 0.05 M acetate buffer, pH 5.5. Ferroxidase-II was eluted immediately following the void volume with 0.05 M acetate buffer, pH 5.5. Ferroxidase-II came off the column as a turbid yellow solution. Ferroxidase-I remained as a small blue-green band at the top of the column. The ferroxidase-II containing solution was then concentrated to 30 ml by ultrafiltration in an Amicon ultrafiltration cell equipped with a Diaflo PM-30 membrane.

For further purification, the concentrated ferroxidase-II solution was applied to an Agarose A-50 M column (4 cm x 55 cm). Agarose A-50 M is a molecular sieving gel for proteins of molecular weight $10 \times 10^4 \rightleftarrows 50 \times 10^6$. Two bands having ferroxidase activity were eluted with 0.05 M acetate buffer, pH 5.5. The first band was yellowish and very turbid and contained 10 - 12% of the ferroxidase-II activity. Approximately 90% of the ferroxidase-II activity was eluted as a second band which was clear yellow in color. This band was collected, concentrated to approximately 30 ml and passed through another Agarose A-50 M column (4 cm x 55 cm). A clear yellow protein band possessing ferroxidase-II activity was eluted from this column. This band was then concentrated to a final protein concentration of approximately 20 mg/ml.

Quantitative Determination of Protein

Protein concentrations were determined by the method of O.H. Lowry (36). This is a spectrophotometric assay combining the use of the biuret and phenol reagents. There are basically two steps which lead to the final color with the protein. In the biuret reaction, the protein reacts with a strongly alkaline, but dilute solution of copper sulfate producing a violet color. The second step involves the reduction of the phosphomolybdic-phosphotungstic reagent (Phenol Reagent) by the copper-treated protein. The reduction of this reagent is due to the tyrosine and tryptophane present in the protein. This method has the advantages of being rapid, sensitive and specific for protein. The limit of detection is 5 μg/ml with a sensitivity 100 times that of the biuret reaction and 10-20 times the value obtained by measurement of the ultraviolet absorption at 280 nm.

In the analysis, duplicate one ml samples of a bovine serum albumin

solution (0.2 mg/ml) and 1 ml of a distilled water blank were placed into colorimetric tubes. The solution of unknown protein concentration was diluted 1:10 (v/v) and 1 ml was placed into a colorimetric tube. Five ml of Lowry B solution (2% $Na_2CO_3$; 0.1 M NaOH; 0.01% $CuSO_4$; 0.02% sodium tartrate) were then added to the tubes which were mixed and allowed to stand ten minutes. After ten minutes, 0.5 ml of phenol reagent (Phipps and Bird, Richmond, Virginia; diluted 1/1 with water) was added to each tube. Maximum color was developed in one hour, at which time, the absorbance of each tube at 600 nm was recorded.

Method of Assay of Enzymic Activity

Ferroxidase assays were carried out using the Gilson Model K-ICC-RP Oxygraph with a Clark-type polarographic oxygen electrode. The basis of the procedure is illustrated in the following equations:

(1) $Fe^{2+} + E_{ox} \rightarrow Fe^{3+} + E_{red}$

(2) $E_{red} + {}^1/_2\ O_2 \rightarrow E_{ox} + H_2O$

The cell containing the electrode system was maintained at 30°C with a Haake FJ thermostatted bath. The instrument was standardized at 30°C assuming the oxygen concentration of air saturated water to be 236 micromolar (35).

The standard polarographic assay procedure for ferroxidase activity was conducted in the manner outlined below. The reaction mixture used for assays contained 7 parts by volume of 0.6 M acetate buffer, pH 6.0 (final concentration 0.2 M), 6 parts by volume of a $4 \times 10^{-4}$ M ferrous ammonium sulfate solution (final concentration 120 micromolar), and 5 parts by volume of distilled water. For each assay, 2 ml of the reaction mixture was pipetted into the reaction vessel and allowed to equilibrate.

Once a steady base line was established, 50 µl of the enzyme preparation was injected and the rate of oxygen consumption was recorded. The specific activity was recorded as micromoles of oxygen consumed per minute per mg protein.

In order to study the enzymic activity with the ferric ion, it was necessary to make a slight modification of the above procedure. For these analyses, a reaction mixture containing 0.6 M acetate buffer, pH 6.0 diluted 1:3 (v/v) was used. A series of standard iron (III) solutions were made and varying amounts of these were injected into the reaction vessel. As before, the specific activity was recorded as micromoles of oxygen consumed per minute per mg protein.

Any variations from the standard procedure will be specifically enumerated in the legends to the figures or tables resulting from these special experiments.

Treatment of Ferroxidase-II with Phospholipase C

Phospholipase C treatments were performed according to the method of Topham and Gaylor (37) and Sung and Topham (32). Four ml of purified ferroxidase-II (18 mg/ml), 0.7 mg of phospholipase C (Type 1) and 0.145 ml of 0.4 M $CaCl_2$ were incubated for two hours at 30°. Controls containing purified ferroxidase-II, $CaCl_2$, but no phospholipase C were incubated under identical conditions. After incubation, the ferroxidase activities of the samples and of the controls were determined.

Spectral Studies of Fe(III) Reduction by Ferroxidase-II

Spectral studies of the enzyme catalyzed reaction were performed using a Perkin-Elmer Model 450 UV-Visible NIR spectrophotometer. The instrument was used in the double beam mode.

These experiments were designed to determine whether Fe(III) was being reduced in a coupled reaction with ferroxidase-II. The basic components of the reference and sample cells were as follows:

Reference cell: reaction mixture (0.2 M Acetate buffer, pH 6.0) containing 1 x $10^{-2}$ M α,α'-dipyridyl or 1 x $10^{-2}$ M 1,10-phenanthroline, 1 x $10^{-3}$M Fe(III), 100 µl of 0.05 M Acetate buffer, pH 5.5

Sample cell: reaction mixture (0.2 M Acetate buffer, pH 6.0) containing 1 x $10^{-2}$ M α,α'-dipyridyl or 1 x $10^{-2}$ M 1,10-Phenanthroline, 1 x $10^{-3}$ M Fe(III), 100 µl(0.91 mg) ferroxidase-II

Control spectra were run to determine the absorbance of the various components of the reference and sample cell.

To determine the absorbance of ferroxidase-II, the reference cell contained the reaction mixture and 100 µl of 0.05 M Acetate buffer, pH 5.5. In the sample cell, 100 µl(0.91 mg) of ferroxidase-II was substituted for the Acetate buffer.

The possible absorbance by Fe(III) and ferroxidase-II was examined by addition of 1 x $10^{-3}$ M Fe(III) to the sample and reference cell used to determine the absorbance of ferroxidase-II.

To look at the absorbance due to the chelating agents and the enzyme, the sample and reference cells included the following:

Reference cell: reaction mixture (0.2 M Acetate buffer, pH 6.0) containing 1 x $10^{-2}$ M α,α'-Dipyridyl or 1 x $10^{-2}$ M 1,10-Phenanthroline, 100 µl of 0.05 M Acetate buffer, pH 5.5

Sample cell: reaction mixture (0.2 M Acetate buffer pH 6.0) containing 1 x $10^{-2}$ M α,α'-Dipyridyl or 1 x $10^{-2}$ M 1,10-Phenanthroline, 100 µl(0.91 mg) ferroxidase-II

Treatment of Ferroxidase-II with Diethyl-Dithio-Carbamate (DTC)

In order to bind the copper associated with ferroxidase-II, the enzyme was treated with the copper chelating agent, DTC. A stock

solution of $10^{-2}$ M DTC was made in 0.05 M acetate buffer pH 5.5. This solution was added to the purified ferroxidase to yield a final DTC concentration of $10^{-3}$ M. After standing, the mixture was dialyzed against acetate buffer to remove excess chelating agent.

Metal Inhibition Studies

To remove trace amounts of contaminating metals, all glassware and containers were washed with concentrated HCl, then rinsed several times with glass-distilled water.

Routinely, the reaction mixture (0.6 M acetate buffer diluted 1→3) was placed in the reaction cell and allowed to equilibrate. After equilibration, 50 µl(1.04 mg) of ferroxidase-II and varying amounts of standard Fe(III) solutions were added and allowed to incubate for two minutes. Next, Fe(II) was added (120 µM final concentration) and the reaction was allowed to proceed. Throughout this procedure, the activity was monitored as micromoles of oxygen consumed per minute.

Extraction of Lipids from Ferroxidase-II

The lipid components of ferroxidase-II were isolated and purified by the procedure of Folch et al (38). Routinely, lipids were extracted from 10 ml of ferroxidase-II by stirring overnight with 200 ml of chloroform/methanol (2:1, v/v). The extract was then washed with 50 ml of distilled water. This resulted in a two phase system with the chloroform (lower) phase containing the extracted lipids. The washing step was repeated until the chloroform layer appeared clear. If an emulsion formed during the washing procedure, solid NaCl was added as a dispersing agent. The chloroform layer was evaporated to 15 ml and an equal volume of 95% ethanol was added. This mixture was evaporated to dryness in a tared

flask under a stream of nitrogen. The flask was then reweighed and the yield was determined.

A stock solution of lipids was prepared by dissolving the extracted lipids in 10 ml of chloroform/methanol (2:1, v/v). For each experiment, a quantity of the stock solution was removed, evaporated to dryness and dissolved in the desired solvent. Controls with the solvent in the absence of the extracted lipids were also run.

# RESULTS

Frequently, end product inhibition is a mechanism by which cells control the rate of certain metabolic reactions. Therefore, the logical starting point in the investigation of the effect of metal ions on ferroxidase-II seemed to be a study of the effect of Fe(III). Thus, Fe(III) was initially examined as a possible inhibitor of ferroxidase-II.

## Interaction of Ferroxidase-II with Fe(III)

Unexpectedly, oxygen consumption was observed immediately following the injection of Fe(III) (Figure 1). This observation suggested that Fe(III) initiated an oxidase type reaction with ferroxidase-II. Since Fe(III) is the highest stable oxidation state of iron, the further oxidation of Fe(III) by ferroxidase-II would be extremely unlikely. Thus, it appeared that Fe(III) must be initiating the oxidation of some substance endogenous in the ferroxidase-II preparation. Additional studies were undertaken to ascertain why Fe(III) initiated oxygen consumption with ferroxidase-II.

## Enzymic Nature of the Fe(III) Reaction

If the Fe(III)-initiated reaction with ferroxidase-II is indeed an enzymic process, then saturation kinetics should be observed for the reaction and heat treatment should result in the loss of the Fe(III)-initiated activity.

A plot (Figure 2) of the specific activity of the Fe(III)-initiated reaction versus the substrate concentration did exhibit saturation kinetics. Treatment of ferroxidase-II at 100° for 5 minutes resulted in the complete loss of the Fe(III)-initiated reaction.

The determination of the basic kinetic parameters of the Fe(III)-initiated reaction seemed to be the next logical step.

FIGURE 1

Time Course of Oxygen Consumption with the Fe(III)-initiated Reaction with Ferroxidase-II

The reaction mixture contained 50 µl (1.04 mg) of purified ferroxidase-II and a final Fe(III) concentration of $1 \times 10^{-3}$ M.

The same activity was observed regardless of the order of addition of iron and ferroxidase-II.

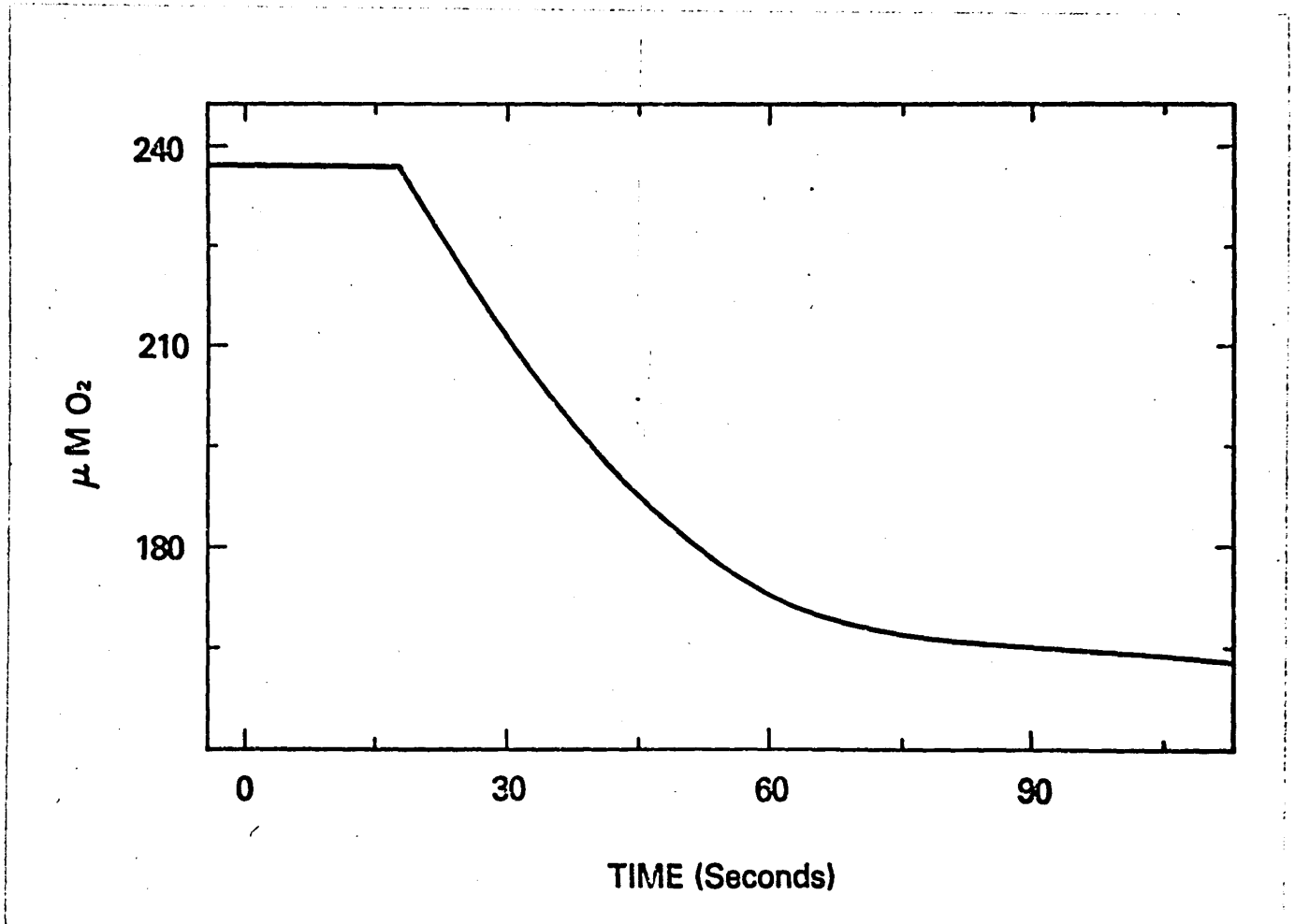
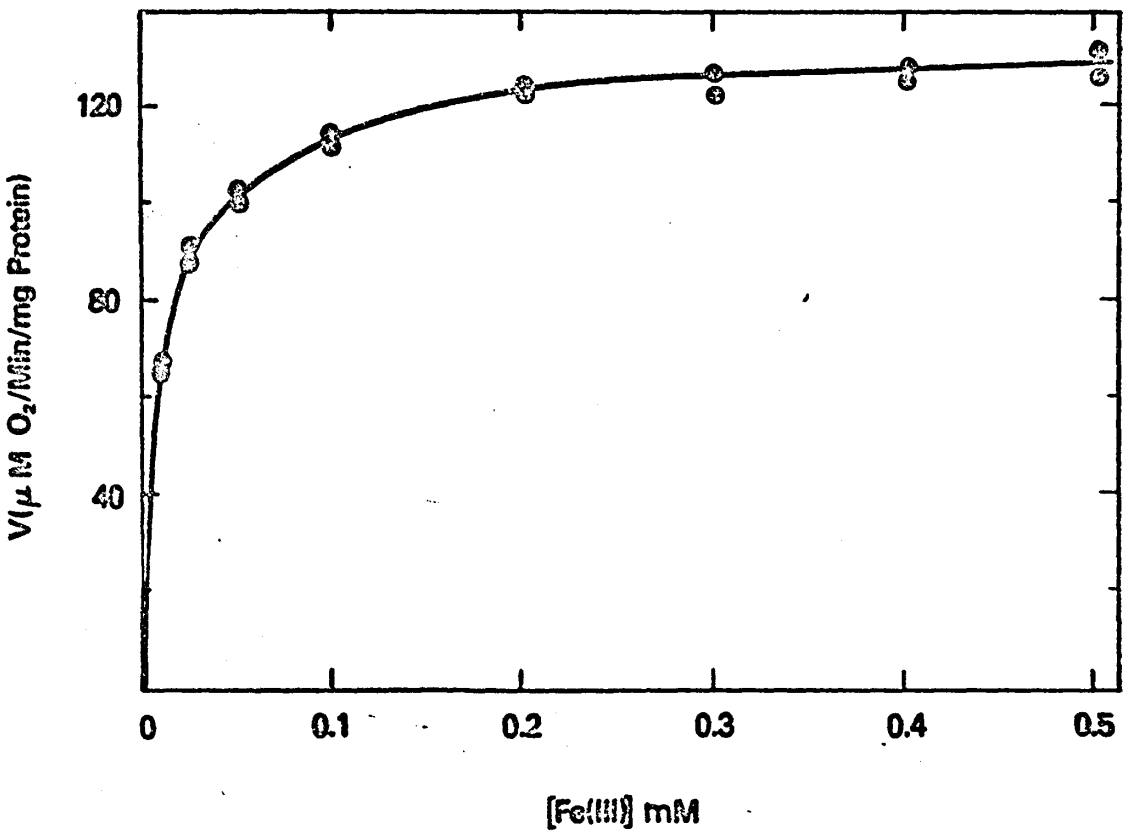

240
210
180
μM O₂
0
30
60
90
TIME (Seconds)

FIGURE 2

Velocity of the Ferroxidase Reaction as a Function of Substrate Concentration

Ferroxidase activities were carried out as described in "Experimental Methods". The reaction mixture contained 50 µl (0.25 mg) of purified ferroxidase-II and varying concentrations of Fe(III). Two entirely separate experiments were done and in each experiment multiple determinations were done at each substrate concentration.


120
80
40
0
0.1
0.2
0.3
0.4
0.5
V(μ M O2/Min/mg Protein)
[Fe(III)] mM

Effect of pH on the Fe(III) Reaction with Ferroxidase-II

The pH optimum for the Fe(III)-ferroxidase-II reaction was determined by measuring the initial rate of oxygen consumption as a function of pH.

Reaction mixtures were adjusted to specific pH values prior to the addition of ferroxidase-II or Fe(III). Since the Fe(III) solutions were prepared in 0.1 N HCl, it was necessary to remeasure the pH following the injection of Fe(III). Maximum oxygen consumption was observed at a pH of 7.0 (Figure 3).

Determination of Steady-State Kinetic Parameters for the Fe(III)-Initiated Reaction

To determine the $K_m$ for Fe(III) for ferroxidase-II, the initial rate of oxygen consumption was measured at various Fe(III) concentrations. An Eadie-Hofstee plot (v/s vs v) yielded a linear relationship (Figure 4). The kinetic parameters calculated from this plot were $K_m$ Fe(III) = 10 μM and $V_{max}$ = 127 μM $O_2$/min/mg protein.

In order to determine the best fitting straight line, a linear regression analysis was performed using the Wang Model 600 programmable calculator in conjunction with the x,y plotter.

The steady-state kinetic parameters obtained for Fe(III) are compared to those previously determined for Fe(II) in Table 1.

Metal Survey

Studies were performed to determine whether other metals were capable of initiating oxygen consumption in the same manner as Fe(III).

As seen in Table 2, no other metal ion tested initiated oxygen consumption with ferroxidase-II.

After each of these ions was added, Fe(III) was introduced into the

FIGURE 3

Effect of pH on the Fe(III) reaction with Ferroxidase-II

Enzymic assays were performed as described in "Experimental Methods". The assay mixture contained 1 x $10^{-3}$ M Fe(III), 50 µl (0.909 mg) of purified ferroxidase-II and 0.2 M acetate buffer pH 3.85 to 7.42 in a final volume of 2 ml. The range of the specific activities of several trials at each pH is shown but the lines are drawn through the average specific activity at each pH.

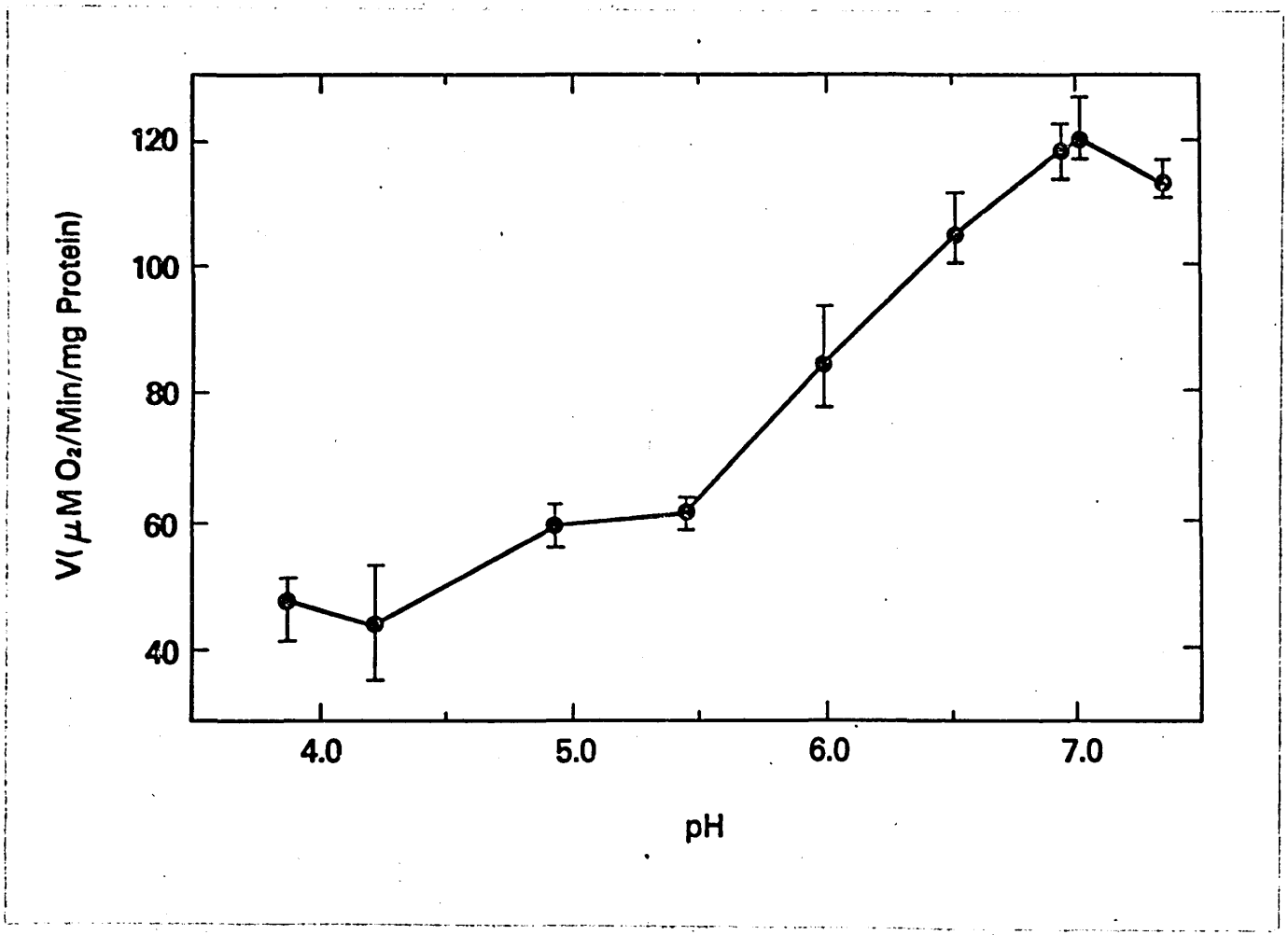

120
100
80
60
40
V(μM O₂/Min/mg Protein)
4.0
5.0
6.0
7.0
pH

FIGURE 4

Eadie-Hofstee Plot of the Steady-State Kinetic Data for Fe(III)

Enzymic assays were performed as described in "Experimental Methods". Each assay mixture contained 50 µl (0.25 mg) of the purified ferroxidase-II solution, 0.2 M acetate buffer, pH 6.0, and concentrations of Fe(III) from 0.01 mM to 0.3 mM. The best fitting straight line was determined by a linear regression analysis performed on the Wang Model 600 programmable calculator in conjunction with the x,y plotter.

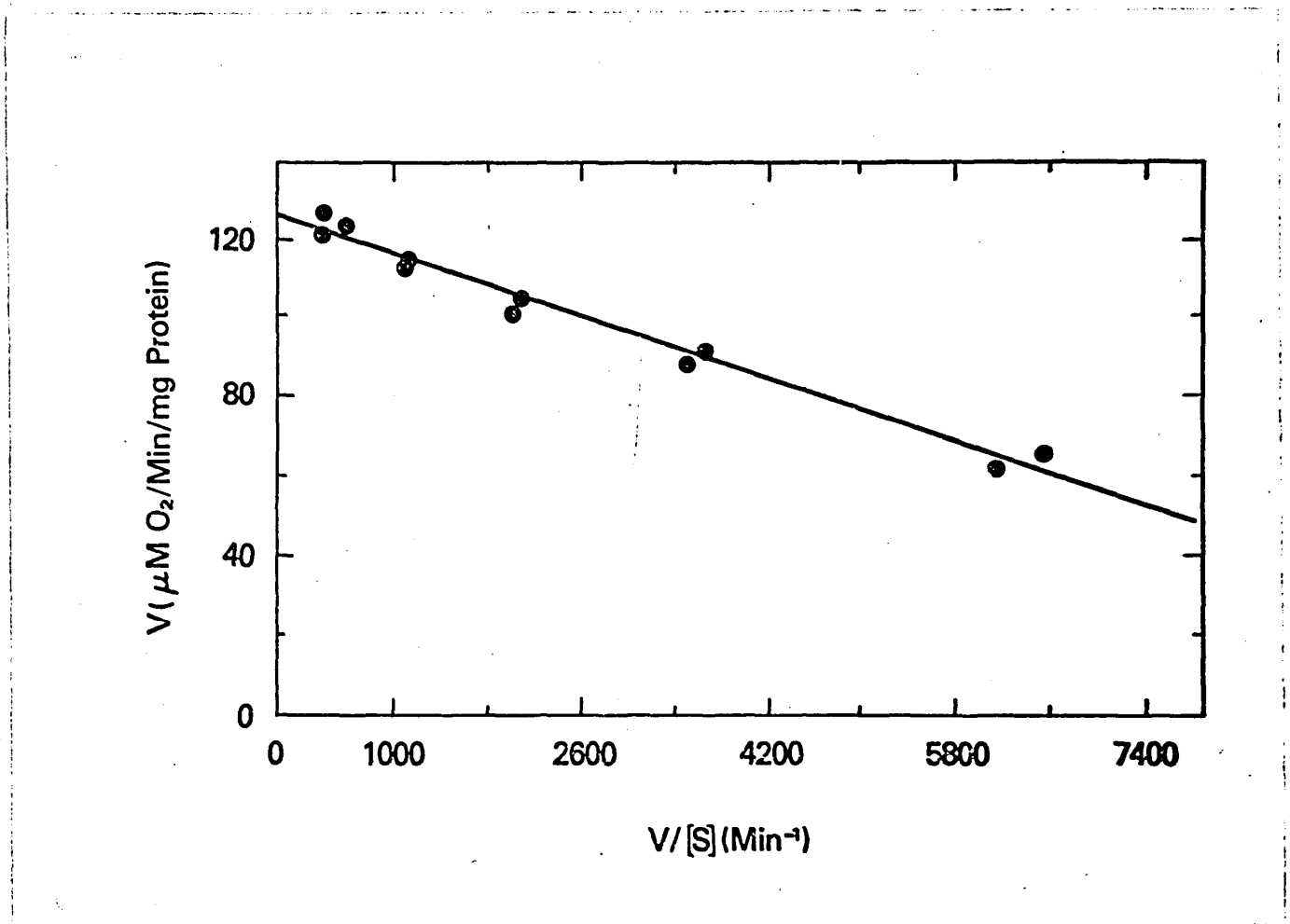

120
80
40
0
0
1000
2600
4200
5800
7400
V(μM O₂/Min/mg Protein)
V/[S](Min⁻¹)

TABLE 1

Comparison of the Kinetic Parameters of Ferroxidase-II with Fe(II) and Fe(III)

| Property | Fe(II)[1] | Fe(III) |
|---|---|---|
| $K_m$ (μM) | 14 | 10 |
| $V_{max}$ (μM $O_2$/min/mg protein) | 102 | 127 |
| pH Optimum | 7.2 | 7.0 |

[1]These values reported by Topham and Johnson (35)

TABLE 2

Effect of Metal Ions on Ferroxidase-II Activity

| Cation ($1 \times 10^{-3}$M) | Specific Activity[1,2] (μmoles $O_2$/min/mg protein) |
|---|---|
| Fe(III) | 100 |
| Cu(II) | 0 |
| Mg(II) | 0 |
| Ni(II) | 0 |
| Al(III) | 0 |
| Co(II) | 0 |
| Mn(II) | 0 |
| Mo(VI) | 0 |

[1]Reaction mixture consists of 0.6 M Acetate buffer (pH 6.0) diluted 1 to 3, 50 μl (0.409 mg) of purified ferroxidase-II and a final cation concentration of $1 \times 10^{-3}$M.

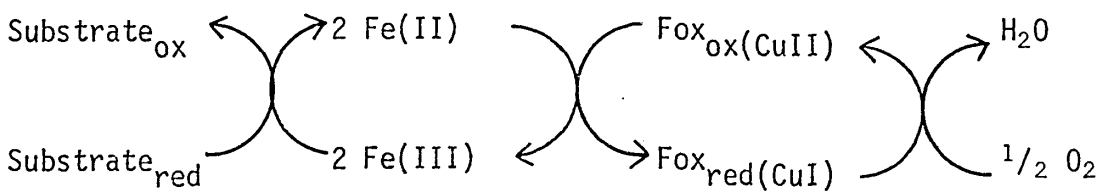
[2]These values represent the specific activity with ferroxidase-II and the cation in the absence of Fe(III). Inhibition by Cu(II) was noted when Fe(III) was introduced into the reaction vessel containing $1 \times 10^{-3}$M Cu(II) and ferroxidase-II.

reaction vessel to determine the effect, if any, of the various metals upon the Fe(III)-initiated reaction. Copper(II) was the only ion found to have a significant effect (Figure 5). As indicated, the presence of Cu(II) in the reaction mixture resulted in a 55% loss in activity with Fe(III). Since copper is an integral part of ferroxidase-II, it may seem odd that inhibition is observed with exogenously added copper. However, it must be remembered that this is an unusually high level of free copper which greatly increases the chances of non-specific binding of copper.

The copper naturally bound to copper-containing enzymes represents only a very small percentage by weight of the enzyme. This copper is normally very tightly bound at specific sites in the enzyme structure. However, many proteins possess the capability of loosely binding copper, particularly if they possess sulfur or nitrogen containing amino acids (1). The binding of copper or other heavy metals at these sites frequently alters the molecular structure of an enzyme and results in its inactivation.

Possible Involvement of Fe(III) with an Endogenous Substrate

The finding that oxygen consumption followed injection of Fe(III) suggested that iron might serve as an intermediary electron carrier for the coupling of electron transfer from an endogenous substrate to ferroxidase-II. A possible mechanism for the Fe(III)-coupled reaction is as follows:

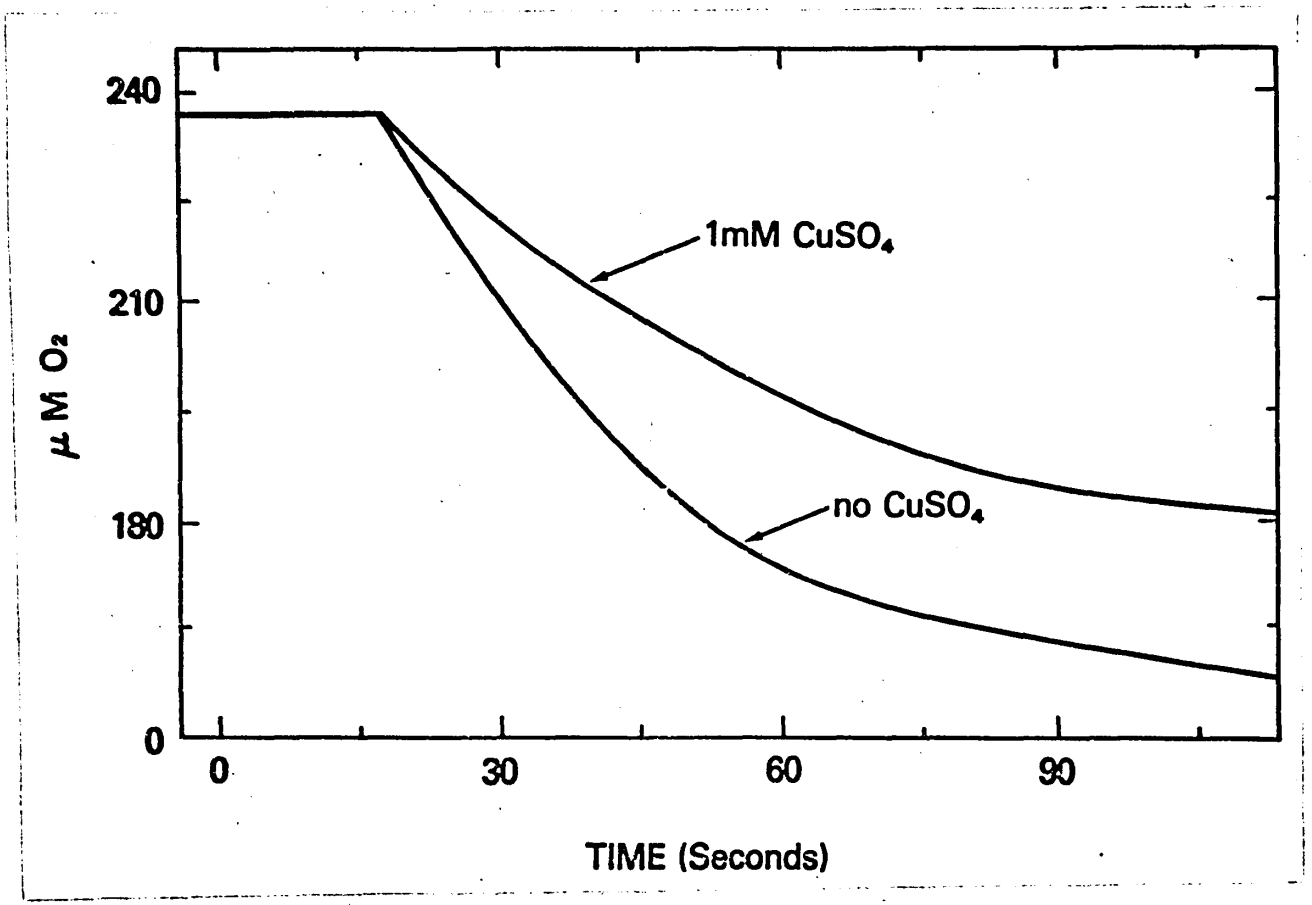



To test whether this mechanism was feasible, it was necessary to show that during the course of the reaction ferric iron was reduced to the ferrous state

FIGURE 5

Effect of Copper(II) upon the Fe(III)-initiated reaction with Ferroxidase-II

Enzymic assays were performed as described in "Experimental Methods". The control assay mixture contained 50 µl (0.909 mg) of purified ferroxidase-II, 0.2 M acetate buffer, pH 6.0, and an Fe(III) concentration of $1 \times 10^{-3}$M. In the second assay mixture, Cu(II) ($1 \times 10^{-3}$ M final concentration) was allowed to incubate with the enzyme prior to the addition of Fe(III).


240
210
180
0
μM O₂
1mM CuSO₄
no CuSO₄
0
30
60
90
TIME (Seconds)

Certain chelating agents, notably α,α'-dipyridyl and 1,10-phenanthroline, complex with ferrous iron. These complexes exhibit characteristic absorption maximum. The phenanthroline complex has an absorption maximum at 510 nm, while the dipyridyl maximum is at 520 nm (39,40). Thus, it should be possible to trap the ferrous ion with 1,10-phenanthroline or α,α'-dipyridyl as it is produced in the proposed reaction scheme. This would prevent reoxidation of the ferrous ion by ferroxidase-II and little, if any, oxygen consumption should be observed.

When ferroxidase-II was incubated with Fe(III) in the presence of 1, 10-phenanthroline, a distinct absorption maximum at 510 nm appeared. The appearance of this peak was indicative of the formation of the Fe(II)-phenanthroline complex. The absorption band at 510 nm increased as a function of the reaction time (Figure 6). Analogous results were obtained when α,α'-dipyridyl was substituted for 1, 10-phenanthroline. In this case, the absorption band appeared at 520 nm, the characteristic $\lambda_{max}$ for the α,α'-dipyridyl ferrous complex (Figure 7). Control spectra were run to ascertain that none of the reaction components absorbed at either 510 nm or 520 nm (Figure 8) Furthermore, no oxygen consumption was observed with reaction mixtures of a composition identical to those used for these spectral studies.

These results strongly support the proposed role of iron as an intermediary electron carrier for the coupling of electron transfer from an endogenous substrate to ferroxidase-II.

Effect of Fe(III) on the Fe(II) rate with Ferroxidase-II and the Effect of Fe(II) on the Fe(III) rate with Ferroxidase-II

In view of our proposed mechanism, it was felt that it would be interesting to study the effect of pretreatment of ferroxidase-II with Fe(III)

FIGURE 6

Iron Chelating Studies Involving 1,10-Phenanthroline

The iron-chelating agent spectra were recorded using a Perkin-Elmer Model 450 UV-visible NIR spectrophotometer. Spectra were recorded at various times after addition of the enzyme.

Reference cell: reaction mixture(0.2 M Acetate buffer, pH 6.0) containing 1 x $10^{-2}$ M 1,10-Phenanthroline, 1 x $10^{-3}$ M Fe(III), 100 µl of 0.05 M Acetate buffer, pH 5.5

Sample cell: reaction mixture(0.2 M Acetate buffer, pH 6.0) containing 1 x $10^{-2}$ M 1,10-Phenanthroline, 1 x $10^{-3}$ M Fe(III), 100 µl(0.91 mg) ferroxidase-II

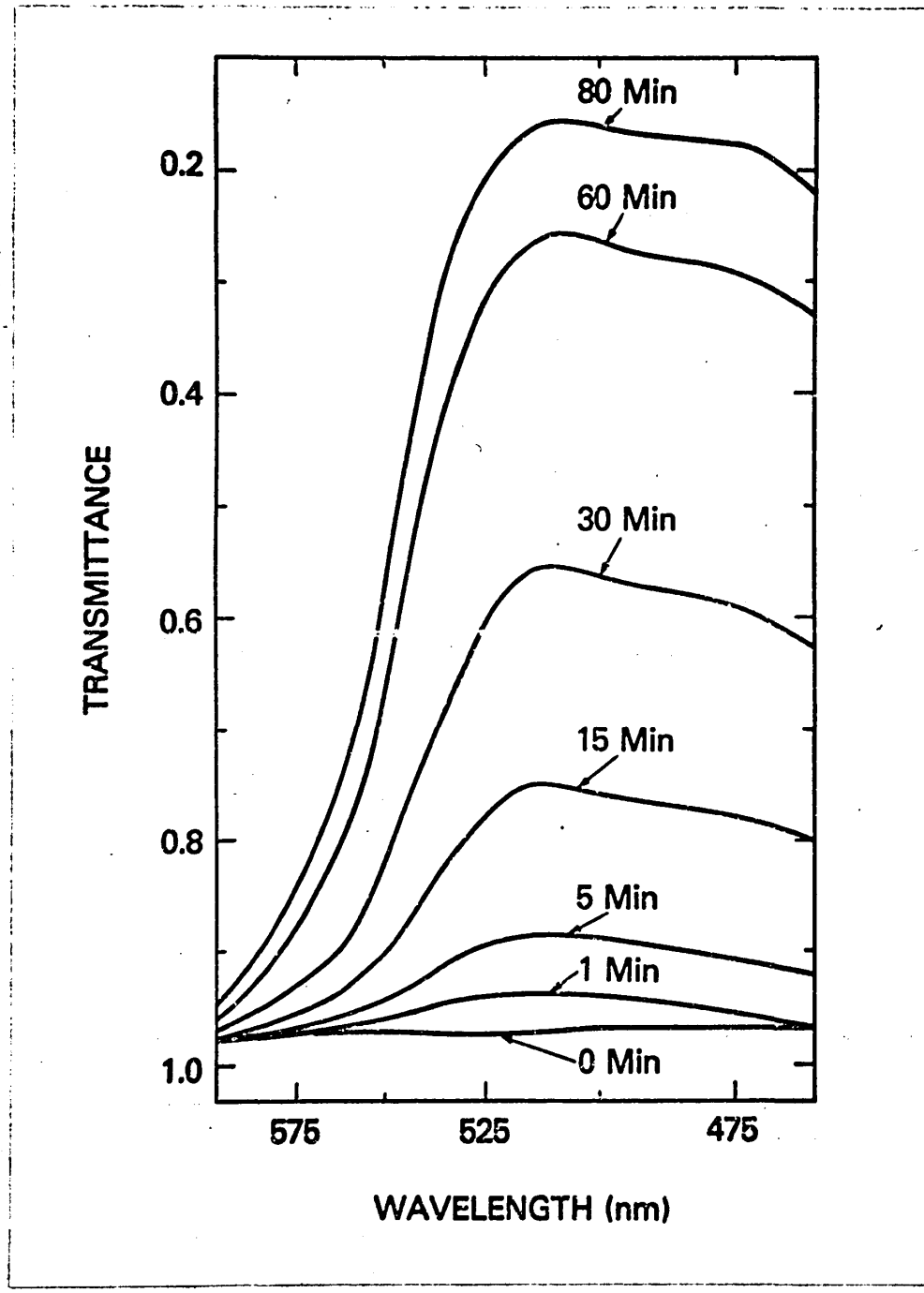

80 Min
60 Min
30 Min
15 Min
5 Min
1 Min
0 Min
0.2
0.4
0.6
0.8
1.0
TRANSMITTANCE
575
525
475
WAVELENGTH (nm)

FIGURE 7

Iron Chelating Studies Involving α,α'-Dipyridyl

The iron-chelating agent spectra were recorded using a Perkin-Elmer Model 450 UV-visible NIR spectrophotometer. Spectra were recorded at various times after addition of the enzyme.

Reference cell: reaction mixture(0.2 M Acetate buffer, pH 6.0) containing 1 x $10^{-2}$ M α,α'-Dipyridyl, 1 x $10^{-3}$ M Fe(III), 100 µl of 0.05 M Acetate buffer, pH 5.5

Sample cell: reaction mixture(0.2 M Acetate buffer, pH 6.0) containing 1 x $10^{-2}$ M α,α'-Dipyridyl, 1 x $10^{-3}$ M Fe(III), 100 µl (0.91 mg) ferroxidase-II

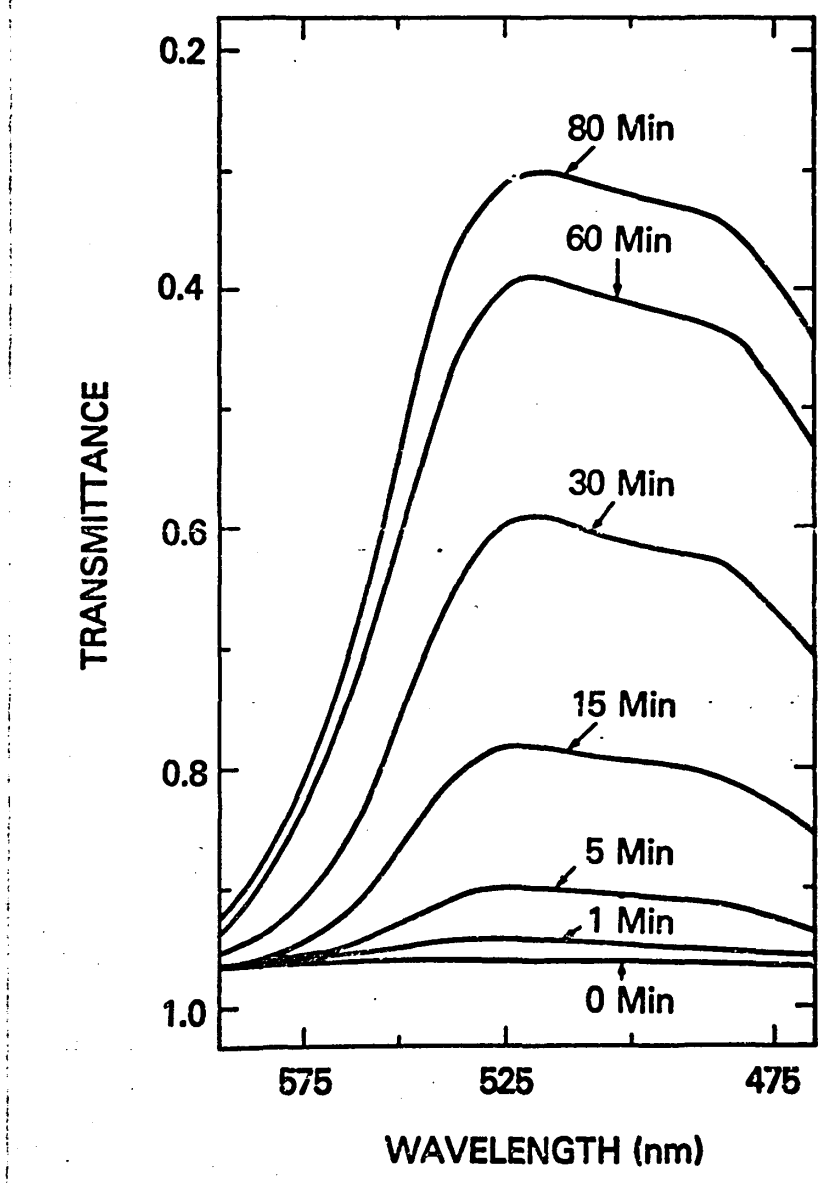

0.2
0.4
0.6
0.8
1.0
TRANSMITTANCE
80 Min
60 Min
30 Min
15 Min
5 Min
1 Min
0 Min
575
525
475
WAVELENGTH (nm)

FIGURE 8

Control Spectra for Studies Involving Iron Chelating Agents

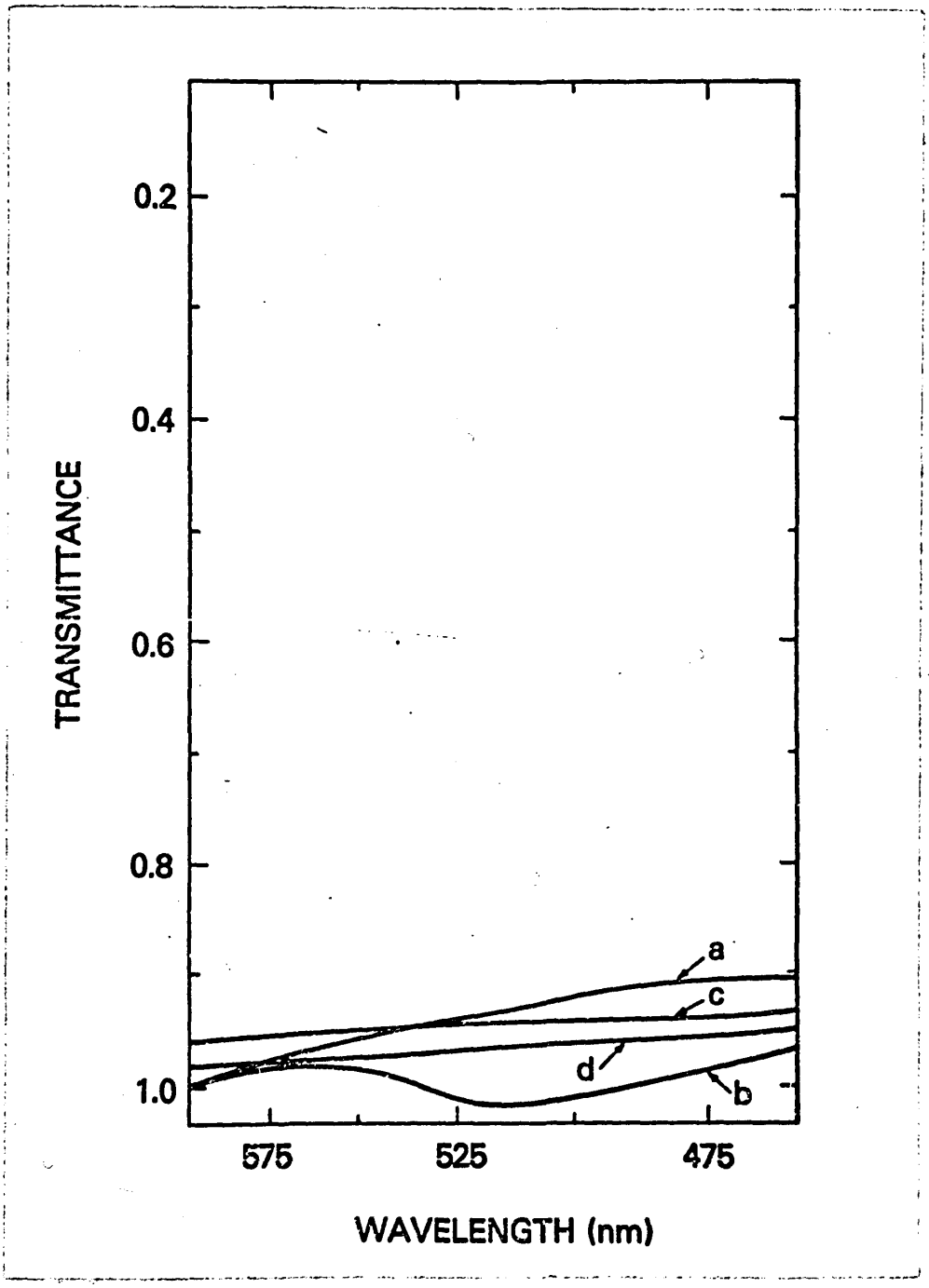
These spectra were recorded using a Perkin-Elmer Model 450 UV-visible NIR spectrophotometer.

Curve A
Reference cell: reaction mixture(0.2 M Acetate buffer, pH 6.0), 100 µl of 0.05 M Acetate buffer, pH 5.5
Sample cell: reaction mixture(0.2 M Acetate buffer, pH 6.0), 100 µl (0.91 mg) ferroxidase-II

Curve B
Reference cell: reaction mixture(0.2 M Acetate buffer, pH 6.0), 100 µl of 0.05 M Acetate buffer, pH 5.5, 1 x $10^{-3}$ M Fe(III)
Sample cell: reaction mixture(0.2 M Acetate buffer, pH 6.0), 100 µl (0.91 mg) ferroxidase-II, 1 x $10^{-3}$ M Fe(III)

Curve C
Reference cell: reaction mixture(0.2 M Acetate buffer, pH 6.0) containing 1 x $10^{-2}$ M α,α'-Dipyridyl, 100 µl of 0.05 M Acetate buffer, pH 5.5
Sample cell: reaction mixture(0.2 M Acetate buffer, pH 6.0) containing 1 x $10^{-2}$ M α,α'-Dipyridyl, 100 µl(0.91 mg) ferroxidase-II

Curve D
Reference cell: reaction mixture(0.2 M Acetate buffer, pH 6.0) containing 1 x $10^{-2}$ M 1,10-Phenanthroline, 100 µl of 0.05 M Acetate buffer, pH 5.5
Sample cell: reaction mixture(0.2 M Acetate buffer, pH 6.0) containing 1 x $10^{-2}$ M 1,10-Phenanthroline, 100 µl(0.91 mg) ferroxidase-I


0.2
0.4
0.6
0.8
1.0
TRANSMITTANCE
575
525
475
WAVELENGTH (nm)
a
c
d
b

upon the rate of Fe(II) oxidation and the effect of pretreatment of ferroxidase-II with Fe(II) upon the Fe(III)-initiated reaction.

Ferroxidase-II was incubated with various levels of Fe(III) (10 μM to 250 μM) for two minutes prior to the introduction of Fe(II) into the reaction vessel. The rate of Fe(II) oxidation was decreased at all Fe(III) concentrations but with Fe(III) concentrations of 50 μM or greater, there was little, if any, oxygen consumption when Fe(II) was added (Figure 9).

Similar observations were made when the enzyme was preincubated with Fe(II) (6 μM to 120 μM) (Figure 10). With 30 μM Fe(II), the Fe(III) rate was reduced by 50% and at those Fe(II) concentrations examined above this, there was no measurable Fe(III) rate.

These findings provide further evidence for the cycling mechanism. According to this mechanism, Fe(III) is reduced to Fe(II) which is in turn reoxidized to Fe(III). If this is true, then the reaction could be initiated by either Fe(II) or Fe(III) and should continue until the endogenous substrate is used up. Thus, if the reaction is allowed to go to completion, additional Fe(II) or Fe(III) would have no effect. As indicated, these findings definitely support the cycling nature of iron.

Treatment of Ferroxidase-II with DTC

Previous studies (41) have demonstrated that the electrons removed during Fe(II) oxidation by ferroxidase-II are passed to oxygen via the copper bound to ferroxidase-II. The mechanism previously illustrated for the coupling of Fe(III) with an endogenous substrate would require that electrons from the endogenous substrate also be passed to oxygen via the copper bound to ferroxidase-II.

DTC specifically binds Cu(II) and prevents its reduction (42). Almost

FIGURE 9

Effect of Fe(III) on the Fe(II) rate with Ferroxidase-II

Enzymic assays were performed as described in "Experimental Methods". In each assay, 50 µl(0.455 mg) of ferroxidase-II and varying concentrations of Fe(III) were allowed to incubate for two minutes prior to the addition of 100 µl of 26.4 x $10^{-4}$M Fe(II)(120 µM). Each point represents the average of three separate experiments. The average specific activity of Fe(II) and ferroxidase-II in the absence of Fe(III) was 133 µmoles $O_2$/min/mg protein.

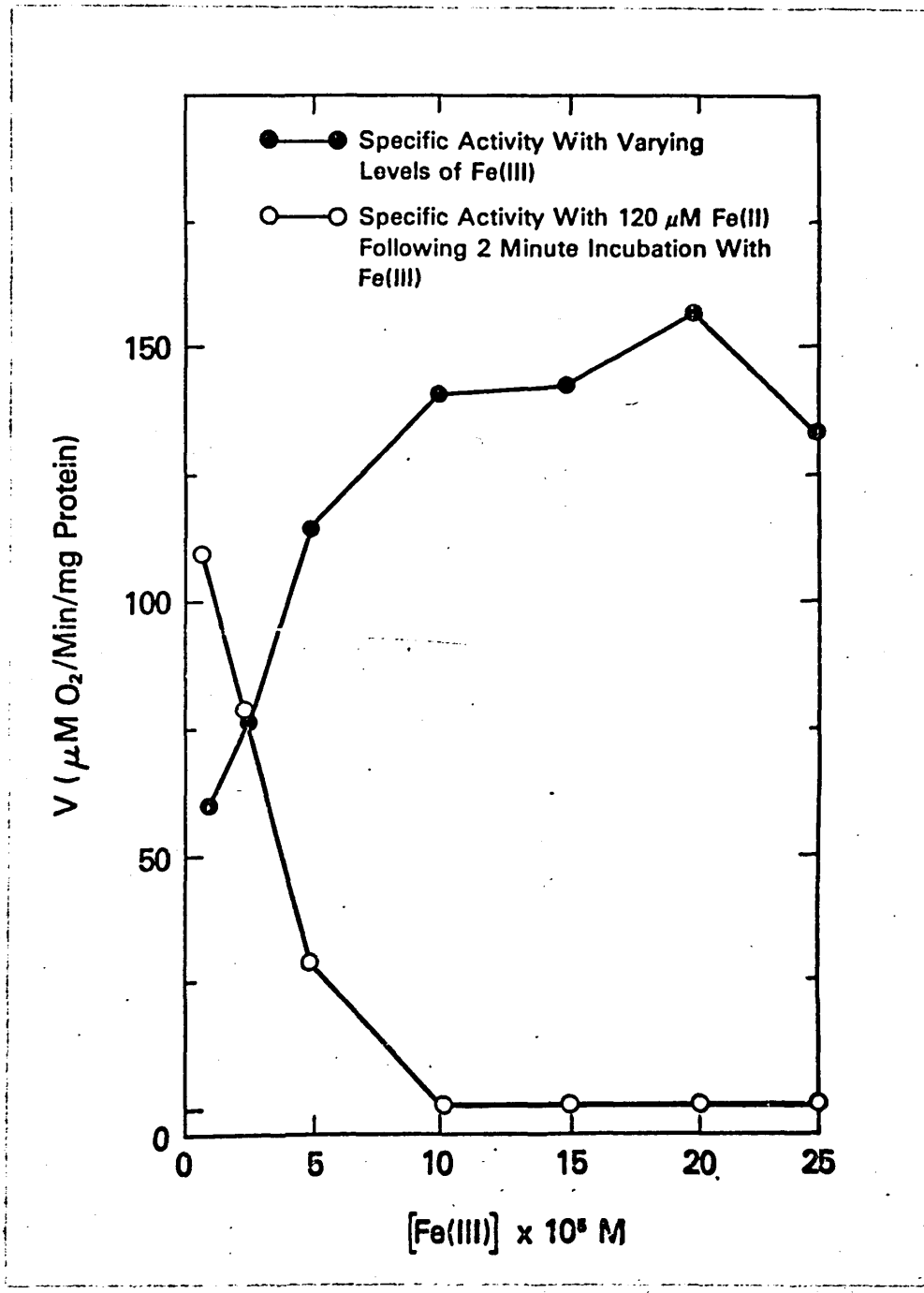
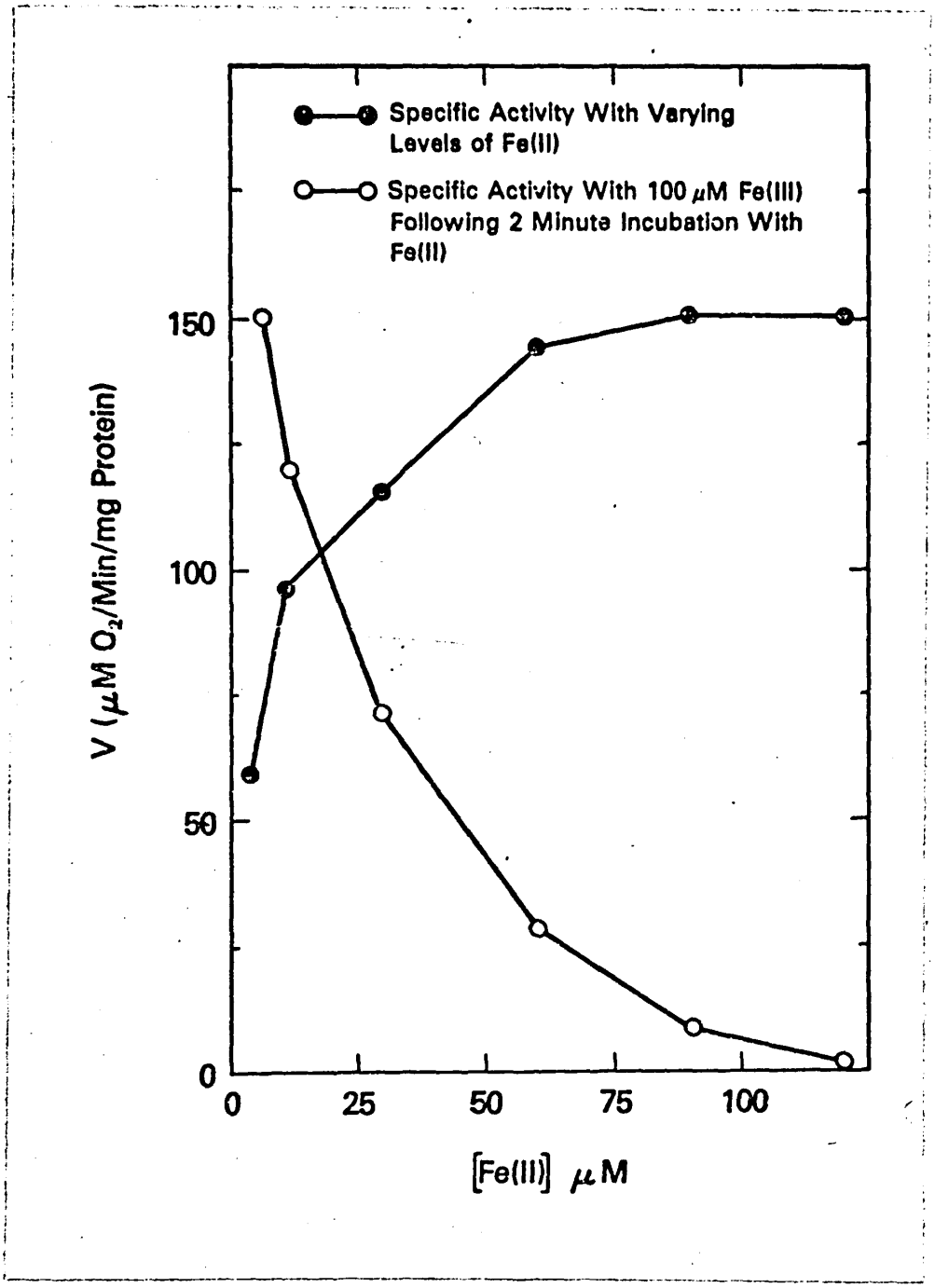

Specific Activity With Varying Levels of Fe(III)
Specific Activity With 120 μM Fe(II) Following 2 Minute Incubation With Fe(III)
V (μM O2/Min/mg Protein)
150
100
50
0
0
5
10
15
20
25
[Fe(III)] x 10⁵ M

FIGURE 10

Effect of Fe(II) on the Fe(III) rate with Ferroxidase-II

Enzymic assays were performed as described in "Experimental Methods". In each assay, 50 µl (0.455 mg) of ferroxidase-II and varying concentrations of Fe(II) were allowed to incubate for two minutes prior to the addition of 0.2 µl of 1 M Fe(III) which yields a final Fe(III) concentration of 100 µM. The increase in the specific activity resulting from the addition of Fe(III) was recorded. The average specific activity of Fe(III) and ferroxidase-II in the absence of Fe(II) was 150 µmoles $O_2$/min/mg protein.


Specific Activity With Varying Levels of Fe(II)
Specific Activity With 100 μM Fe(III) Following 2 Minute Incubation With Fe(II)
V (μM O2/Min/mg Protein)
150
100
50
0
0
25
50
75
100
[Fe(II)] μM

total loss of Fe(III)-initiated activity was observed with the DTC treated enzyme (Table 3). These results suggest that if electrons originate from an endogenous substrate via an Fe(III)-coupled reaction they also ultimately pass through the ferroxidase-II bound copper in route to oxygen.

Search for Identity of Endogenous Substrate

After determining the kinetic parameters of the Fe(III)-initiated reaction with ferroxidase-II and obtaining evidence that indicated that Fe(III) could serve as an intermediary electron carrier from the endogenous substrate to the copper of ferroxidase-II, the next step was to attempt to identify the endogenous substrate responsible for this activity.

Catecholamines as Potential Substrates

Ferroxidase-II was purified from blood serum so, it seemed logical to examine some components of blood as potential endogenous substrates.

Walaas et al (43) found that ferroxidase-I had some oxidase activity with the catecholamines: epinephrine, norepinephrine and dopamine. To further differentiate between ferroxidase-I and II, and in the hope of identifying the endogenous substrate with ferroxidase-II, the catecholamines were examined to see if they could serve as a substrate for ferroxidase-II. In addition to the catecholamines mentioned, serotonin and ascorbic acid were also studied as potential substrates. Each compound was made up in the reaction mixture to which the ferroxidase-II and the Fe(III) were injected.

None of the substances tested were found to stimulate the ferroxidase activity. Thus, it appeared that none of the catecholamines were capable of serving as a substrate for ferroxidase-II.

TABLE 3

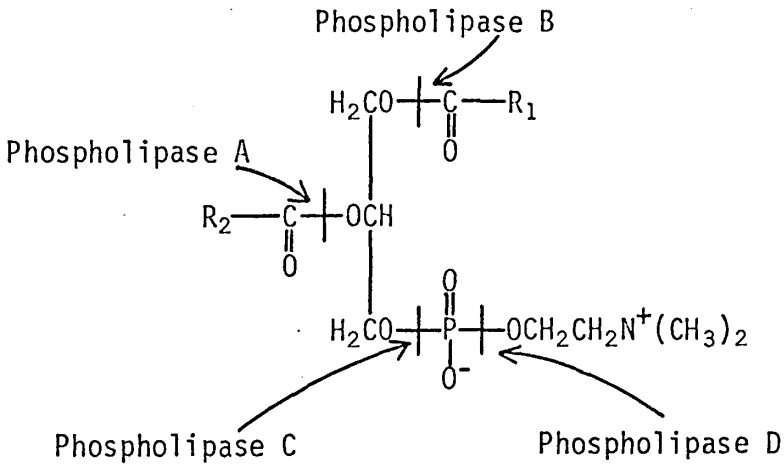
Effect of DTC on Oxygen Consumption Initiated by Fe(III)[a]

| | Specific Activity ($\mu M$ $O_2$/min/mg protein) | |
|---|---|---|
| | Fe(II) ($1.2 \times 10^{-4}$ M) | Fe(III) ($1 \times 10^{-2}$ M) |
| Control | 102.8 | 83.9 |
| DTC-Treated | 28.4 | 7.3 |
| % Inhibition | 72.4 | 91.3 |

[a]DTC treatments and enzyme assays were performed as described in "Experimental Methods"

Treatment of Ferroxidase-II with Phospholipase C

Recent work by C.S.M. Sung (32) showed that ferroxidase-II was a lipoprotein having cholesterol as well as phospholipids tightly associated with it. She observed a very large decrease in the ferroxidase activity of samples which had been treated with Phospholipase C. Phospholipases are enzymes which catalyze the hydrolysis of phospholipids at the specific points illustrated below (44):




Phospholipase C hydrolyzes the bond between phosphoric acid and glycerol to form a 1,2-diglyceride and a water-soluble phosphorylcholine.

Sung's (32) observation that ferroxidase activity was lost after phospholipase treatment indicated that the lipids were essential for the maintenance of enzymic activity. This led to the idea that the endogenous substrate for the Fe(III)-initiated ferroxidase reaction might be a lipid or at least bound in the lipid matrix of the enzyme.

To test this hypothesis, three different ferroxidase-II preparations were treated with Phospholipase C and examined for loss of Fe(III)-initiated activity. As seen in Table 4, hydrolysis with Phospholipase C resulted in a dramatic loss of activity with both Fe(II) and Fe(III). To completely remove the hydrolyzed lipids, portions of the Phospholipase C treated

TABLE 4

Effect of Phospholipase C treatment on Ferroxidase-II Activity

| Treatment | Specific Activity ($\mu$moles $O_2$/min/mg protein) | |
|---|---|---|
| | $1 \times 10^{-3}$ M Fe(III) | $1.2 \times 10^{-4}$ M Fe(II) |
| None | 88.5 | 139.2 |
| Phospholipase C treated[1] | 22.3 | 21.2 |
| Phospholipase C treated, passed over Agarose A-5m[1] | 0 | 0 |

[1]Similar losses in activity were observed after each step with all three enzyme preparations.

enzyme were passed over an Agarose A-5m column (2.5 cm x 27 cm). This procedure resulted in a total loss of activity with Fe(II) as well as Fe(III). These findings suggest that the endogenous substrate may be a lipid or bound in the lipid matrix of the enzyme.

Examination of Lipid Components of Ferroxidase-II

The finding that the phospholipase C treatment of ferroxidase-II resulted in the loss of activity with the Fe(III)-initiated reaction suggested that the endogenous substrate might be lipid in nature. To further investigate this, a purified lipid extract was prepared from ferroxidase-II (see experimental), dissolved in various solvents then introduced into the reaction vessel with Fe(III) and ferroxidase-II. This was designed to determine the extent, if any, of stimulation of the Fe(III)-initiated reaction by the addition of these lipids which had been extracted from ferroxidase-II.

Purified Lipids in Normal Reaction Mixture

The lipid extract was insoluble in the normal reaction mixture(0.6 M acetate buffer, pH 6.0) so the solution was homogenized to form a uniform suspension. The results of this experiment (Table 5) indicate that the addition of the lipids as a buffer suspension caused no increase in activity.

Although there appeared to be no stimulation, it is possible that the lipids were unable to participate in the reaction because they were insoluble in the reaction system. Thus, different solvent systems were examined.

Purified Lipids in Triton X-100

The lipid extract was readily soluble in a 10% solution of Triton

TABLE 5

Examination of Lipid Components of Ferroxidase-II

as Possible Substrates[1,2]

| | Specific Activity (μMoles $O_2$/min/mg protein) | | | |
|---|---|---|---|---|
| | Trial 1 | Trial 2 | Trial 3 | Average |
| Fe(III), Ferroxidase-II | 70.4 | 67.5 | | 68.9 |
| 0.145 mg lipid extract, Fe(III), Ferroxidase-II | 71.8 | 67.5 | 71.8 | 69.9 |
| 1.45 mg lipid extract, Fe(III), Ferroxidase-II | 67.5 | | | 67.5 |
| Fe(III), delipidized Ferroxidase-II[3] | 0 | | | 0 |
| Fe(III), delipidized Ferroxidase-II[3], 0.145 mg lipid extract | 0 | | | 0 |

[1]Normal reaction mixture (0.2 M Acetate buffer, pH 6.0) used as solvent
[2]Lipid insoluble, homogenized to form uniform suspension
[3]has been through Agarose A-5m

X-100 but there was too much oxygen consumption due to the auto-oxidation of the Triton solution. Because of this problem, the Triton system could not be used in these studies.

Purified Lipids in 50% Glycerol

The lipid extract was suspended in a 50% glycerol solution but there was no increase in activity noted.

Purified Lipids in n-Hexane

The lipid extract was also found to be soluble in hexane. Additions of lipids in a hexane solution caused a slight increase in oxygen consumption but, this was attributed to the hexane rather than the lipids.

Purified Lipids in Diethyl Ether and Tetrahydrofuran

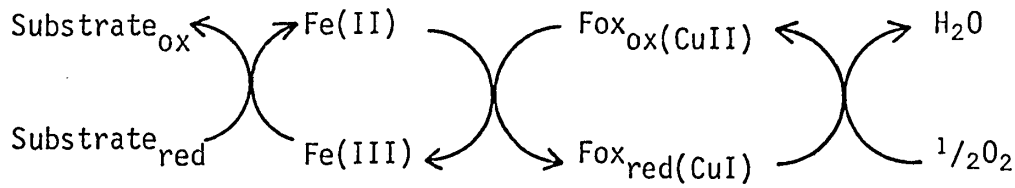
Both diethyl ether and tetrahydrofuran were found to be excellent solvents for the lipid extract; however, when these solvents were injected into the reaction vessel with ferroxidase-II and Fe(III), total inhibition of ferroxidase activity was observed. Since tetrahydrofuran and diethyl ether inactivated the enzyme, they could not be used in these studies.

# DISCUSSION

Ferric iron was first examined to determine its inhibitory effect upon ferroxidase-II; however, quite surprisingly, an Fe(III)-initiated oxidase reaction with ferroxidase-II was observed. Because of this unexpected finding, a thorough investigation of this Fe(III)-initiated reaction was undertaken.

The finding that saturation kinetics were observed with the Fe(III)-initiated reaction and that heat treatment resulted in the loss of this activity suggested that the oxidase reaction was an enzymic process.

To explain the Fe(III)-initiated reaction, a mechanism involving iron as an intermediary electron carrier for the coupling of electron transfer from an endogenous substrate to ferroxidase-II was proposed:




Several pieces of experimental evidence led to the formulation of this mechanism and support it as a feasible explanation of the Fe(III)-initiated oxidation reaction.

(1) Spectral studies with Fe(II) chelating agents demonstrate that ferric iron is reduced to the ferrous state in the presence of ferroxidase-II.

(2) The trapping of the ferrous ion by these chelating agents would prevent its reoxidation and halt oxygen consumption. No oxygen consumption was observed when these chelating agents were included in the reaction mixture at concentrations sufficient to completely trap all of the ferrous iron produced.

(3) This mechanism suggests that Fe(II) and Fe(III) are participating at the same site on the enzyme. The kinetic parameters of ferroxidase-II with Fe(III) and Fe(II) were found to be quite similar. The $K_m$ for each was quite low and their maximal

(3) velocities were nearly equal. The pH optima found with Fe(III) and Fe(II) were comparable and the fact that they are in the vicinity of neutrality indicates that they would be compatible with the pH of blood.

(4) The mechanism would suggest that the reaction of the endogenous substrate with ferroxidase-II could be initiated by either Fe(II) or Fe(III) and this reaction would continue until oxidation of the endogenous substrate was complete. A two minute preincubation of the enzyme with either Fe(II) or Fe(III) was found to considerably reduce further oxygen consumption when additional Fe(III) or Fe(II) was added.

(5) The mechanism would also imply that the copper associated with ferroxidase-II is actively involved in the shuttle of electrons from the endogenous substrate to oxygen. The potent inhibition of the Fe(III)-initiated oxygen consumption observed with diethyl-dithio-carbamate(DTC), a copper specific chelating agent, strongly suggests that the copper associated with ferroxidase-II must be involved in this reaction.

(6) The enzymic reaction was initiated specifically by iron and substitution by other transition elements resulted in no oxygen consumption. Thus, iron must be ideally suited to participate in the coupled reaction with the endogenous substrate. This indicates that the site on the enzyme at which this reaction takes place must have some features which make it compatible only with iron. Indeed the reduction potential of protein-bound iron has been found to be more negative than that of protein-bound copper(45). Thus, it would seem logical for iron to be the initial acceptor of electrons and to pass these electrons to copper which in turn would transfer the electrons to oxygen.

Considerable evidence suggested that the proposed mechanism was feasible. Thus, investigations concerning the isolation and identification of the endogenous substrate(or source of endogenous reducing power) were initiated. This presented quite a problem since the substrate appeared to be very tightly bound to the enzyme. The substrate remained associated with the enzyme through an organic solvent precipitation, an ion exchange chromatographic step, ultrafiltration and two gel filtration columns.

These purification steps should have removed any impurities or loosely bound substances.

Initially, various oxidizable components of human blood were examined in the hopes of finding one which would stimulate the Fe(III)-initiated reaction. None of the substances studied caused significant increases in activity, so it was decided to approach the problem by an alternative method.

The loss in activity following the hydrolysis of the phospholipids associated with ferroxidase-II suggested that the endogenous substrate was a lipid or was bound in the lipid matrix of the enzyme. This prompted studies with lipid extracts from ferroxidase-II, but numerous problems were encountered.

Attempts to restore the activity of delipidized ferroxidase-II were unsuccessful; however, this failure can be explained in several ways. Perhaps the most obvious answer is that the phospholipase C treatment caused an irreversible alteration in the enzyme's three dimensional structure thus rendering it inactive. If this were the case, no activity should be observed. Difficulties were also encountered in finding a common solvent for the lipid extract and the enzyme. Since the lipids are insoluble in an aqueous mixture, it was necessary to find a solvent which would expose them to the enzyme. Unfortunately, all of the solvents in which the enzyme and lipids were mutually soluble were found to inactivate the native enzyme. Because of this, our studies were done using suspensions of the lipids rather than solutions. This does not assure good interaction between the enzyme and lipids, so the fact that no activity was observed does not conclusively prove that the substrate was not present in the lipid extract.

Assuming that the phospholipase C treatment might be irreversibly altering the enzyme's three dimensional structure, a third approach for identifying the endogenous substrate was attempted. Rather than adding the lipid extract to the delipidized ferroxidase-II, it was added to native ferroxidase-II. This approach suffered from problems similar to those encountered with the delipidized enzyme. Here again, a suitable solvent for the enzyme and substrate was not found. This lack of a common solvent turned out to be the major stumbling block in the studies with the lipid extracts.

This is an interesting area which might be explored further, but to pursue the theory that the endogenous substrate is a lipid, it would be necessary to effect a separation of the individual lipids present in the lipid extract. Then, it would be possible to make more concentrated solutions of the individual lipids and examine them to see if they increase the activity of the Fe(III)-initiated reaction.

Even if the lipid responsible for this activity were isolated, it is possible that no increase in activity would be observed. It should be remembered that in the native enzyme the substrate is already attached to the enzyme. It is likely that the enzyme and exogenous lipid would not come together in such a manner as to duplicate the binding of the enzyme and substrate in the native complex.

Recently, a quinone-like substance, capable of being oxidized and reduced, has been isolated from ferroxidase-II. Although the substance has not yet been definitely identified, it appears to be part of the lipid matrix and is extremely similar to quinones found in living systems. Based upon its chromatographic (column and TLC) behavior, spectral data, and chemical tests, the substance is very much like ubiquinone (46). As of

this date, sufficient quantities have not been obtained for definitive identification or for substrate studies.

Once sufficient quantities have been isolated, it would be interesting to compare its structure before and after treatment with iron. It should be possible to determine whether there is an alteration in its structure and whether or not it is being oxidized.

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