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# Ferroxidase-II: a blood serum lipo-protein

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# Ferroxidase-II: A Blood Serum Lipoprotein

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## BIOGRAPHICAL SKETCH

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# ABSTRACT

Ferroxidase-II was definitely identified as a lipoprotein. Cholesterol and phospholipids remained tightly associated with the ferroxidase-II protein following extensive purification. Quantitative analysis showed that it is a high-density lipoprotein. Thin-layer chromatographic analyses indicated that phosphatidylcholine accounts for the majority of the bound phospholipid with lysophosphatidylcholine and sphingomyelin accounting for the remaining phospholipid.

Treatment of purified ferroxidase-II with phospholipase C or A resulted in a loss of ferroxidase activity which paralleled the hydrolysis of phospholipid. Phospholipid D treatment also resulted in the loss of ferroxidase activity, yet the loss was not as great as with other phospholipases. A lipid-depleted form of ferroxidase-II was prepared by acetone extraction, or gel-filtration following treatment with phospholipase C. However, hydrolysis, not removal, of the lipid was sufficient for the loss of ferroxidase activity.

Disc-gel electrophoresis and protein elution patterns from gel-filtration columns of the native and lipid-depleted ferroxidase-II demonstrated that no gross structural changes occur during lipid-depletion. The loss of copper accompanied lipid-depletion. The loss of copper explained the inactivation of ferroxidase-II occurring on hydrolysis and removal of lipids. Reconstitution studies with lipid-depleted ferroxidase-II indicated that the changes occurring during hydrolysis and removal of the lipids were not readily reversible.

# LITERATURE REVIEW

Although copper has been a useful metallic element for thousands of years, its presence in living matter was not discovered until about a century and a half ago. The amount required by an organism is minuscule; the adult human body, for example, contains only about 100 milligrams of copper (1). Yet, it has been found to be involved in life processes to a surprising extent. Evidently copper, along with iron and a few other trace metals, became incorporated in living matter at an early stage in the evolution of life, and it has remained as a vital trace element ever since.

As with most of the essential transition metals, the importance of copper arises from the fact that it is a required constituent of numerous essential proteins and enzymes. Those copper enzymes whose catalytic functions are well understood are listed in Table I (2).

Copper possesses three chemical properties which account for its effectiveness as a biological agent (1).

(a) Copper ions react strongly with amino acids or proteins and consequently form very stable chelates with biologically active substances.

(b) Copper is an exceptionally effective catalytic agent. Its catalytic activity may be enhanced and made more specific when the ion is incorporated in a protein to form a copper enzyme.

(c) Copper's ionization states have flexible properties that make it particularly suitable for oxidative metabolic functions.

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 anthropod 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 |

Interest in the biological role of copper has greatly increased as the recognition of its role in a number of key physiological processes has developed. These include its importance in elastin and collagen formation which prevent aneurisms, soft bones, and other defects (3,4). It is also an important component of cytochrome oxidase, the terminal oxidase of the electron transport chain of mitochondria (5). Finally, copper also appears to serve a vital role in the formation of hemoglobin, the oxygen carrying protein of blood (6,7,8).

In 1927, Warburg and Krebs observed that mammalian blood sera possessed a fairly constant copper content (9). Twenty years later, Holmberg and Laurell demonstrated that at least 90% of normal serum copper was in the globulin protein fraction and that the increase in serum copper observed during pregnancy and infections was also associated with this protein fraction (10).

In 1948, Holmberg and Laurell isolated from mammalian plasma an intensely blue, copper-containing protein which they named "caeruloplasmin" (11). They found that this newly identified protein had a molecular weight of 151,000, contained 8 copper atoms per molecule, and belonged to the plasma protein class of α-globulin. The α-globulin nature of ceruloplasmin explained Holmberg and Laurell's earlier finding that 90% of the copper of serum was associated with the globulin protein fraction (10).

Ceruloplasmin has been reported to have oxidase activity *in vitro* towards many substrates. Holmberg and Laurell found oxidase activity with p-phenylenediamine and several other organic compounds (12). During his investigation of the effects of inorganic ions on the oxidase activity of purified human ceruloplasmin with N,N-dimethyl-p-phenylenediamine, Curzon found that the system was very sensitive to metals, both inhibition and activation were observed (13).

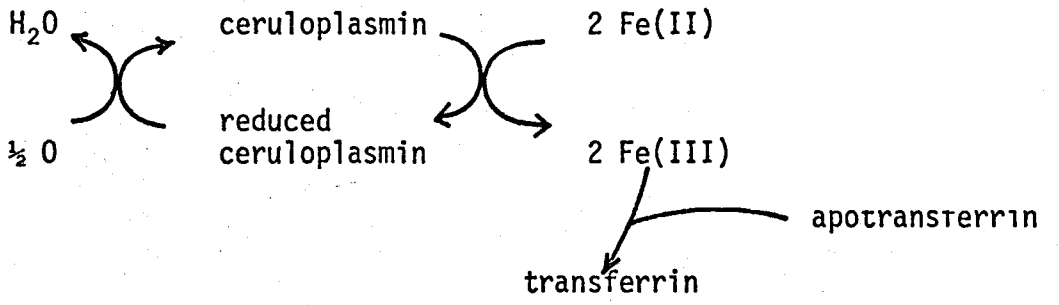
The most marked activating effect was found with ferrous iron. Curzon and O'Reilly's experiments (14) showed that ceruloplasmin was able to oxidize ferrous to ferric iron and this explained the activating effect of iron on the ceruloplasmin catalyzed oxidation of a number of organic compounds. Curzon concluded that any substance which is oxidizable by ferric iron is potentially oxidizable by the coupled iron-ceruloplasmin system. The effectiveness of this system and its ability to compete with a direct ceruloplasmin-substrate interaction vary widely from substrate to substrate (15). Curzon and Vallet (16) suggested that the conflicting results in the literature on the activity of ceruloplasmin against 5-hydroxytryptamine and adrenaline may to some extent be explicable in terms of coupled iron-ceruloplasmin oxidation system.

The recognition of anemia in the copper-deficient animal was reported in 1928 (6). In copper-deficient pigs, it was eventually revealed as a severe anemia, both hypochromic and microcytic (7,18). Lahey, *et al.* (7) compared blood analyses of five-day-old pigs fed a milk diet supplemented with iron and litter-mate controls on the same diet supplemented with both iron and copper. He observed that serum copper fell rapidly. This was followed by a decrease in serum iron, erythrocyte copper, and eventually by a dramatic reduction in red cell volume (7). In copper-deficient rats, Owen and Hazelrig observed a decrease in plasma ceruloplasmin and copper. The decrease in ceruolplasmin was followed by a slower but steady decline in liver copper and in hemoglobin (8). This led Cartwright, *et al.* to pursue the idea of a role for copper in the biosynthesis of hemoglobin. This role could be effected at one of the three main lines of hemoglobin biosynthesis: the biosynthesis of protoporphyrin or heme, the utilization of iron, or the biosynthesis of

globin. Early hopes to find a copper-dependent step in heme biosynthesis were abandoned by Lee, *et al.* (19). They found that as anemia developed in copper-deficient swine, there was a 2-3 fold increase in the activity of heme biosynthetic enzymes, δ-aminolevulinic acid synthetase, α-keto-glutaric acid-dependent glycine decarboxylase, and heme synthetase. They concluded that the anemia of copper deficiency was not the result of defective heme biosynthesis and copper was not a co-factor in any of these reactions. There is also no evidence for any impairment in globin biosynthesis. Thus it seemed most reasonable to assume that copper is essential for the proper utilization of iron (19).

While a connection between iron and copper metabolism has been appreciated for over 40 years, no authentic molecular mechanisms had been proposed until Osaki, *et al.* reported on the possible significance of the ferroxidase activity of ceruloplasmin in normal human serum and proposed that this catalytic activity was involved in promoting the rate of iron saturation of transferrin in the plasma and in iron utilization (20,21). It is generally believed that the iron entering the blood stream from the intestine or liver storage sites is mostly in the ferrous form (22). In the plasma, Fe(II) is rapidly oxidized to Fe(III), and is incorporated into the specific iron-binding protein, transferrin, which can bind two atoms of Fe(III) per each protein molecule, forming a red Fe(III)-protein. When Fe(II) is added to apotransferrin, oxygen is required for the formation of the red complex, and the rate of color formation depends on the rate of Fe(II) oxidation to Fe(III) by molecular oxygen (22). Koechlin also mentioned that the rate of complex formation between ferrous ion and apotransferrin was enhanced by certain catalytic factors present in plasma (23). From Osaki and his coworkers' experiments, a biological role for ceruloplasmin in iron metabolism may ensue from its

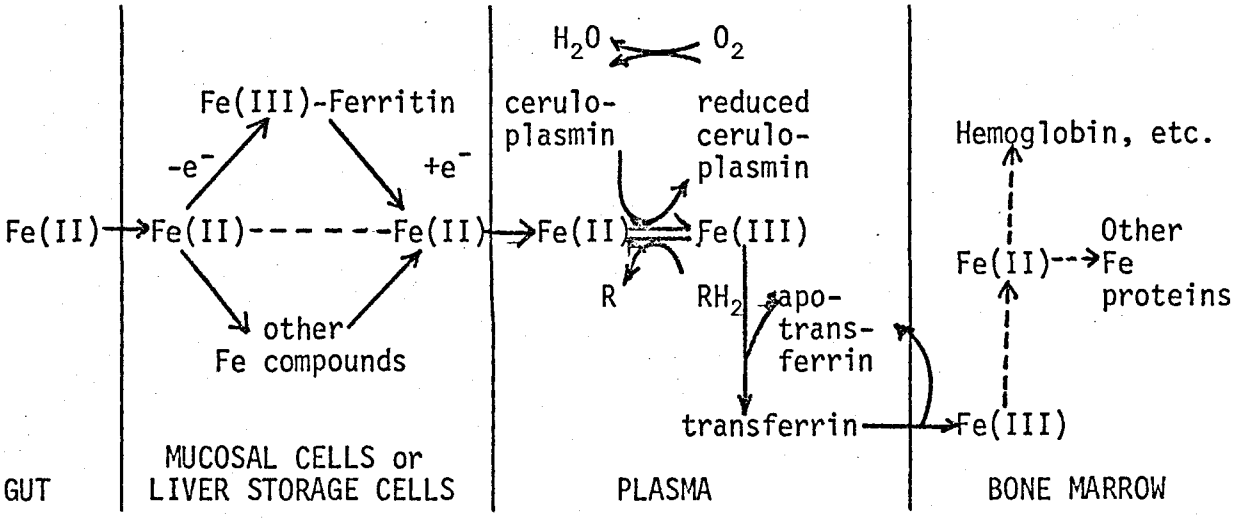
catalytic activity in converting Fe(II) to Fe(III), thereby promoting the rate of incorporation of Fe(III) into apotransferrin (20), as schematically presented below.




The ceruloplasmin-catalyzed rate of Fe(II) oxidation under conditions that might be expected to prevail in human serum is 10 to 20 times faster than the nonenzymic oxidation. The nonenzymic rate would not account for the daily turnover of iron in the plasma (20).

Any erythroid stimulation leads to a faster turnover of iron *in vivo*, and erythroid suppression has the opposite effect (24). Only the reticulocytes are capable of utilizing the Fe(III) bound to transferrin, although both the reticulocytes and matured red cell can take up free Fe(III). Katz and Jandl (24) stated: "Thus, transferrin, in some manner, directs the entry of iron into those cells which are still actively synthesizing hemoglobin and prevents its presumable needless accumulation by the matured cell." These facts supported the generally accepted idea that any Fe(II) absorbed from the intestine or removed from liver storage sites is oxidized and incorporated into apotransferrin in the plasma, and transferred to the marrow. Transferrin appears to be the protein which exclusively supplies iron to the marrow. The availability of iron to the reticulocytes of the bone marrow can be rate-limiting. The rate of formation of Fe(III) from Fe(II) in plasma and of transferrin formation could play a significant role in the overall turnover of iron. The fact that ceruolplasmin can increase this rate under physical conditions

suggests that this is a possible biological function for ceruloplasmin, as is schematically shown below (9,20).




$RH_2$ = Ascorbate
R = Dehydroascorbate

Prevailing theory of iron metabolism showing the possible role of ceruloplasmin promoting iron utilization.

There is now considerable evidence from both *in vitro* and *in vivo* systems which supports the proposal that ceruloplasmin is the key link between iron and copper metabolism (25,26,27,28,29). Roeser, *et al.* demonstrated that the injection of ceruloplasmin into the blood stream of copper-deficient pigs resulted in a rapid rise in the plasma iron concentration (25). This rise in plasma iron concentration was followed by a gradual rise in the concentration of hemoglobin of the blood stream. Osaki, *et al.* perfused livers from copper-deficient pigs with saline solutions containing ceruloplasmin and observed a rapid mobilization of stored iron from the liver into the perfusing solution (26,27). No mobilization of iron was observed with perfusing solution devoid of ceruloplasmin.

While the name ceruloplasmin, coined by its discoverers, Holmberg and Laurell (11), will always retain its historical importance, Osaki, *et al.* anticipated that the name "Ferroxidase" may be more useful than

designating this enzyme as a sky-blue substance from plasma (20).

One major objection was raised to the idea that ceruloplasmin is the key link between copper and iron metabolism. This objection arose from studies of iron metabolism in Hepatolenticular Degeneration, more commonly referred to as Wilson's Disease (33). In this disease the serum is largely depleted of ceruloplasmin, and then copper diffuses into the tissues, where it may accumulate to a high level, particularly in the liver and brain. It can produce severe mental illness and death. If iron metabolism is dependent on ceruloplasmin, we might expect a disturbance in iron metabolism in Wilson's Disease. However, most Wilson's Disease subjects investigated were found to have low normal or normal levels of iron transport (30). In attempting to correlate the aryldiamine oxidase activity with the ferroxidase activity of sera from Wilson's Disease patients, it was observed that these sera had more ferroxidase than expected (30,31). Lee, *et al.* (32) proposed citrate as an alternative source of ferrous iron-oxidizing activity in low ceruloplasmin serum. Frieden and Osaki suggested the possibility of an alternative protein ferroxidase, quite different from ceruloplasmin, which might substitute for the normal enzyme in Wilson's Disease sera (30). In 1970, Topham and Frieden identified, purified, and partially characterized the alternative protein ferroxidase from normal human sera and Wilson's Disease sera (33). They named this non-ceruloplasmin ferroxidase protein "Ferroxidase-II".

Ferroxidase-II differed from ceruloplasmin in many respects as illustrated in Table II(33).

Three of the most important differences were that Ferroxidase-II:

(a) was yellow rather than blue as ceruloplasmin.

(b) it was not inhibited by azide.

(c) it exhibited no p-phenylenediamine oxidase activity.

TABLE II

PROPERTIES DISTINGUISHING FERROXIDASE-II FROM CERULOPLASMIN

| PROPERTY | CERULOPLASMIN | FERROXIDASE-II |
|---|---|---|
| Inhibition with 1 mM Azide | > 98% | None |
| Apparent Km values for Fe(II) | 2 | 1 |
| Specific activity ($A_{460}$ per 10 min per mg of protein) | 30 | 4.9 |
| p-Phenylenediamine oxidase activity | Yes | None |
| Elution after chromatography[a] | .2 to .3 M 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 .05 M Acetate buffer, pH 5.5, with or without .2 to .3 M NaCl

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

The ferroxidase-II protein in human serum, which contributes appreciably to the rate of Fe(III)-transferrin formation, was shown to be a constituent of blood plasma Cohn fraction IV-1 (33).

Topham and Frieden's work indicated that while the ceruloplasmin level of Wilson's Disease serum decreases very dramatically, ferroxidase-II is less affected. The total ferroxidase activity of Wilson's Disease sera is approximately 5 $\sim$ 10% of that of normal sera. They found only about 10% of the total ferroxidase activity of normal sera was necessary in order to observe maximum iron mobilization responses from liver (33). Thus, total ferroxidase of Wilson's Disease serum will be enough for normal or low normal iron metabolism (33,31). However, without ferroxidase-II which accounts for 30% of the total ferroxidase in Wilson's Disease sera, this would not be possible. Ferroxidase-II also accounts for the fact that p-phenylenediamine oxidase activity (another oxidase activity of ceruloplasmin) did not correlate with ferroxidase activity in Wilson's Disease sera and in sera from some other animals (26,30,31).

# INTRODUCTION

From data obtained from gel filtration and disc-gel electrophoresis (33), it would appear that ferroxidase-II must have a molecular weight between 800,000 to 2,000,000 which is much larger than the value of 151,000 for ceruloplasmin (34). Purified ferroxidase-II was also subjected to immunoelectrophoresis. A single major precipitation arc was observed when cross-reacted with whole anti-human serum and stained with buffalo-black (33) or oil red, a stain for lipoproteins (35). The ferroxidase-II protein migrated only a small distance toward the anode and it had a mobility similar to that of serum lipoproteins. (Ceruloplasmin under the same conditions, migrated much further toward the anode.)

To date, no one has reported an enzymic function for a blood lipoprotein. The molecular weight, immunoelectrophoretic mobility, and staining characteristics of ferroxidase-II suggested that lipid components were tightly bound to the enzyme following extensive purification.

Thus, this thesis sought to investigate the possible lipoprotein nature of ferroxidase-II. The basic approach was the following:

(1) To establish quantitatively that lipids were associated with the enzyme.

(2) To identify the nature of the lipids associated with the enzyme.

(3) To determine if these lipids were essential to the maintenance of the catalytic function (ferroxidase activity) of the enzyme.

(4) If so, to determine how these lipids might be involved in the maintenance of catalytic activity.

(5) To develop methods for preparing a lipid-depleted form of the enzyme and to attempt to reconstitute the enzyme by the addition of specific lipids to the lipid-depleted form of the enzyme.

# EXPERIMENTAL PROCEDURES

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

Pure ferroxidase-II was prepared from Cohn fraction IV-1 of human plasma basically as described by Topham and Frieden (33). 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, it represents an excellent starting material for the purification of ferroxidase-II. Routinely, 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, for 4 hours at 4° with gentle continuous stirring. The resulting extract was centrifuged at 25,000 x gravity, 4° for 30 minutes to remove any undissolved materials. The supernatant from this centrifugation was passed through a thin layer of fine glass wool to remove the floating lipid layer and then dialyzed in the cold for 24 hours with 2 liters of 0.05 M acetate buffer, pH 5.5 to remove any small molecular weight impurities from the Cohn IV-1 extract. The dialyzed Cohn IV-1 extract was recentrifuged at 25,000 x gravity for 30 minutes to remove any material which precipitated during the dialysis.

Ceruloplasmin (ferroxidase-I) and ferroxidase-II differ greatly in charge at pH 5.5. At this pH, ceruloplasmin is highly negatively charged; however, ferroxidase-II bears little charge. Thus, these two proteins of human plasma exhibiting ferroxidase activity may be completely separated by chromatography on an anion exchange resin, DEAE-sephadex A-50. The entire dialyzed extract of Cohn IV-1 (100 ml) was applied to a column (4 cm x 15 cm) of DEAE-sephadex A-50. The column was washed with 0.05 M acetate buffer, pH 5.5. Ferroxidase-I (ceruloplasmin) remained as a small blue band at the top of the column.

Ferroxidase-II was eluted from the column with 0.05 M acetate buffer, pH 5.5. The resulting yellowish eluant containing ferroxidase-II was concentrated to 30 ml by ultrafiltration in an Amicon ultrafiltration cell.

To further purify ferroxidase-II, the concentrated solution was applied to a column (4.5 cm x 53 cm) of Agarose A-50 M, a molecular sieving material for proteins of mol. wt. $10 \times 10^4 \gtrsim 50 \times 10^6$. Three bands of protein were eluted from the column with 0.05 M acetate buffer, pH 5.5. The first band was turbid and presumably contained denatured protein material (33). The second of these bands was clear yellow and possessed 90% of the ferroxidase-II activity. The third band was colorless and contained no ferroxidase-II activity. The second band was collected and concentrated by ultrafiltration to a value of 20 ml and passed through another Agarose A-50 M column (3 cm x 45 cm). A single light yellow protein band possessing ferroxidase-II activity was eluted from this column.

Method of Assay of Enzymic Activity

Ferroxidase assays were carried out spectrophotometrically measuring $Fe^{3+}$-transferrin formation at 460 nm as previously reported (31,33). The basis of this assay procedure is illustrated in the following equations:

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

(2) $Fe^{3+} + ApoTf \rightarrow Fe^{3+}Tf_{(460\ nm)}$

(3) $E_{red} + \frac{1}{2}O_2 \rightarrow E_{ox} + H_2O$

$E_{ox}$ = oxidized ferroxidase-II; $E_{red}$ = reduced ferroxidase-II; ApoTf = iron-free transferrin. The oxidation of iron by ferroxidase-II is rate-limiting (28,29).

Quartz spectrophotometric cuvets, 1.5 ml in capacity and 1 cm in path length, contained 0.35 ml of a 0.6 M acetate buffer, pH 6.0; 0.25 ml of the 2% (w/v) apotransferrin solution; 0.30 ml of the $1 \times 10^{-4}$ M ferrous ammonium sulfate solution; 0.10 ml of distilled water, and 0.10 ml of the enzyme preparation. The time course of absorbance change at 460 nm resulting from the formation of transferrin from apotransferrin and Fe(II) was recorded with a Beckman Acta-III spectrophotometer equipped with 0.1 absorbance scale. The specific activity was recorded as the absorbance per 10 minute per mg protein. (See "Results")

Quantitative Determination of Protein, Phospholipid, and Cholesterol

Protein concentrations were determined by the method of Lowry (36). This method combines the use of the biuret and phenol reagents. In the biuret reaction, solutions containing protein are made strongly alkaline with sodium hydroxide and very dilute copper sulfate is added. A purplish to pinkish violet color is obtained. The phenol reagent consists of phosphomolybdic and phosphotungstic acids. The final blue color obtained in the Lowry method depends on a combination of the biuret reaction and the reduction of the phosphomolybdic-phosphotungstic reagent by tyrosine and tryptophan present in the protein. This method is rapid, sensitive (limit of detection, 5 µg/ml; 10 to 20 times more sensitive than simply measuring the ultraviolet absorption at 280 nm, 100 times more sensitive than the biuret method), and specific for protein. Specifically, duplicate standards of 1 ml of a bovine serum albumin solution (0.2 mg/ml) and a blank containing distilled water were prepared. One-tenth ml of solution of unknown protein concentration was diluted to 1 ml with distilled water. Five ml of Lowry B solution (2% $Na_2CO_3$; 0.1 M NaOH; 0.5% $CuSO_4$; 1% sodium tartrate) were added to each tube. The tubes

were mixed and allowed to stand 10 minutes. To each tube was added 0.5 ml of phenol reagent (Phipps and Bird, Richmond, Virginia). Maximum color developed in 1 hour, at which time, the absorbance of each tube at 600 nm was recorded.

Lipid phosphorus was determined by Bartlett's method (37). This method is based on the determination of inorganic phosphate in perchloric acid digests of the lipid. Perchloric acid under reflux rapidly oxidizes lipids with quantitative release of phosphorus as inorganic phosphate. The inorganic phosphate is allowed to react with ammonium molybdate to form phosphomolybdic acid, which is reduced and then determined spectrophotometrically. As little as 0.01 micromole of phosphorus may be detected. Specifically, samples containing up to 0.15 micromole of phosphorus were digested for 20 minutes with 0.4 ml of 70 $\sim$ 72% perchloric acid in 18 x 150 mm test tubes. Duplicate standards of 0.3 ml of 0.5 mM $KH_2PO_4$ (0.15 micromole of phosphorus) plus 0.4 ml of perchloric acid and a blank of perchloric acid alone were prepared. After digestion, the tubes were cooled and 2.4 ml of ammonium molybdate reagent and 2.4 ml of diluted (1:12, v/v) reducing agent (30 g $NaHSO_3$, 6 g $Na_2SO_3$, and 0.5 g 1,2,4-aminonapthol sulfonic acid per 250 ml) were added. The samples were mixed and heated in a boiling water bath for 10 minutes. The tubes were allowed to cool and the absorbance of each tube was recorded at 830 nm. An average molecular weight (775 g per mole) of phospholipid was used in the calculation of phospholipid contents.

A simple rapid micro technique described by Zak (38) was used to determine cholesterol concentrations. The technique is based on the precipitation of protein from the sample by the addition of an acetic acid solution of ferric chloride, leaving the liberated cholesterol in the supernatant solution. A diluted aliquot of the supernatant solution

is treated with concentrated sulfuric acid which yields a color that is stable, reproducible, and obeys Beer's law over a wide range of analysis (50 µg to 700 µg cholesterol per ml). The colored complex and the mechanism of its formation are not completely understood at present. However, the mechanism of the reaction and formation of color does appear to involve oxidation and dehydration of cholesterol. Specifically, the following reagents were prepared:

(a) Ferric chloride stock reagent: 840 mg of $FeCl_3 \cdot 6 H_2O$ was dissolved in glacial acetic acid and diluted to 100 ml with glacial acetic acid.

(b) Ferric chloride precipitating reagent: Reagent (a) was diluted 1:10 (v/v) with glacial acetic acid.

(c) Ferric chloride blank and diluting reagent: 8.5 ml of reagent (a) was diluted to 100 ml with glacial acetic acid.

(d) Cholesterol stock standard: 100 mg of pure cholesterol was dissolved in glacial acetic acid and diluted to 100 ml with glacial acetic acid.

(e) Cholesterol working standard: 2.5 ml of reagent (d) was added to 2.1 ml of reagent (a) and diluted to 25 ml with glacial acetic acid. (Prepared just prior to performing the analysis.)

For the preparation of the standard cholesterol curve, 0, 1, 2, 3 ml of reagent (e) were diluted to 4 ml with reagent (c) and 0.1 ml of 0.05 M acetate buffer, pH 5.5 was added to each tube. Then, from each tube, 3 ml of the aliquot was withdrawn and 2 ml of concentrated sulfuric acid was layered on the liquid surface, mixed well, and permitted to come to room temperature. The absorbance of each tube at 560 nm was measured. The absorbance *vs.* concentration was plotted and

a linear relationship observed. For the determination of cholesterol content in ferroxidase-II preparations, 0.1 ml of the sample was pipetted into 4 ml of the reagent (b) and mixed well. After 3 minutes, the mixture was filtered into a test tube. Two ml of concentrated sulfuric acid was mixed with a 3 ml of the aliquot of the clear yellow filtrate. The solution was allowed to come to room temperature. The absorbance was recorded at 560 nm and the cholesterol content was calculated from the standard curve.

Qualitative Identification of Lipids by Thin-Layer Chromatography

Lipid components were isolated from 2 ml of the pure ferroxidase-II preparations by overnight extraction with 40 ml of chloroform:methanol (2:1, v/v). The extracts were washed with 8 ml of distilled water by the procedure of Folch, *et al.* (39). The lipid components were contained in the chloroform (lower) phase. Separation of the aqueous and chloroform phases during washing was greatly facilitated by the addition of a small quantity of solid NaCl. The chloroform phase was concentrated to a volume of 1 ml under a stream of $N_2$.

Individual lipids were separated by thin-layer chromatography. Thin-layer plates coated with silica gel H were purchased from Analtech, Co., Inc., Newark, Del. Plates were activated at least 1 hour at 110° C before chromatography (37).

(a) Identification of Individual Phospholipids:

Solutions containing 6 mg/ml of each of the following standards were prepared in chloroform:methanol (2:1, v/v). Phosphatidylcholine (PC), lysophosphatidylcholine (LPC), phosphatidylserine (PS), phosphatidylethanolamine (PE), sphingomyelin (SPh), cholesterol (C), and cholesterol acetate (CE). The standards (5 ∿ 10 µl) were applied to

the plate individually and as a mixture. Samples (50 µl) of the lipid extracts of ferroxidase-II were also applied to the plate. Chloroform:methanol:acetic acid:water (25:15:4:2, v/v) was used as the developing solvent. To detect lipid spots following development, the plates were allowed to dry and then placed in a tank saturated with iodine vapor. Lipids appeared as intense brown spots in the iodine vapor.

(b) Identification of Individual Neutral Lipids:
Solutions containing 6 mg/ml of each of the following neutral lipids were prepared in chloroform:methanol (2:1, v/v) cholesterol (C), cholesterol acetate (CE), monoglyceride (monopalmitin, MG), 1,2-diglyceride (1,2-dipalmitin, 1,2-DG), 1,3-diglyceride (1,3-dipalmitin, 1,3-DG), triglyceride (tripalmitin, TG), free fatty acid (palmitic acid, FA). The standards (10 µl) were applied to the plate, individually and as a mixture. Samples of the lipid extract of ferroxidase-II were also applied. Hexane:diethylether:acetic acid (20:80:1, v/v) was the developing solvent. Following development, the plates were allowed to dry, sprayed with 50% sulfuric acid, and placed in an oven at 180° C for at least 2 hours. The sulfuric acid charred the lipids which then appeared as intense black spots.

Treatment of Ferroxidase-II with Phospholipases

Phospholipase A Treatments: These treatments were performed as described by Topham and Gaylor (40). Thirty-five µl of a phospholipase A solution (from *Vipera russelli*, 2 mg protein/ml, 7.7 units/mg protein; one unit

will hydrolyze 1 μmole of L-α-Lecithin to lysolecithin and a fatty acid per min., pH 6.5, at 37°), 0.5 ml of a 0.4 M $CaCl_2$ solution, and 2 ml of a purified ferroxidase-II preparation were incubated at 30° with gentle shaking for various time intervals. Controls containing 0.5 ml of 0.4 M $CaCl_2$, 2 ml of purified ferroxidase-II, and 35 μl of 0.05 M acetate buffer, pH 5.5, instead of phospholipase A were incubated at 30°. The ferroxidase activity of each sample as well as the control and the extraction and separation of the individual phospholipids from each sample were carried out as previously described.

Phospholipase C Treatments: These treatments were performed according to the procedure reported by Topham and Gaylor (40). Two ml of purified ferroxidase-II, 0.02 ml of 0.4 M $CaCl_2$, and 0.5 mg of phospholipase C (10 units/mg, 1 unit liberates 1.0 μmole of water-soluble organic phosphate from egg yolk lecithin per minute) were incubated at 30° with gentle shaking for various time intervals. Controls containing purified ferroxidase, $CaCl_2$, but no phospholipase C was incubated at 30°. The ferroxidase activities of the samples and of the controls were determined, the lipids extracted and washed, and the lipid phosphorus contents determined as previously described.

Phospholipase D Treatments: Two ml of purified enzyme, 0.02 ml of 0.4 M $CaCl_2$, and 100 μl of a phospholipase D solution (1 mg protein/ml) were incubated at 30° for various time intervals. Controls containing all components but 100 μl of 0.05 M acetate buffer, pH 5.5, instead of 100 μl phospholipase D were incubated at 30°. The ferroxidase activities of the samples and of the controls were determined, the lipids were extracted and washed, and the individual phospholipids separated as previously described.

Attempt to Prepare a Lipid-Depleted Enzyme by Acetone Extraction

A modified form of the procedure of Moore and Gaylor was used (41). Ten ml of the purified enzyme was added in dropwise manner to 30 ml of prechilled (-15°) acetone, stirred for 30 minutes and the temperature maintained at -15°. Instead of the filtration procedure suggested by Moore and Gaylor (41), centrifugation was used to harvest the protein precipitated by the acetone. The acetone-layer was decanted. A second 30 ml portion of prechilled acetone was added to the residue, stirred and centrifugated. The acetone-layer again was decanted. The resulting powder was dried under a gentle stream of $N_2$, dessicated and stored in a vial, and kept in the freezer until used.

The acetone powder (3.8 mg) was dissolved in 1 ml of 0.05 M acetate buffer, pH 5.5, and the ferroxidase activity and the phospholipid per mg protein were determined.

Attempt to Prepare Lipid-Depleted Enzyme by Phospholipase Treatment Followed by Gel-Filtration

(a) Phospholipase A Treatment and Gel-Filtration:

Four ml of the purified ferroxidase-II were treated with phospholipase A for 2 hours as previously described. Following treatment, 2 ml of the preparation was subjected to gel-filtration on a column (0.9 x 12 cm) of Sephadex G-100. The protein was eluted from the Sephadex G-100 with 0.05 M acetate buffer, pH 5.5. The fractions from the column containing protein were combined. The ferroxidase activity, protein concentration, and phospholipid content of the preparation were determined as previously described, prior to the treatment with phospholipase A and following treatment with phospholipase A and gel-filtration on sephadex columns.

Controls containing all components except phospholipase A were carried through the same procedure.

(b) Phospholipase C Treatment and Gel-Filtration:
Thirty ml of purified ferroxidase-II, 0.3 ml of 0.4 M $CaCl_2$, and 3.8 mg of phospholipase C were incubated for two hours at 30°. Following the incubation, a 20 ml sample of the treated material was applied to a column (3 x 45 cm) of Agarose A-50 M (a gel-filtration material with a greater capacity and larger fractionation range than the sephadex gels). The eluting solvent was 0.05 M acetate buffer, pH 5.5. Eighty 5.5 ml fractions were collected utilizing a Gilson Model 80 micro-fractionator. The protein concentration of every other fraction was determined as previously described. Two protein bands were eluted from the column. The fractions composing each of these separate bands were combined, concentrated by ultrafiltration, and the ferroxidase activity, protein concentration, phospholipid content and cholesterol content determined as previously described. A sample of purified ferroxidase-II which had not been treated with phospholipase C was applied to the same Agarose A-50 M column and subjected to gel-filtration in an identical manner to the treated sample.

Disc-Gel Electrophoresis

Disc-gel electrophoresis was performed by the simplified procedure described by Davis (42). Standard, uniform bore, straight glass tubes (120 mm x 4 mm) were filled with a 4% polyacrylamide separating gel to a height of 60 mm, and layered with 0.025 M Tris-glycine buffer, pH 8.8.

Sufficient solid sucrose was added to the ferroxidase-II preparations to obtain a 5% sucrose solution. Twenty-five µl of the dense sucrose-ferroxidase-II solution was carefully allowed to drop through the buffer solution to the top of the separating gel. The gels were subjected to 5 ma per gel at pH 8.8, 0.025 M tris-glycine buffer for 35 minutes. After electrophoresis the gels were stained with Coomassie Blue, a protein stain, as described by Chrambach, *et al.* (43).

Copper Determination

Copper contents of ferroxidase-II preparations were determined by the method of Wharton and Rader (44). This method involves the treatment of a protein solution with glacial acetic acid to liberate bound copper, reduction of the copper with mercaptoacetic acid, chelation of Cu(I) by bathocuproine (4,7-diphenyl-1,10-phenanthroline), precipitation of the protein with 95% ethanol, and removal of the precipitated Cu-free protein by centrifugation. The Cu(I)-bathocuproine complex is colored and the intensity of the color is directly proportional to the Cu concentration in the range of 5 to 70 nanomoles of Cu per ml. Specifically, 0.4 ml of the appropriate ferroxidase-II solution, 0.1 ml of a 5% (w/v) solution of mercaptoacetic acid, 0.5 ml of 0.1% (w/v) solution of bathocuproine in glacial acetic acid, 0.5 ml of 95% ethanol were mixed vigorously for 5 minutes. Blanks containing 0.4 ml of 0.05 M acetate buffer, pH 5.5 instead of the ferroxidase-II solution were prepared in the same manner. A standard curve was prepared with samples containing 10, 25, 50 and 100 nanomoles of Cu and this standard curve used for the calculation of Cu contents of various ferroxidase-II preparations. All samples were read at 479 nm.

Reconstitution

Attempts were made to reconstitute the enzymic activity of lipid-

depleted ferroxidase-II prepared by acetone extraction or phospholipase C treatment followed by gel-filtration.

The lipid-depleted enzyme from acetone extraction (3.8 mg) was dissolved in 1 ml of 0.05 M acetate buffer, pH 5.5. To 0.35 ml of this solution was added 0.1 mg of phosphatidylcholine to reestablish the phospholipid to protein ratio present in the preparation before lipid-depletion. The solution was incubated for 2 hours at 30°. Following incubation, the specific activity of the solution was determined.

Two tenths ml of a glycerol solution of phosphatidylcholine (1.68 mg/ml) was added to 2 ml of the lipid-depleted enzyme prepared by phospholipase C treatment followed by gel-filtration. This quantity of phosphatidylcholine was equivalent to the phospholipid present in the enzyme preparation prior to lipid-depletion. The resulting solution was incubated for 2 hours at 30°. Following incubation, the solution was dialyzed overnight with 0.05 M acetate buffer, pH 5.5, to remove any non-protein bound phosphatidylcholine. The phospholipid content, protein content, and specific activity of the dialyzed preparation were determined. A control containing 2 ml of the lipid-depleted enzyme, 0.2 ml of pure glycerol, but no phosphatidylcholine was incubated and dialyzed in an identical manner. The specific activity of the control was determined. The addition of this quantity of pure glycerol did not significantly affect the activity of the enzyme.

Reconstitution was also attempted by the addition of phosphatidylcholine and cholesterol and by the addition of phosphatidylcholine, cholesterol, and copper to the lipid-depleted enzyme. Two mg of phosphatidylcholine and 1 mg of cholesterol were suspended in 1 ml of 0.05 M acetate buffer, pH 5.5 by gentle homogenization. To 1 ml of the lipid-depleted enzyme (prepared by phospholipase C treatment and gel-

filtration) was added 0.15 ml of the lipid suspension to reestablish the phospholipid and cholesterol contents present before lipid depletion. To another 1 ml sample of the lipid-depleted enzyme was added 0.3 ml of the lipid suspension to yield twice the phospholipid and cholesterol contents present before lipid-depletion. Both samples were incubated for 2 hours at 30°. Following incubation, the specific activity of each sample was determined.

Two mg of phosphatidylcholine and 1 mg of cholesterol were dissolved in glycerol. The previous experiment was repeated with the glycerol solution of lipid replacing the buffer suspension of lipid.

In a third experiment, 0.15 ml and 0.3 ml of the buffer solution of phosphatidylcholine and cholesterol were added to 1 ml samples of the lipid-depleted enzyme preparation. One tenth ml of a $CuSO_4$ solution (500 μmoles of Cu/ml) was added to the sample containing 0.15 ml of the buffer suspension of lipids and 1 ml of the lipid-depleted enzyme preparation. Two tenths ml of the $CuSO_4$ solution was added to the sample containing 0.3 ml of the buffer suspension of lipids and 1 ml of the lipid-depleted enzyme preparation. These samples were incubated for 2 hours at 30°. Following incubation the specific activity of each sample was determined.

# RESULTS

## Quantitative Determination of Lipids Bound to Ferroxidase-II

The molecular weight, immunoelectrophoretic mobility, and staining properties reported for Ferroxidase-II by Topham and Frieden (33) suggested that this serum enzyme might be a lipoprotein. Most of the lipid bound to blood serum lipoproteins is in the form of the neutral lipid, cholesterol, and the polar phospholipids (46). Thus, the phospholipid and cholesterol contents of six separately purified ferroxidase-II preparations of high specific activity were determined. The ferroxidase-II preparations contained lipid both in the form of cholesterol and phospholipid. All six preparations had a consistent ratio of protein, phospholipid, and cholesterol (Table 1). The purification procedure for ferroxidase-II involves extensive dialysis, ion-exchange chromatography, ultrafiltration, and gel-filtration which should result in the loss of any unbound or loosely bound lipids from the ferroxidase-II preparations. Thus, these lipid components must be tightly associated with the purified enzyme.

## Qualitative Identification of Individual Phospholipid and Neutral Lipids

Thin-layer chromatographic analyses were carried out to ascertain which individual phospholipids and neutral lipids were bound to ferroxidase-II. Phosphatidylcholine accounts for the majority of the bound phospholipid with small amounts of sphingomyelin and lysophosphatidylcholine accounting for the remaining phospholipid (Figure 1). Cholesterol, its esters, and other neutral lipids move with the solvent front in this particular developing solvent system (chloroform:methanol: acetic acid:water - 25:15:4:2, v/v). Therefore, in order to identify the neutral lipid components a second solvent system (hexane:diethyl ether:acetic acid = 20:80:1, v/v) was employed. Spots for cholesterol,

TABLE 1

Quantitative Determination of Lipids Bound to Ferroxidase-II

| Enzyme Preparation No. | Specific Activity ($A_{460nm}$/10 min/mg protein) | Protein (mg/ml) | Phospholipid (mg/ml) | Cholesterol (mg/ml) | Phospholipid: Cholesterol: Protein |
|---|---|---|---|---|---|
| 1 | 2.79 | 4.65 ± .02 | 0.72 ± .005 | 0.47 ± .06 | 1.5:1:10 |
| 2 | 2.03 | 4.70 ± .05 | 0.60 ± .03 | 0.34 ± .01 | 1.3:0.7:10 |
| 3 | 3.31 | 7.40 ± .02 | 0.92 ± .03 | 0.72 ± .03 | 1.3:1:10 |
| 4 | 4.72 | 3.72 ± .02 | 0.43 ± .002 | 0.38 ± .04 | 1.2:1:10 |
| 5 | 3.79 | 3.82 ± .02 | 0.56 ± .01 | 0.45 ± .03 | 1.4:1.2:10 |
| 6 | 2.87 | 5.11 ± .03 | 0.68 ± .003 | 0.53 ± .004 | 1.4:1.1:10 |

FIGURE 1

Qualitative Identification of Individual Phospholipid of Ferroxidase-II

Thin-layer chromatographic analyses were carried out as described in "Experimental Procedures". The developing solvent system was $CHCl_3$:$CH_3OH$:$CH_3COOH$:$H_2O$ (25:15:4:2, v/v).

1-1. Rf of standards ------- C(.989); PE(.659); PS(.512); PC(.182); Sph(.122); LPC(.089).

Rf of sample ------- Spot #1(.989); Spot #2(.234); Spot #3(.124); Spot #4(.077).

1-2. Rf of standards ------- CE(.976); C(.935); PE(.574); PS(.475); PC(.418); Sph(.344); LPC(.230).

Rf of sample ------- Spot #1(.984); Spot #2(.916); Spot #3(.457); Spot #4(.356); Spot #5(.272).

C = Cholesterol

CE = Cholesterol acetate

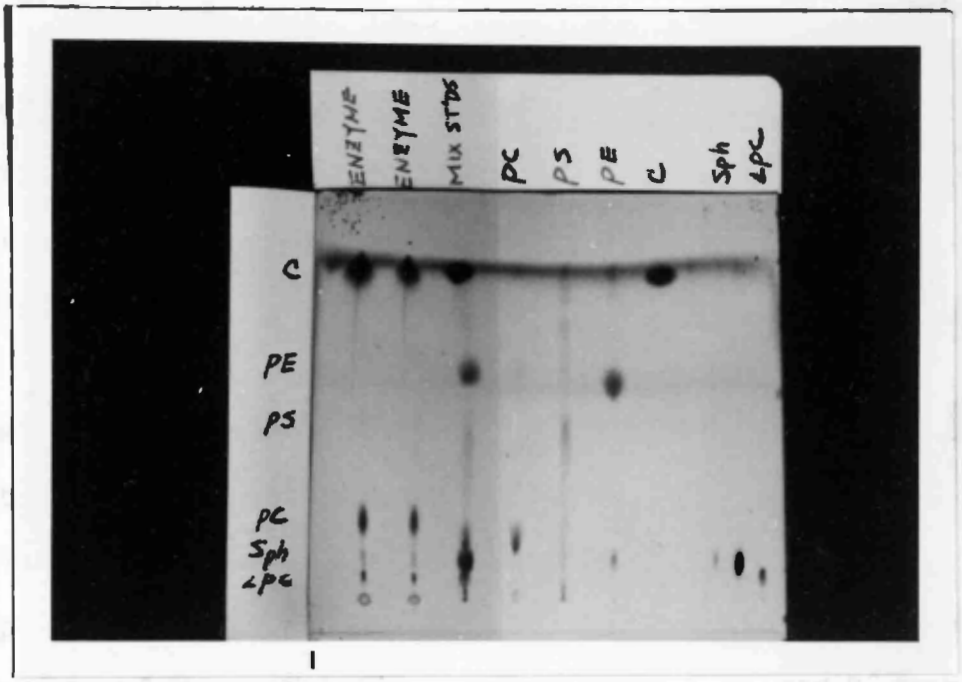
LPC = Lysophosphatidylcholine

PC = Phosphatidylcholine

PE = Phosphatidylethanolamine

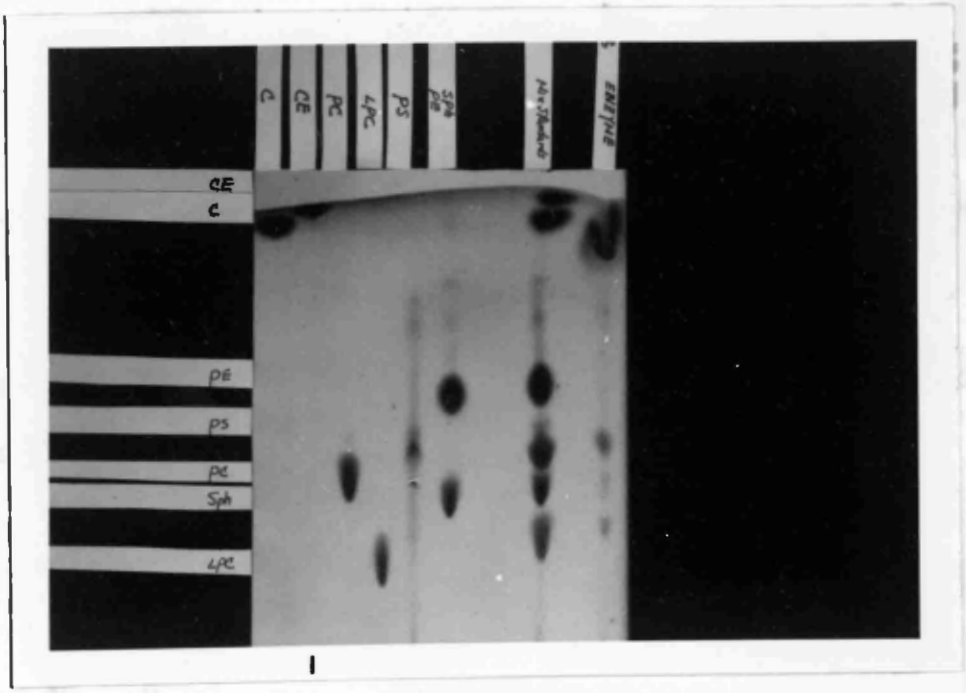
PS = Phosphatidylserine

Sph = Sphingomyelin


ENZYME
ENZYME
MIX STDS
PC
PS
PE
C
Sph
LPC
C
PE
PS
PC
Sph
LPC

1

1 — 1


C
CE
PC
LPC
PS
Sph
CE
MIXTURE
ENZYME
CE
C
PE
PS
PC
Sph
LPC

1

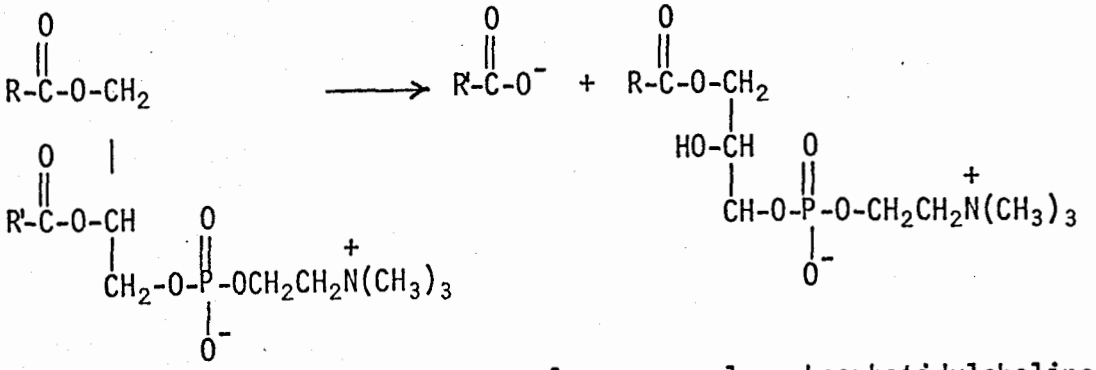
1—2

cholesterol esters, and fatty acids were obtained with this solvent system (Figure 2).

Treatment of Ferroxidase-II with Phospholipases

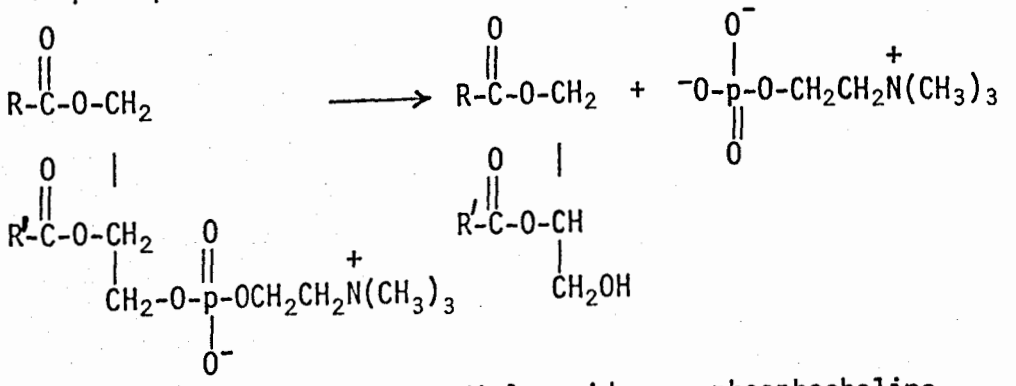
Several enzymes attached to the inner membrane of the mitochondria of the cell, appear to have an absolute requirement for specific phospholipids for the maintenance of enzymic activity (47). It would be quite interesting if the enzymic activity of ferroxidase-II, a blood serum lipoprotein, exhibited a similar requirement. To determine whether the phospholipids bound to ferroxidase-II were essential for the maintenance of enzymic activity, purified ferroxidase-II preparations were treated with phospholipases, enzymes normally manufactured in the pancreas which catalyze the hydrolysis of phospholipids. Phospholipase A, C, and D, each catalyze the hydrolysis of phospholipid at a specific but different position as illustrated below:

(a) Phospholipase A hydrolysis:



phosphatidylcholine fatty acyl group lysophosphatidylcholine

(b) Phospholipase C hydrolysis:



phosphatidylcholine 1,2-diglyceride phosphocholine

FIGURE 2

Qualitative Identification of Individual Neutral Lipids of Ferroxidase-II

Thin-layer chromatographic analyses were carried out as described in "Experimental Procedures". The developing solvent system was hexane:diethylether:acetic acid (20:80:1, v/v).

2-1. Rf of standards ------- TG(.932); CE(.831); FA(.729); 1,3-DG(.712); 1,2-DG(.644); C(.526); MG(.237).

Rf of sample ------- Spot #1(.864); Spot #2(.757); Spot #3(.7); Spot #4(.522).

2-2. Rf of standards ------- TG(.825); CE(.736); FA(.640); 1,3-DG(.632); 1,2-DG(.552); C(.474); MG(.123).

Rf of sample ------- Spot #1(.745); Spot #2(670); Spot #3(632); Spot #4(.482).

C = Cholesterol

CE = Cholesterol acetate

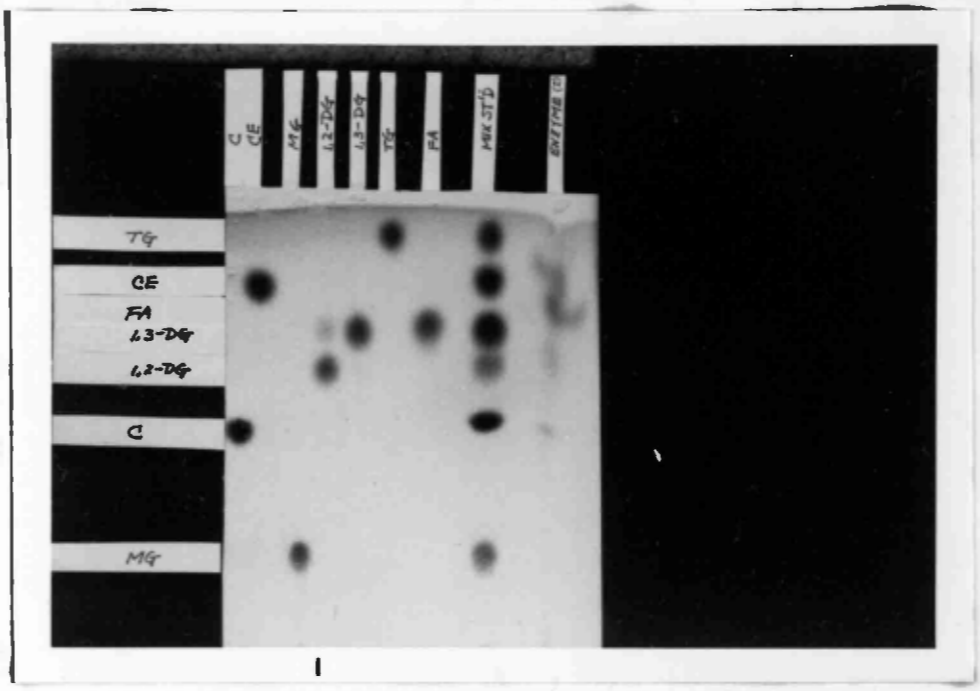
1,2-DG = 1,2-dipalmitin (1,2-diglyceride)

1,3-DG = 1,3-dipalmitin (1,3-diglyceride)

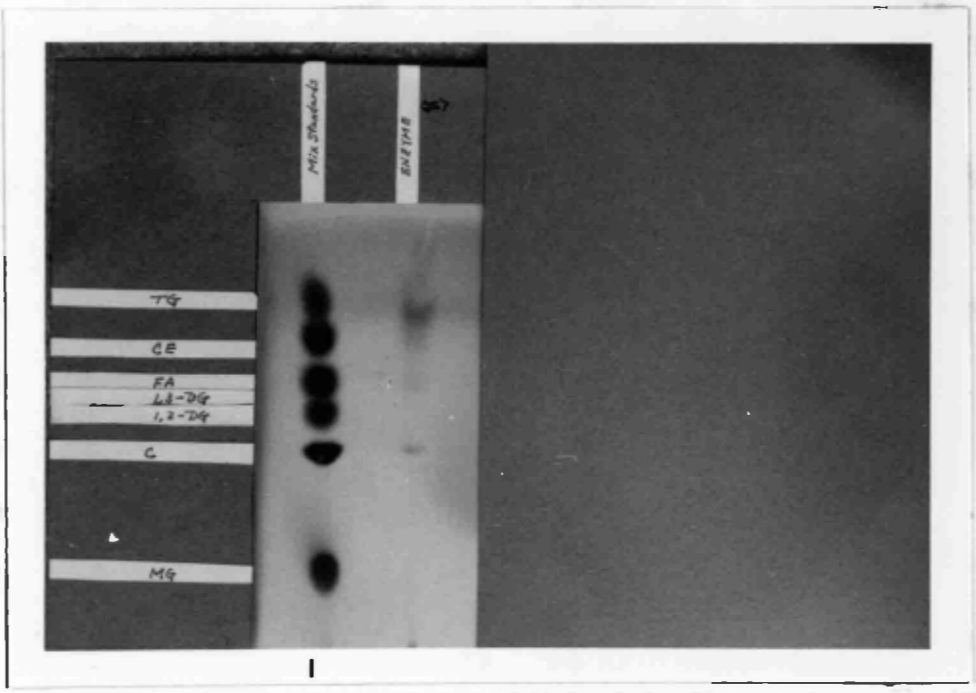
FA = Palmitic acid (free fatty acid)

MG = Monopalmitin (monoglyceride)

TG = Tripalmitin (triglyceride)

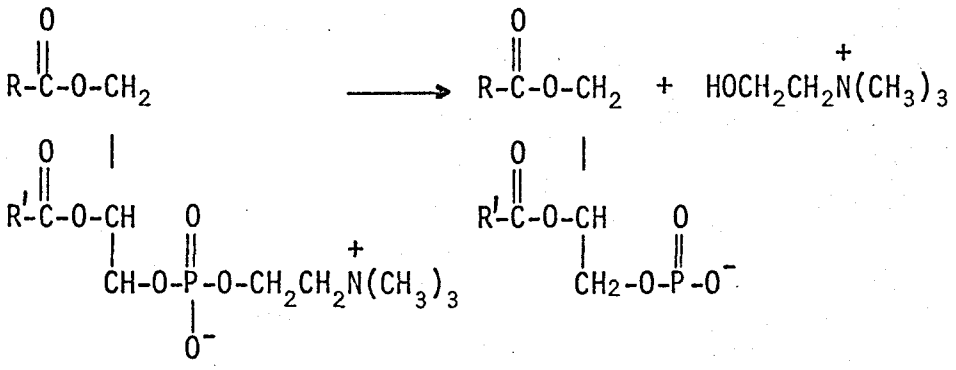

C
CE
MG
1,2-DG
1,3-DG
TG
FA
MIX STD
ENZYME
TG
CE
FA
1,3-DG
1,2-DG
C
MG

2 — 1


Mix Standards
ENZYME
TG
CE
FA
1,3-DG
1,2-DG
C
MG

2 — 2

(c) Phospholipase D hydrolysis:



phosphatidylcholine phosphatidic acid choline

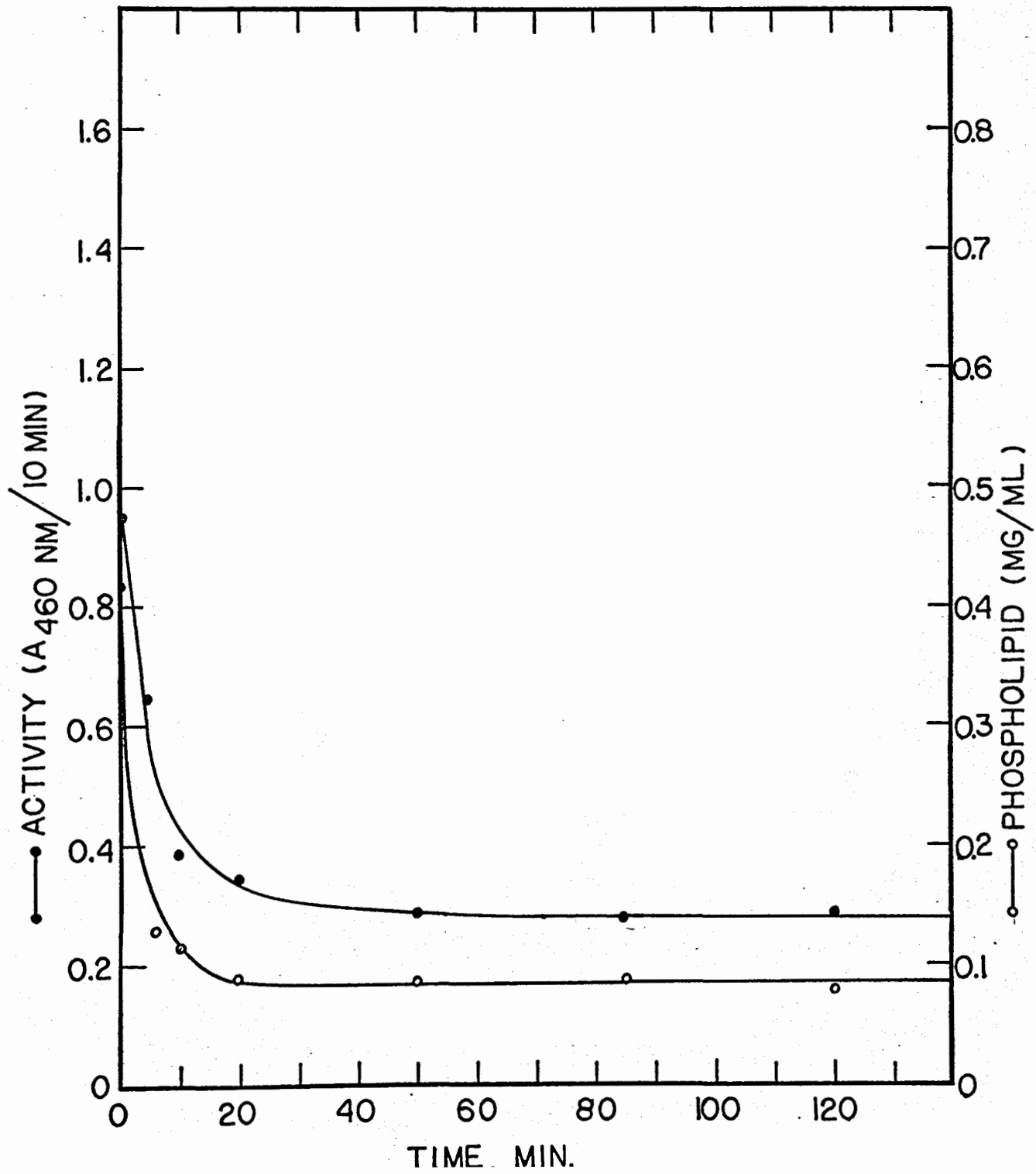
Phospholipase C hydrolysis of phosphatidylcholine results in the formation of a 1,2-diglyceride and water-soluble phosphocholine. Thus, phospholipid hydrolysis could be monitored simply by following the disappearance of lipid bound phosphorus from chloroform:methanol extracts of treated ferroxidase-II preparations. Treatment of purified ferroxidase-II with phospholipase C resulted in a progressive loss of ferroxidase activity which paralleled the hydrolysis of phospholipid (Figure 3-1 and 3-2). In the first 10 minutes of treatment, the activity and phospholipid content decreased dramatically; little change in activity or phospholipid content was observed after 1 hour of treatment. No ferroxidase activity was lost from the control samples incubated without phospholipase C. The loss in activity and hydrolysis of phospholipid following 80 minutes of treatment were compared for several ferroxidase-II preparations (Table 2). The loss of activity and hydrolysis of phospholipid were comparable for all four preparations examined.

Treatment of purified ferroxidase-II with phospholipase A also resulted in the progressive loss of ferroxidase activity (Figure 4). The activity declined dramatically during the first 10 minutes of treatment. No loss in ferroxidase activity was observed in the control flasks without phospholipase A. Phospholipase A hydrolysis of phosphatidylcholine

FIGURE 3-1 and 3-2

Time Course of Loss of Ferroxidase Activity and Hydrolysis of Phospholipid of Ferroxidase-II with Phospholipase C

The phospholipase C treatment of ferroxidase-II was carried out as described in "Experimental Procedures". Ferroxidase activities and phospholipid contents of ferroxidase-II were determined at several time intervals.


1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ACTIVITY ($A_{460}$ NM/10 MIN)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
PHOSPHOLIPID (MG/ML)
0
20
40
60
80
100
120
TIME MIN.

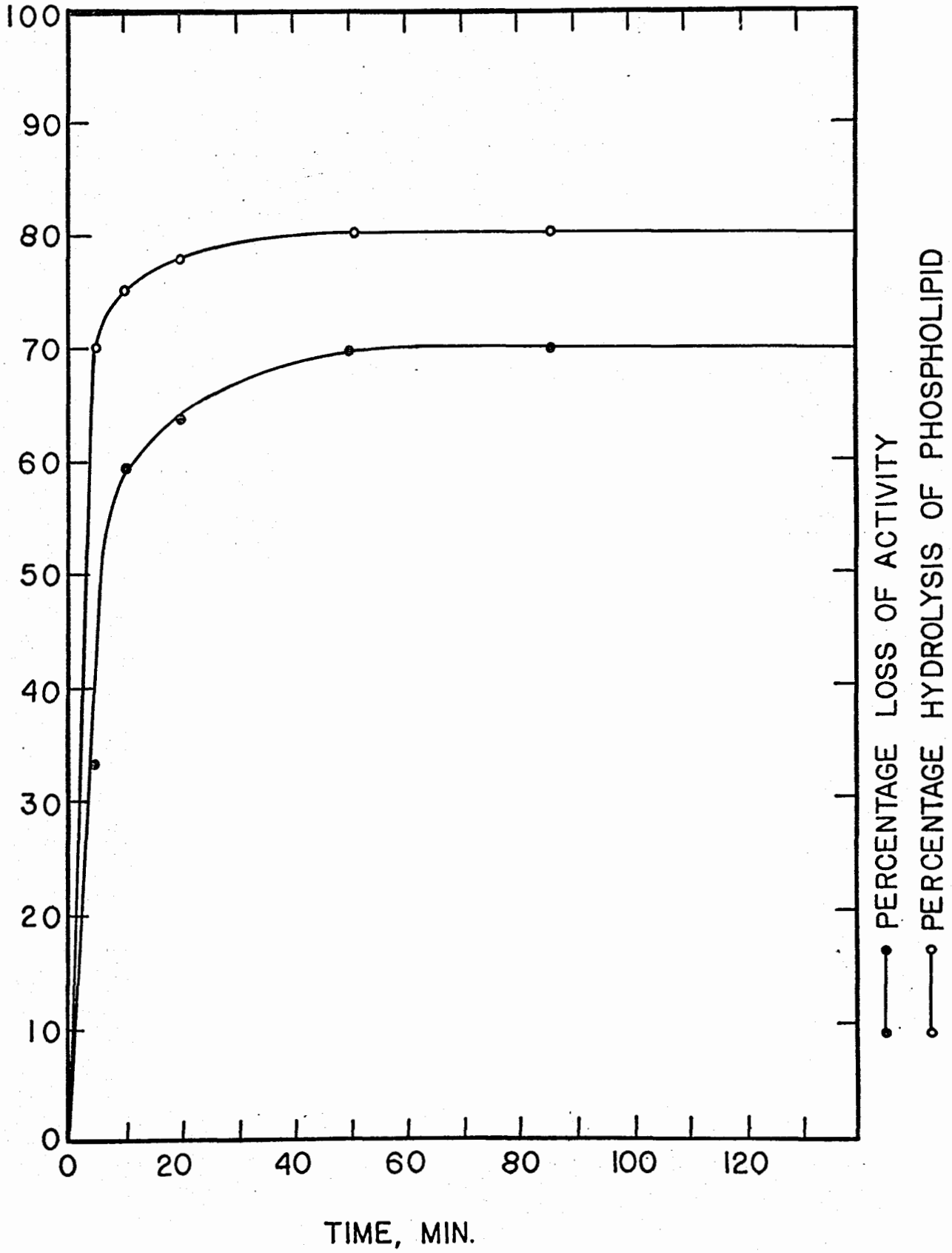

100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
TIME, MIN.
PERCENTAGE LOSS OF ACTIVITY
PERCENTAGE HYDROLYSIS OF PHOSPHOLIPID

TABLE 2

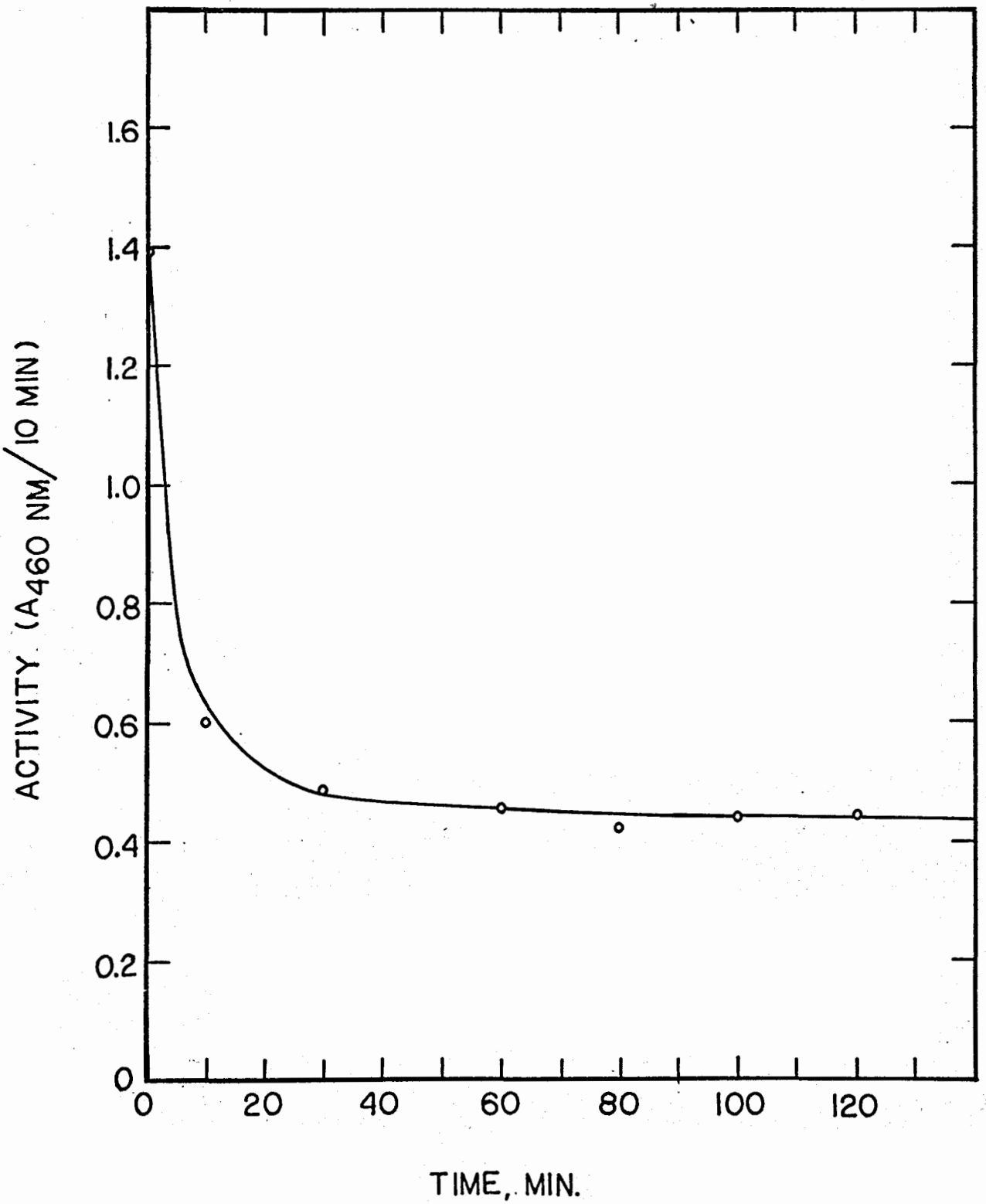
Comparison of Loss of Activity and Phospholipid Hydrolysis with Phospholipase C Treatment for Several Ferroxidase-II Preparations

| Enzyme Preparation No. | Specific Activity | Time of Treatment (min) | Activity ($A_{460nm}$/10 min) | | Phospholipid (mg/ml) | |
|---|---|---|---|---|---|---|
| | | | | % Loss | | % Hydrolysis |
| 7 | 3.17 | 0 | 1.34 | - | 0.47 ± .002 | - |
| | | 80 | 0.38 | 71 | 0.14 ± .004 | 70 |
| 8 | 3.31 | 0 | 1.30 | - | 0.67 ± .003 | - |
| | | 80 | 0.36 | 72 | 0.15 ± .001 | 78 |
| 9 | 2.79 | 0 | 0.91 | - | 0.48 ± .005 | - |
| | | 80 | 0.17 | 82 | 0.10 ± .003 | 77 |
| 10 | 2.03 | 0 | 0.95 | - | 0.43 ± .005 | - |
| | | 80 | 0.29 | 70 | 0.09 ± .005 | 80 |

FIGURE 4

Time Course of Loss of Ferroxidase Activity of Ferroxidase-II with Phospholipase A

The phospholipase A treatment of ferroxidase-II was carried out as described in "Experimental Procedures". The ferroxidase activity of the samples was determined at several time intervals.


ACTIVITY (A460 NM/10 MIN)
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
20
40
60
80
100
120
TIME, MIN.

results in the formation of lysophosphatidylcholine and a fatty acyl group. Thus, phospholipid hydrolysis could not be monitored simply by following the disappearance of lipid phosphorus in the chloroform: methanol extracts of treated samples. Phospholipid hydrolysis was monitored by thin-layer chromatography. Disappearance of the spot for phosphatidylcholine and intensification of the spot for lysophosphatidylcholine was observed on thin-layer chromatography of the lipid extracts from treated samples (Figure 5). The loss of ferroxidase activity following 2 hours treatment with phospholipase A was compared for several different ferroxidase-II preparations (Table 3). The loss in activity was comparable with all preparations tested.

Treatment of purified ferroxidase-II with phospholipase D also resulted in the loss of activity (Figure 6). However, the extent of inactivation at similar time intervals was not nearly as great as observed in treatment with phospholipase C or A. Hydrolysis of phosphatidylcholine with phospholipase D results in the formation of phosphatidic acid and choline. Thus, phospholipid hydrolysis could not be monitored simply by following the disappearance of lipid phosphorus in the chloroform:methanol extracts of treated samples. Phospholipid hydrolysis was monitored by thin-layer chromatography (Figure 7). Little decrease in the intensity of the spot for phosphatidylcholine was observed suggesting that phospholipase D was not nearly as effective for the hydrolysis of the phospholipids bound to ferroxidase-II as phospholipase C or A. The extent of inactivation after 40 minutes treatment with phospholipase D was determined for two separate ferroxidase-II preparations. The loss of activity was comparable for the two preparations (Table 4).

FIGURE 5

Thin-Layer Chromatography of Phospholipids of Ferroxidase-II Before and After Phospholipase A Treatment

The extracted samples after phospholipase A treatment were applied to thin-layer chromatographic plates as described in "Experimental Procedures".

On the plate:

Column #1: Mixture of standards were applied C, CE, PE, PS, PC, Sph, LPC.

Column #2, #3: Extracted sample of purified ferroxidase-II. PC was the most intense spot.

Column #4, #5, #6, #7, #8: Lipid extracts after 10, 20, 40, 80, 160 minutes of phospholipase A treatment.

Column #9: Spots of standard PC and LPC.

TABLE 3

Comparison of Loss of Activity with Phospholipase A Treatment

for Several Ferroxidase-II Preparations

| Enzyme Preparation No. | Specific Activity | Time of Treatment (hrs) | Activity ($A_{460nm}$/10 min) | % Loss of Activity |
|---|---|---|---|---|
| 7 | 3.17 | 0 | 1.39 | - |
| | | 2 | 0.48 | 66 |
| 8 | 3.31 | 0 | 1.03 | - |
| | | 2 | 0.44 | 58 |
| 9 | 4.72 | 0 | 1.03 | - |
| | | 2 | 0.36 | 65 |

FIGURE 6

Time Course of Loss of Ferroxidase Activity with Phospholipase D Treatment

The phospholipase D treatment of ferroxidase-II was carried out as described in "Experimental Procedures". The ferroxidase activities of treated samples were determined at several time intervals.

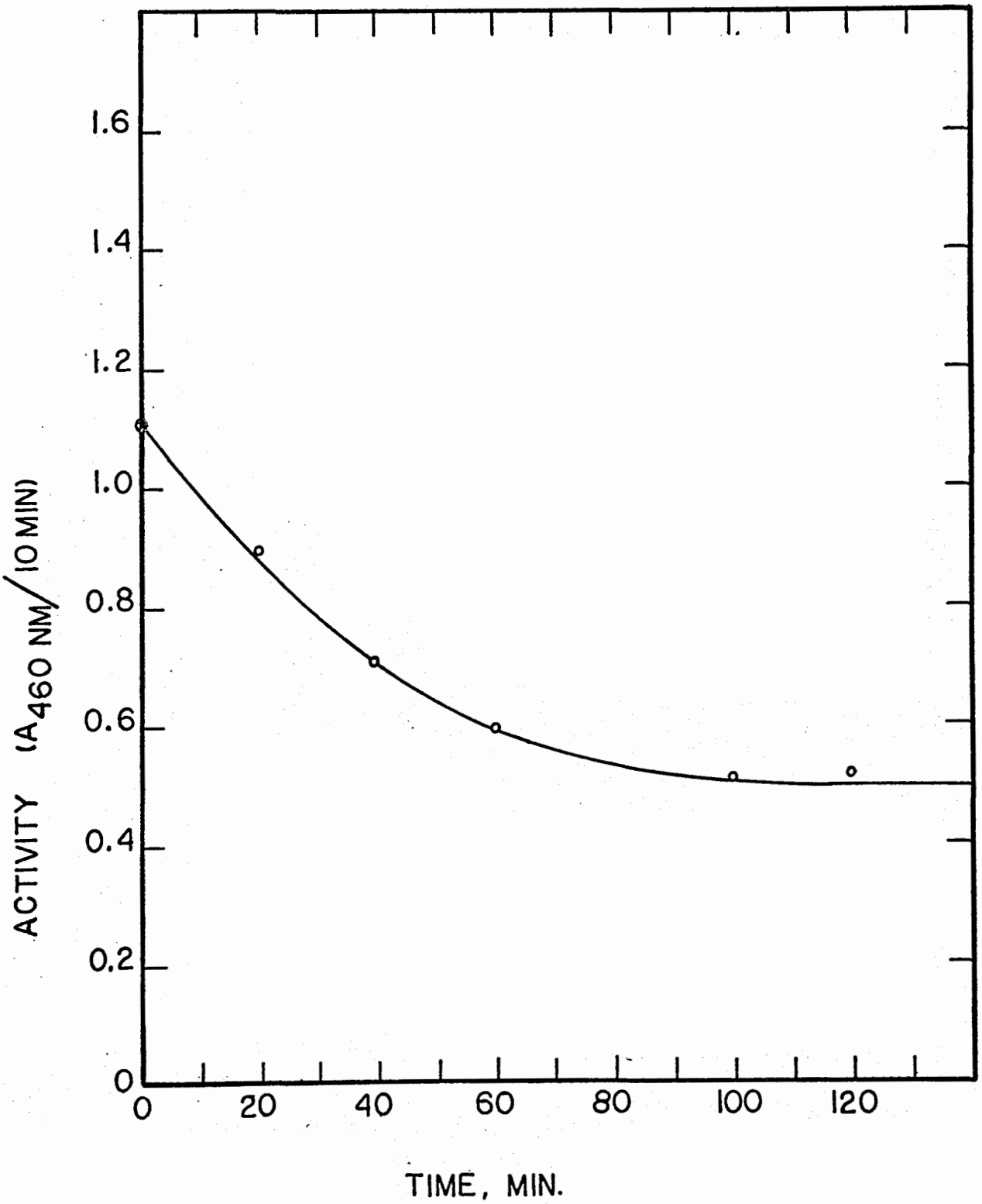

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
ACTIVITY (A460 NM/10 MIN)
0
20
40
60
80
100
120
TIME, MIN.

FIGURE 7

Thin-Layer Chromatographic Analysis of Phospholipids of Ferroxidase-II Before and After Phospholipase D Treatment

The extracted samples after phospholipase D treatment were subjected to thin-layer chromatographic analysis as described in "Experimental Procedures".

On the plate:

Column #1: Mixture of standards (C, CE, PE, PS, PC, Sph, LPC and phosphatidic acid).

Column #2-#7: Extracted samples of phospholipase D treatment of ferroxidase-II after 10, 20, 40, 60, 80, 160 minutes.

Column #8: Spot of standard phosphatidic acid.

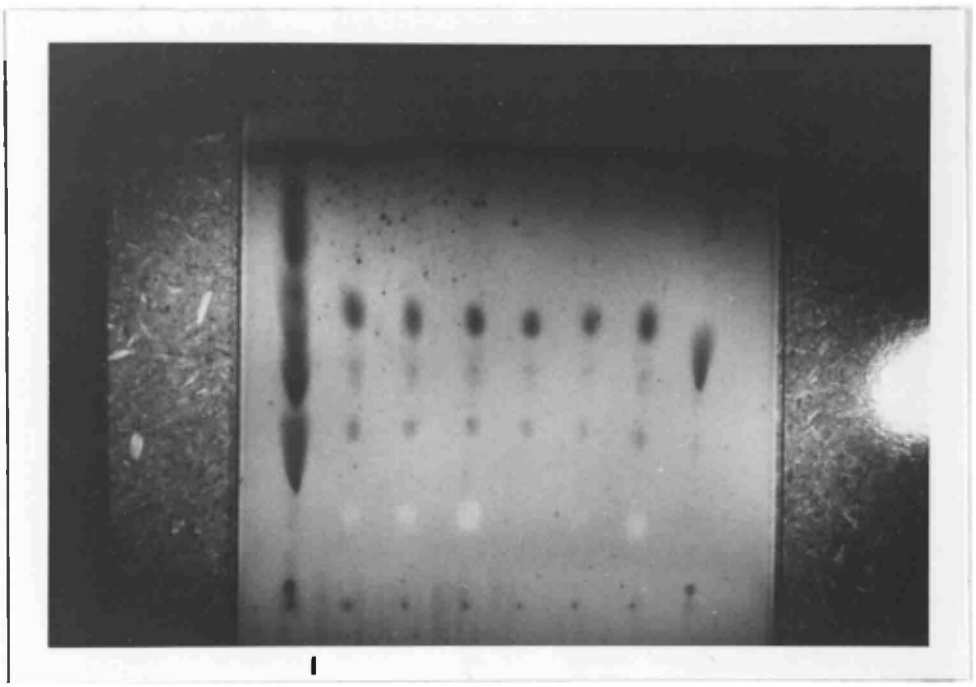


TABLE 4

Comparison of Loss of Activity with Phospholipase D Treatment

for Several Ferroxidase-II Preparations

| Enzyme Preparation No. | Specific Activity | Time of Treatment (min) | Activity | % Loss of Activity |
|---|---|---|---|---|
| 8 | 3.31 | 0 | 0.984 | - |
| | | 40 | 0.672 | 32 |
| 9 | 4.72 | 0 | 1.10 | - |
| | | 40 | 0.55 | 35 |

Attempts to Prepare A Lipid-Depleted Form of Ferroxidase

The loss of activity observed when ferroxidase-II preparations were treated with phospholipases, particularly phospholipase C or A, indicated that intact phospholipids were essential for the maintenance of maximum enzymic activity. It was felt that the preparation of a lipid-depleted form of the enzyme might provide clues regarding the specific role that phospholipids serve for ferroxidase-II. Furthermore, if a lipid-depleted form of the enzyme could be successfully prepared, it might be possible to reconstitute the enzymic activity by the addition of specific lipids and thus to establish which individual lipids were most essential.

Frequently, lipids have been dissociated from lipoproteins simply by extraction with acetone at ice temperature (-15°). The acetone extraction, if successful, results in the removal of lipids and the precipitation of the protein without significant denaturation. Acetone extractions of purified ferroxidase-II preparations resulted in the formation of a very finely divided precipitate which could not easily be isolated by filtration. The precipitate was collected by centrifugation; however, the precipitate represented less than 10% by weight of the protein originally present in the preparation before acetone extraction. A portion of the powder obtained was redissolved in 0.05 M acetate buffer, pH 5.5, and the protein content, phospholipid content, and the specific activity were determined (Table 5). The phospholipid to protein ratio decreased dramatically indicating that considerable phospholipid had been removed during the acetone extraction; however, the acetone extract had little ferroxidase activity. A sample of the powder obtained during acetone extraction was saved for reconstitution experiments to be discussed later.

TABLE 5

Acetone Extraction of Ferroxidase-II

| Treatment | Specific Activity ($A_{460nm}$/10 min/mg protein) | Protein (mg/ml) | Phospholipid (mg/ml) | $\frac{\text{Phospholipid}}{\text{Protein}}$ |
|---|---|---|---|---|
| None | 3.77 | 3.82 ± .02 | 0.56 ± .01 | 0.15 |
| Redissolved Acetone Extract | 0.14 | 1.45 ± .05 | 0.04 ± .001 | 0.03 |

Because of the poor yield obtained during acetone extraction, other methods for the preparation of a lipid-depleted form of the enzyme were sought. The intact phospholipids bound to ferroxidase-II were not removed by the gel-filtration techniques employed during the purification of ferroxidase-II. As previously described, the treatment of purified ferroxidase-II with phospholipase A or C resulted in the formation of hydrolysis products of the bound phospholipids. It seemed possible that these hydrolysis products might be more easily removed from ferroxidase-II than intact phospholipids. Thus, the preparation of a lipid-depleted form of the enzyme was attempted by subjecting phospholipase A or C treated ferroxidase-II to gel-filtration. The loss of phospholipid hydrolysis products was monitored by the determination of total phosphorus (lipid and non-lipid phosphorus in the ferroxidase-II preparation before treatment and after treatment and gel-filtration) (See Bartlett Method; "Experimental Procedures"). It was felt that the phospholipase treatment and gel-filtration represented a milder procedure in terms of protein denaturation than subjecting the enzyme to organic solvent extraction.

Ferroxidase-II preparations were treated with phospholipase A for 2 hours and applied to a column of Sephadex G-100. All fractions collected from the column containing protein were pooled. The protein content, phospholipid content, and specific activity of the pooled sample were determined. As observed in previous experiments, phospholipase A treatment resulted in a dramatic decrease in the specific activity of the ferroxidase-II preparation, but the phospholipid:protein ratio following gel-filtration was only decreased by 24% (Table 6). This indicated that the majority of the hydrolysis products of the phospholipids remained bound to the enzyme following gel-filtration.

TABLE 6

Lipid-Depletion of Ferroxidase-II by Phospholipase A Treatment

Followed by Gel-Filtration

| Treatment | Specific Activity ($A_{460nm}$/10 min/mg protein) | Protein (mg/ml) | Phospholipid (mg/ml) | $\frac{\text{Phospholipid}}{\text{Protein}}$ | % Loss of Phospholipid |
|---|---|---|---|---|---|
| None | 2.53 | 3.76 ± .03 | 0.74 ± .004 | 0.198 | - |
| Treatment with Phospholipase A for 2 hrs. followed by gel-filtration | 0.51 | 1.57 ± .02 | 0.24 ± .004 | 0.153 | 24 |

Similar experiments were performed with phospholipase C. A purified ferroxidase-II preparation was treated 2 hours with phospholipase C and then subjected to gel-filtration on a column of Agarose A-50 M. Following gel-filtration, all column fractions containing protein were pooled and the protein content, phospholipid content, and specific activity of the pooled sample were determined. The specific activity of the preparation following phospholipase C treatment and gel-filtration was drastically reduced. The phospholipid:protein ratio was reduced by 87% indicating that the majority of the phospholipid originally bound to the ferroxidase-II protein had been removed (Table 7). It was possible that the loss of the bound phospholipid following hydrolysis with phospholipase C and gel-filtration might be accompanied by a loss of bound cholesterol. Thus, the experiment was repeated with a second purified ferroxidase-II preparation and the cholesterol:protein ratio in addition to the phospholipid:protein ratio was determined before treatment and after phospholipase C treatment and gel-filtration (Table 8). The phospholipid:protein ratio was reduced by 83% which compared well to 87% observed with the first preparation. The cholesterol:protein ratio was also markedly reduced indicating that indeed bound cholesterol as well as phospholipid had been removed from the ferroxidase-II protein. The hydrolysis of phospholipids by phospholipase C results in the formation of a 1,2-diglyceride and phosphocholine. Thus, a spot for 1,2-diglyceride should appear on thin-layer chromatography of a lipid extract of phospholipase C treated samples before gel-filtration. Following gel-filtration this spot should disappear if indeed the phospholipid hydrolysis products are being removed during the gel-filtration procedure. The appearance of a spot for 1,2-diglyceride was observed in lipid extracts of phospholipase C treated samples prior to

TABLE 7

Lipid-Depletions of Ferroxidase-II by Phospholipase C Treatment

Followed by Gel-Filtration

| Treatment | Specific Activity ($A_{460nm}$/10 min/mg protein) | $\frac{\text{Phospholipid}}{\text{Protein}}$ | % Loss of Phospholipid Based on Protein |
|---|---|---|---|
| None | 2.97 | 0.15 | - |
| 2 hrs. with Phospholipase C followed by Agarose A-50 M gel-filtration | 0.09 | 0.020 | 87 |

TABLE 8

Lipid-Depletion of Ferroxidase-II by Phospholipase C Treatment

Followed by Gel-Filtration

| Treatment | Specific Activity ($A_{460nm}$/10 min/mg protein) | Phospholipid/Protein | | Cholesterol/Protein | |
|---|---|---|---|---|---|
| | | ratio | % Loss | ratio | % Loss |
| None | 1.73 | 0.12 | - | 0.08 | - |
| 2 hrs. with Phospholipase C followed by Agarose A-50 M gel-filtration | 0 | 0.02 | 83 | 0.03 | 63 |

gel-filtration. This spot was not observed with lipid-extracts of phospholipase C treated samples following gel-filtration (Figure 8).

Investigations Concerning Possible Structural Changes and Loss of Copper During Lipid-Depletion

The treatment of ferroxidase-II with phospholipase C followed by gel-filtration provided a method for the preparation of a lipid-depleted form of the enzyme in high yield. The removal of bound lipid could possibly alter the structure of ferroxidase-II and this alteration in structure could result in the loss of activity. To determine if any large change in the structure of ferroxidase-II had occurred during lipid depletion, the elution patterns of the native and lipid-depleted ferroxidase-II protein from an Agarose A-50 M column were compared. The elution patterns for native and lipid-depleted ferroxidase-II were extremely similar (Figure 9). However, with the lipid-depleted ferroxidase-II preparation a very small turbid band of protein (< 15% of the total protein) was eluted prior to the major protein band. This minor band could represent a small amount of denatured protein which may have been formed during the gel-filtration or incubation procedures. The major protein band obtained with the lipid-depleted sample had an elution volume identical to that of the native enzyme. This would indicate that no gross changes in the three-dimensional shape of the protein occurred during the lipid-depletion procedure. Native and lipid-depleted ferroxidase-II were also subjected to disc-gel electrophoresis (a technique which separates proteins in the basis of size, shape, and charge simultaneously). The electrophoretic mobilities of the native and lipid-depleted enzyme were identical (Figure 10). The electrophoretic results also indicated that no gross change in the three-dimensional shape had occurred during the lipid-depletion procedure.

FIGURE 8

Neutral Lipid Components of Ferroxidase-II Before and After Lipid-Depletion by Phospholipase C Treatment and Gel-Filtration

Thin-layer chromatographic analyses were carried out as described in "Experimental Procedures". The developing solvent system was hexane:diethylether:acetic acid (20:80:1, v/v).

8-1. Rf's of standards ------- TG(.932); CE(.831); FA(.729); 1,3-DG(.712); 1,2-DG(.644); C(.526); MG(.237)

Rf's of extracted ferroxidase-II ------- Spot #1(.894); Spot #2(.787); Spot #3(7); Spot #4(.532).

Rf's of extracted sample after phospholipase C treatment followed by gel-filtration ------- Spot #1(.816); Spot #2(.702); Spot #3(.64); Spot #4(.517).

8-2. Rf's of standards ------- TG(.825); CE(.736); FA(.64); 1,3-DG(.632); 1,2-DG(.552); C(.474); MG(.123).

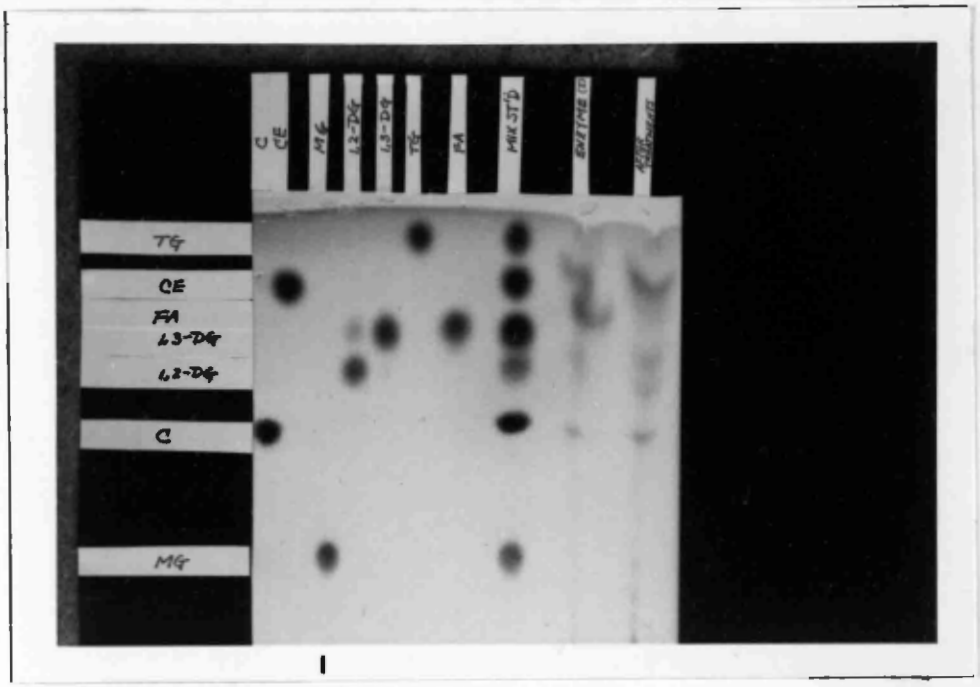
Rf's of extracted ferroxidase-II (enzyme) ------- Spot #1(.799); Spot #2(.745); Spot #3(.632); Spot #4 (.482).

Rf's of extracted sample of ferroxidase-II after phospholipase C treatment (ECT) ------- Spot #1(.702); Spot #2(.526); Spot #3(.456).

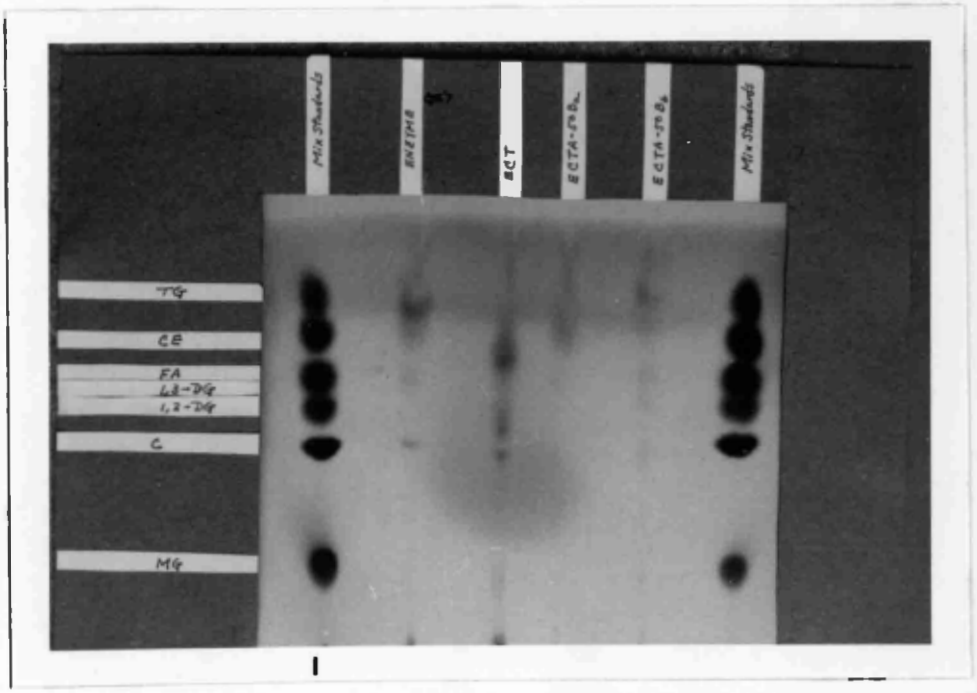
Rf's of extracted sample of ferroxidase-II after phospholipase C treatment followed by gel-filtration

(1) Band a (ECTA-50 Ba) ------- Spot #1(.768)

(2) Band b (ECTA-50 Bb) ------- Spot #1(.797); Spot #2(.655); Spot #3(.584); Spot #4(.469).


C
CE
MG
1,2-DG
1,3-DG
TG
FA
MIX STD
TG
CE
FA
1,3-DG
1,2-DG
C
MG

8 — 1


Mix Standards
ENZYME
ACT
EGTA-Fr 1
EGTA-Fr 4
Mix Standards
TG
CE
FA
1,3-DG
1,2-DG
C
MG

8 — 2

FIGURE 9

Comparison of Protein Elution Patterns for Native and Lipid-Depleted Ferroxidase-II

The protein elution patterns of gel-filtration of native and lipid-depleted ferroxidase-II were determined as described in "Experimental Procedures".

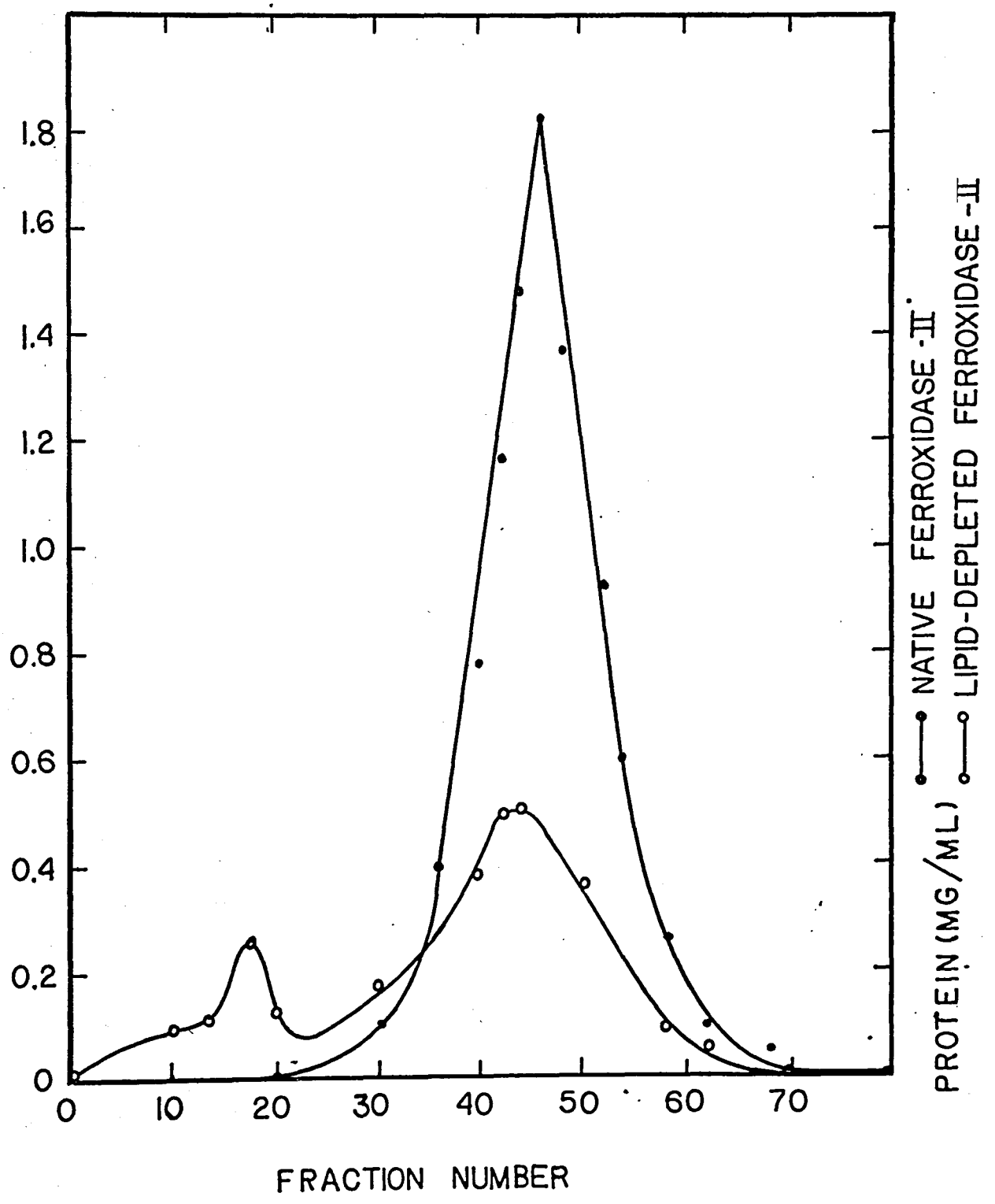
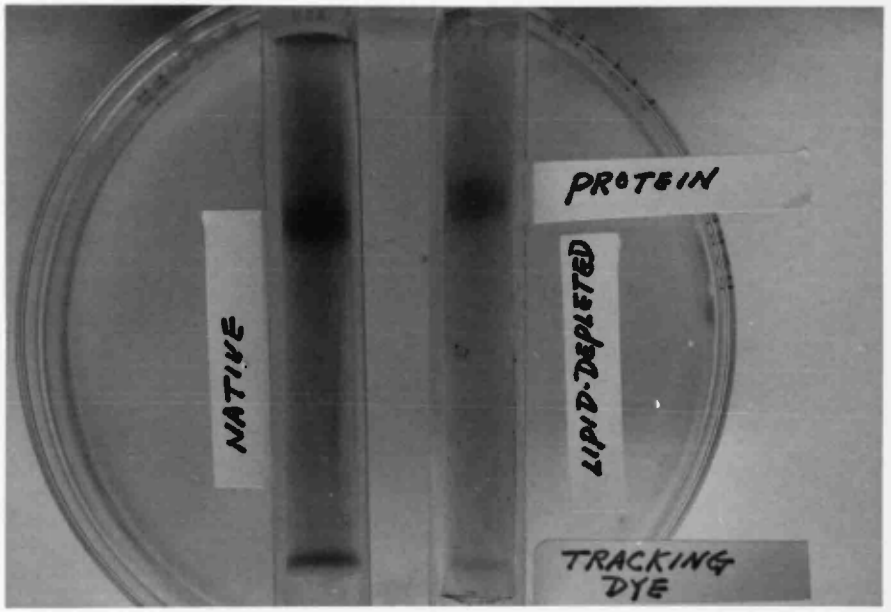

1.8
1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
60
70
FRACTION NUMBER
PROTEIN (MG/ML)
NATIVE FERROXIDASE -II
LIPID-DEPLETED FERROXIDASE -II

FIGURE 10

Comparison of Electrophoretic Mobilities of the Native and Lipid-Depleted Enzyme Preparations

Disc-gel electrophoresis was performed as described in "Experimental Procedures". The electrophoretic mobilities of native and lipid-depleted ferroxidase-II were compared.


NATIVE
PROTEIN
LIPID-DEPLETED
TRACKING DYE

Copper remains tightly associated with ferroxidase-II following purification (33) and this copper associated with ferroxidase-II has been shown to be essential for its ferroxidase activity (48). The removal of lipid components from ferroxidase-II might in some manner effect the afinity of the enzyme for copper. Thus, the copper content, phospholipid content, cholesterol content, and specific activity before and after lipid-depletion were compared for a purified ferroxidase-II preparation. As expected from previous experiments, a dramatic loss in the phospholipid and cholesterol bound to the ferroxidase-II was observed following phospholipase C treatment and gel-filtration. A dramatic loss of bound copper accompanied the loss of the lipid components (Table 9).

Reconstitution Attempts

A dramatic decrease in the specific activity of purified ferroxidase-II preparations was observed when bound phospholipids were hydrolyzed by phospholipase treatment or removed by acetone extraction or phospholipase C treatment followed by gel-filtration. This indicated that the lipid must be essential for the maintenance of the catalytic activity of ferroxidase-II. Reconstitution experiments were performed to attempt to restore the activity of the lipid-depleted enzyme by the addition of specific lipids or specific lipids and copper.

The addition of phosphatidylcholine to the acetone extracted enzyme did not result in a significant reconstitution of ferroxidase activity (Table 10). The addition of phosphatidylcholine to ferroxidase-II depleted of lipid by phospholipase C and gel-filtration also did not result in a significant reconstitution of the ferroxidase activity (Table 11). Furthermore, little phosphatidylcholine appeared to bind the lipid-depleted enzyme.

TABLE 9

Lipid and Copper Depletion of Ferroxidase-II by Phospholipase C Treatment

Followed by Gel-Filtration

| Treatment | | Specific Activity ($A_{460nm}$/10 min/mg protein) | Phospholipid / Protein | Cholesterol / Protein | Copper Content (nanomoles/mg protein) |
|---|---|---|---|---|---|
| None | | 2.59 | 0.15 | 0.09 | 10.29 |
| Agarose A-50 M Gel-filtration after phospholipase C treatment | BAND 1 | 0 | 0 | 0 | 0 |
| | BAND 2 | 0.08 | 0.05 | 0.03 | 0.54 |

TABLE 10

Addition of Phosphatidylcholine to Acetone-Extracted Ferroxidase-II

| Treatment | Activity ($A_{460nm}$/10 min) | Protein (mg/ml) | Specific Activity ($A_{460nm}$/10 min/mg protein) | Recovery of Specific Activity |
|---|---|---|---|---|
| None | 1.42 | 3.82 ± .02 | 3.77 | - |
| Following acetone extraction | 0.02 | 1.45 ± .01 | 0.14 | - |
| Reconstitution after acetone extraction | 0.05 | 1.45 ± .01 | 0.34 | 9.0% |

TABLE 11

Addition of Phosphatidylcholine to Lipid-Depleted Ferroxidase-II

| Treatment | $\frac{\text{Phospholipid}}{\text{Protein}}$ | Specific Activity ($A_{460nm}$/10 min/mg protein) | Percentage of the original Specific Activity |
|---|---|---|---|
| None | 0.17 | 2.36 | 100% |
| 2 hrs. with Phospholipase C followed by gel-filtration | 0.07 | 0.25 | 10.6% |
| 2 hrs. of incubation of lipid-depleted enzyme with phosphatidyl-choline in glycerol solution | 0.10 | 0.08 | 3.4% |

The treatment of ferroxidase-II with phospholipase C followed by gel-filtration resulted in the loss of cholesterol and copper as well as phospholipid. Thus, it was felt that better reconstitution might be achieved by including cholesterol and copper in the reconstitution medium. However, neither the addition of cholesterol nor copper improved the recovery of ferroxidase activity (Table 12). These results suggested that the removal of the lipids from ferroxidase-II promoted a change in the enzyme which prevented the rebinding of exogeneously added lipids or copper.

TABLE 12

Addition of Phosphatidylcholine, Cholesterol, and Copper to Lipid-Depleted Ferroxidase-II

| Treatment | | Specific Activity ($A_{460nm}$/10 min/mg protein) | Percentage of the Original Specific Activity |
|---|---|---|---|
| None | | 2.59 | 100% |
| 2 hrs. with Phospholipase C Treatment followed by Agarose A-50 M gel-filtration | | 0.08 | 3.1% |
| 2 hrs. incubation of lipid and copper depleted enzyme with acetate buffer suspension of phosphatidylcholine and cholesterol | Adding back to the original ratio of phospholipid, and cholesterol to protein | 0.00 | - |
| | Adding back twice the original ratio | 0.11 | 4.2% |
| 2 hrs. incubation of lipid and copper depleted enzyme with glycerol solution of phosphatidylcholine and cholesterol | Adding back to the original ratio of phospholipid, and cholesterol to protein | 0.00 | - |
| | Adding back twice the original ratio | 0.11 | 4.2% |
| 2 hrs. incubation of lipid and copper depleted enzyme with acetate buffer suspension of phosphatidylcholine, cholesterol, and copper | Adding back to the original ratio of phospholipid, cholesterol, and copper to protein | 0.00 | - |
| | Adding back twice the original ratio | 0.15 | 5.8% |

# DISCUSSION

It was recognized many years ago that there was a close relationship between lipids and proteins in many natural sources. In fact, many proteins appear to complex lipid components very strongly. As data accumulated on the chemical nature of these complexes, now termed lipoproteins, it was evident that the linkage was not of a covalent but rather of an associated type. Many type associations, based on the different polarity regions of lipids and proteins are possible. Nonpolar groups in the side chains of the amino acids of the protein may form hydrophobic bonds with the long saturated or unsaturated carbon chains of the triglyceride, fatty acids, phospholipids or sterols. Polar groups in the amino acid side chains may also hydrogen bond groups of similar polarity, particularly the hydroxy functional groups present in sterols and phospholipids. The negatively charged phosphate groups present in the phospholipid may also form strong ionic bonds with positively charged amino groups present in the side chain of the basic amino acids of the protein.

To date, the complete detailed three dimensional structure of a single lipoprotein has not yet been determined. However, the lipoproteins from blood have received considerable attention. These studies have shown that there are several different types of lipoproteins and that these may be categorized conveniently on the basis of their density (46). The low-density lipoproteins contain approximately 80% by weight of lipid and 20% by weight of protein. The low-density lipoproteins contain large quantities of bound triglycerides, cholesterol (or its esters), but small quantities of bound phospholipids. In contrast, the high-density lipoproteins contain greater than 50% by weight of protein and less than 50% by weight of lipid. The high-density lipoproteins contain large

quantities of bound phospholipid and cholesterol (or its esters) but very small quantities of bound triglycerides.

Topham and Frieden's work (33) indicated that ferroxidase-II could possibly be a serum lipoprotein. The present study definitely establishes the lipoprotein nature of ferroxidase-II. Ferroxidase-II was found to be approximately 80% by weight of protein and 20% by weight of lipid. Phospholipid and cholesterol (or its esters) accounted for most of the lipid of ferroxidase-II. Phosphatidylcholine constituted the majority of the phospholipid and lysophosphatidylcholine and sphingomyelin accounted for the remaining phospholipid. The relative quantities of lipids and protein and the predominance of phospholipids and cholesterol would place ferroxidase-II in the high-density class of blood serum lipoproteins.

The lipoprotein nature of ferroxidase-II is quite interesting from another point of view. It represents the discovery of the first example of a serum lipoprotein that possesses an enzymic activity. Furthermore, intact phospholipids appear to be essential for maintenance of enzymic activity. Hydrolysis of the phospholipid with either phospholipase A or C resulted in a loss of activity which paralleled the hydrolysis of phospholipid. Treatment with phospholipase D resulted in some loss of activity but the loss was not nearly as complete as observed with phospholipase A or C. The larger size of phospholipase D may prevent it from coming in contact with the majority of the phospholipid bound to ferroxidase-II and thus account for the much lower degree of inactivation and phospholipid hydrolysis. Phospholipases A and C hydrolyze phosphatidylcholine at different but at specific positions. The nature of the hydrolysis products is also quite different. Hydrolysis with phospholipase A results in the loss of a non-polar fatty acyl group at the 2

position with the formation of lysophosphatidylcholine (See "Results"). In contrast, hydrolysis with phospholipase C results in the loss of phosphocholine, the polar head group, with the formation of a 1,2-diglyceride. The hydrolysis of either the non-polar tail or the polar head group was sufficient for the loss of activity which would suggest that no single structural feature of the phospholipid is solely important for the maintenance of enzymic activity. Rather, it would appear that both polar and non-polar groups may be involved.

The lipid components could be removed from ferroxidase-II by acetone extraction or phospholipase C treatment followed by gel-filtration. However, hydrolysis not removal of the phospholipid components was sufficient for loss of ferroxidase activity.

The loss of ferroxidase activity upon hydrolysis of the phospholipid associated with ferroxidase-II could be explained in several ways. The phospholipid could be important for the maintenance of the native structure of ferroxidase-II and hydrolysis of the bound phospholipid could result in an alteration in the three dimensional structures which would result in inactivation of ferroxidase-II. However, the elution pattern of native and lipid-depleted ferroxidase-II from gel-filtration columns were very similar. The mobilities of the nature and lipid-depleted ferroxidase-II upon disc-gel electrophoresis, a technique for the simultaneous separation of proteins on the basis of size and charge, were also similar. These results would suggest that no gross changes in the three dimensional shape of ferroxidase-II occurred following hydrolysis and removal of the bound phospholipid. However, small subtle changes in the structure of ferroxidase-II following hydrolysis and removal of lipids can not be ruled out. To detect small structural changes would require the crystallization of both native and lipid-depleted ferroxidase-II

and x-ray crystallographic analyses of both. The crystallization of ferroxidase-II, in fact of any lipoprotein, has not yet been achieved. Even if crystalline ferroxidase-II were available, its very large molecular weight would make an x-ray analyses quite complex. Furthermore, considerable uncertainty concerning the fair comparison of crystalline and solution structures for native and lipid-depleted ferroxidase-II would still exist.

Another possible explanation of the loss of activity following the hydrolysis of phospholipid was explored. The hydrolysis and loss of the bound lipids could be accompanied by a loss of copper from ferroxidase-II. *In vivo* and *in vitro* studies have shown that copper plays a functional role in the catalytic activity of ferroxidase-II (48). The hydrolysis and removal of the bound lipid of ferroxidase-II was accompanied by the loss of copper. The hydrolysis of the phospholipids could cause a subtle change in the structure of ferroxidase-II which would, in turn, reduce the affinity of ferroxidase-II for copper. The reduction of the affinity of ferroxidase-II for copper could result from the movement of groups in the protein (possibly side-chain amino or sulfur groups) so that they are no longer in the proper alignment for forming a strong ligand complex with copper. It is also possible that the polar groups of the phospholipids (the oxygen of the phosphate group, the nitrogen of the choline group) could be directly involved in binding of copper to the enzyme. The hydrolysis of phospholipids could weaken the ability of the groups to participate in cooperation with the groups in the protein in the binding of copper. The exact manner in which the lipid, copper, and protein are associated with one another has not been ascertained. However, these studies would indicate that the association among them is indispensable to the catalytic function of ferroxidase-II.

Attempts to reconstitute the lipid-depleted form of ferroxidase-II by the addition of phosphatidylcholine; phosphatidylcholine and cholesterol; and phosphatidylcholine, cholesterol, and copper resulted in little regeneration of ferroxidase activity. This suggests that any structural change and change in affinity for copper occurring during hydrolysis and removal of bound lipid are not readily reversible.

Ceruloplasmin and ferroxidase-II have been found in the sera of all animals investigated to date and these proteins are the sole source of ferroxidase activity in these animal sera. A detailed comparison of some of the chemical properties of these two proteins was made in tabular form in the "Literature Review". However, it is interesting to compare these proteins from a slightly different point of view. Both proteins contain extremely tightly bound copper atoms. In addition, ceruloplasmin contains some bound carbohydrate residues and is thus classified as a glycoprotein. Cartwright, *et al.* (25) demonstrated from *in vivo* studies that the loss of these carbohydrate residues resulted in a loss in the ability of ceruloplasmin to mobilize iron from liver storage cells. In contrast, ferroxidase-II contains no bound carbohydrate residues but it contains bound lipid components and is thus considered a lipoprotein. The studies described in this thesis have demonstrated that the lipid components of ferroxidase-II are essential for the maintenance of its ferroxidase activity. Cartwright, *et al.* (25) have suggested that possibly the carbohydrate residues of ceruloplasmin may in some manner promote the specific binding of some of the circulating ceruloplasmin to the surface of the liver storage cell. This bound ceruloplasmin would be ideally located for promoting the oxidation of any Fe(II) passing from the liver storage cell into the blood stream. It is possible that the lipid components of ferroxidase-II could promote the specific

binding of ferroxidase-II to the surface of the liver storage cell. This appears particularly feasible because the membrane composing the liver cell surface is rich in phospholipids and cholesterol. Thus, the liver cell membrane represents a hydrophobic environment which could bind the hydrophobic-lipid rich region of ferroxidase-II. Verification of this, however, must await further experimentation.

In summary, ferroxidase-II represents a very interesting and complex enzyme. It not only contains tightly bound copper but also tightly bound lipid components and both appear essential for the maintenance of the catalytic and physiological function of the enzyme.

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