University of Richmond
UR Scholarship Repository

Master's Theses

Student Research

9-1982

Isolation and characterization of the serum ferroxidase inhibitor

Melissa Page Calisch

Follow this and additional works at: http://scholarship.richmond.edu/masters-theses

Recommended Citation

Calisch, Melissa Page, "Isolation and characterization of the serum ferroxidase inhibitor" (1982). *Master's Theses.* Paper 486.

This Thesis is brought to you for free and open access by the Student Research at UR Scholarship Repository. It has been accepted for inclusion in Master's Theses by an authorized administrator of UR Scholarship Repository. For more information, please contact scholarshiprepository@richmond.edu.

ISOLATION AND CHARACTERIZATION OF THE SERUM FERROXIDASE INHIBITOR

A THESIS
SUBMITTED TO THE DEPARTMENT OF CHEMISTRY
OF THE GRADUATE SCHOOL OF
THE UNIVERSITY OF RICHMOND
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR THE DEGREE OF
MASTER OF SCIENCE

by

MELISSA PAGE CALISCH

APPROVED: Richard W. Topham

SEPTEMBER 1982 W. Allan Powell

William H. Myers

J. M. Jayne

Stuart C. Clough

Robert Mathews

LIBRARY
UNIVERSITY OF RICHMOND
VIRGINIA

BIOGRAPHICAL SKETCH

Melissa Page Calisch was born on April 20, 1953 in Richmond, Virginia to Page Dabney and Elliott Woolner Calisch. She completed her primary and secondary education in Richmond.

After high school, she attended Westhampton College of the University of Richmond where she majored in Chemistry. In June, 1974 she attended the School of Medical Technology of the Medical College of Virginia from which she received the Bachelor of Science degree in May of 1975. In August of 1975 she received the Bachelor of Science degree from the University of Richmond.

In October 1975, the author began work as a chemist for the Department of Pharmacy of the Medical College of Virginia. In July 1976 she transferred to the Department of Clinical Pathology where she worked as a medical technologist until September of 1977 when she became a full time research assistant for Dr. Richard W. Topham in the Department of Chemistry at the University of Richmond. In January of 1978 she re-entered the University of Richmond as a special student in the Department of Chemistry. In May of 1980 she became a degree candidate under the direction of Dr. Richard W. Topham. This work was supported by USPHS Grant 1 R01AM20148 from the National Institute of Arthritis, Metabolism and Digestive Diseases.

ACKNOWLEDGEMENTS

The author wishes to express her appreciation to Dr. Richard W. Topham for his direction and assistance throughout this course of study and for his help in the preparation of this thesis. Deepest respect and gratitude is also expressed to Dr. Topham for his constant moral support and for the excellent example he set as a professional, devoted teacher, and loyal friend. Appreciation is also expressed to the members of the Chemistry Department for their instruction and encouragement.

A special thanks is extended to Mrs. Joyce Pierce, Mrs Dorothy Ogden, and Mrs. Betsy Word for their excellent assistance in the preparation of the typescript. Deepest appreciation is also expressed to her parents and her fiance' for their understanding and encouragement throughout this course of study.

TABLE OF CONTENTS

Biographical Sketch i
Acknowledgements ii
Table of Contents iii
List of Tables v
List of Figures vi
Abstract 1
History 2
Introduction 18
Materials and Methods 20
Results 27
    Isolation and Purification of the Rabbit Ferroxidase Inhibitor 28
    Homogeneity of the Purified Inhibitor 35
    Estimation of the Molecular Weight 37
    Comparison of the Lipid and Copper contents of Ferroxidase II and the Ferroxidase Inhibitor 37
    Range of Inhibition of the Purified Ferroxidase Inhibitor 42
    In vivo studies of the Rabbit Ferroxidase Inhibitor 42
    Evidence for the Pressence of a Human Ferroxidase Inhibitor 48
    Purification of the Human Ferroxidase Inhibitor 48
    Estimation of the Molecular Weight 55
    Electrophoretic Comparisons 59
    Colorimetric Determination of Albumin 59

Carbohydrate Analysis 64

Immunodiffusion Studies 64

Cross Reactivities 64

Mechanism of Inhibitor 64

Discussion 75

Bibliography 84

List of Tables

Table	Title	Page
I	Purification of Rabbit Ferroxidase II	19
II	Purification of Rabbit Ferroxidase II Inhibitor	36
III	Chemical Comparison of Rabbit Ferroxidase II and Rabbit Ferroxidase II Inhibitor	43
IV	Effect of Diet on the Serum Content of Ferroxidase Activity and the Ferroxidase Inhibitor	45
V	Comparison of the Total Activity of Ferroxidase II in Whole Human Serum and After Elution from Sephadex G - 200	49
VI	Purification of the Human Serum Ferroxidase Inhibitor	56
VII	Comparison of the Inhibitory Potencies of the Purified Ferroxidase Inhibitor and Authentic Human Albumin	67
VIII	Comparison of the Inhibitory Activity of the Pepsin Generated Peptide Fragments Eluted from Sephadex G - 75	74

List of Figures

Figure	Title	Page
1	Physiological Role of Ceruloplasmin in Iron Metabolism	12
2	Elution Profile of Protein from G - 200 Sephadex	29
3	Elution Profile of Protein from Spehadex G - 200	31
4	Elution Profile of Protein from DEAE Sephadex A-50-120	33
5	Elution Profile of Protein from DEAE Sephadex A-50-120	33
6	Purity of the Ferroxidase Inhibitor as Determined by Polyacrylamide Gel Electrophoresis	38
7	Determination of the Molecular Weight of the Purified Rabbit Ferroxidase Inhibitor	40
8	Effect of Repetitive Bleeding on the Serum Content of Ferroxidase Activity and the Ferroxidase Inhibition	46
9	Elution Profile of Protein from G - 200 Sephadex	50
10	Elution Profile of Protein from DEAE - Sepharose CL - 6B	53
11	Determination of the Molecular Weight of the Purified Human Ferroxidase Inhibitor	57
12	Comparison of the Electrophoretic Mobilities of the Purified Ferroxidase Inhibitor and Authentic Human Albumin in Non - SDS and SDS Systems	60
12A	Comparison of the Electrofocusing Behavior of the Purified Ferroxidase Inhibitor and Authentic Human Albumin	62

13 Comparison of the Purified Ferroxidase Inhibitor and Authentic Human Albumin with Anti - Human Albumin and Anti - Human Whole Serum in an Immunodiffusion System 65

14 Elution Profile of Protein from G - 75 Sephadex 70

15 Elution Profile of Protein from G - 25 Sephadex 72

16 Schematic Summary of Iron Metabolism 78

ABSTRACT

Strong evidence suggests that the serum ferroxidases (ceruloplasmin and ferroxidase II) promote the formation of Fe-III transferrin and thereby stimulate the turnover of iron from tissue stores. Ceruloplasmin is the major ferroxidase in human serum; whereas, ferroxidase II accounts for an increased proportion of the activity in less highly developed animals. A large fold increase in total ferroxidase II activity was observed when both human and rabbit sera were subjected to gel-filtration on sephadex G-200. This indicated that whole serum might contain a potent inhibitor of ferroxidase II. Such an inhibitor has been isolated and purified to homogeneity by a combination of gel-filtration and ion exchange chromatography. It has a molecular weight of 64,000-67,000. The molecular weight, chromatographic behavior, electrophoretic mobility, electrofocusing pH, carbohydrate content, and reactivity with anti-human albumin in an immmodiffusion system indicate that the ferroxidase inhibitor could be serum albumin. Furthermore, commercial human serum albumin exhibits an inhibitory activity with both serum ferroxidases that is equivalent to the ferroxidase inhibitor purified from whole rabbit and human serum. Serum albumin can be fragmented into several smaller peptides, one of which contains a specific binding site for copper. It was this fragment that had inhibitory activity towards the serum ferroxidases.

"In vivo" studies demonstrated that the content of the ferroxidase inhibitor in serum decreased when iron mobilization was accelerated from tissue stores by either dietary manipulation or repetitive bleeding. Furthermore, an inverse relationship was observed between the total serum content of the inhibitor and the total serum ferroxidase activity. By modulating the activity of the serum ferroxidases, serum albumin could participate in the regulation of the efflux of iron from tissue stores.

HISTORY

Copper was discovered in living matter only a century and a half ago. The adult human contains only about 100mg of copper and while organisms may vary in their dependence for other elements, they all seem to require a certain amount of copper. Because it is an essential constituent of many enzymes, copper plays a major role in the overall metabolic picture. There are several reasons for copper's effectiveness as a biological agent. First of all, copper reacts with amino acids and proteins more strongly than most other metals.do, and consequently it forms very stable chelates with the biologically active substances. In copper containing enzymes, the copper is so strongly embedded that no amount of dialysis will separate the copper from the protein; the copper ion can only be released by more drastic measures that alter the structure of the protein or by exposure to an even stronger chelating agent. Secondly, copper serves as a very effective catalyst whose activity seems to be enhanced when it is embedded in an enzyme. Lastly, copper can exist in three states: as the free neutral atom, as the cuprous ion (with one electron removed), and as the cupric ion (with two electrons removed). The two ionic states are easily interconverted by the addition or release of an electron which thereby gives copper greater versatility as an electron accepter or an electron donor. Moreover, compounds containing singly ionized copper, are easily oxidized by oxygen from the air; consequently, copper enzymes that act as oxidative catalysts, are promptly recycled by reoxidation to repeat their function (1).

Biological copper is almost never found in any compound less complex than a protein. Very little free ionic copper (in the form of salts) is ever found in the body, even in the blood-stream. When radioactive copper is injected in humans in the form of a copper salt, it first appears bound to the serum protein albumin. It is then absorbed by the liver and reappears in the blood-stream bound to ceruloplasmin, (a serum protein first isolated by the Swedish biochemists,Holmberg and Laurell in 1947, (2)). Ninty-eight per cent of the copper in human serum is concentrated in ceruloplasmin (1).

The biological role of copper has been difficult to determine. Its presence in the body went undetected until recent times, mainly due to the rarity of copper deficiency in animals. Copper deficiency in humans has never been observed, since we are supplied with an abundance of copper in our water and food, including contributions from copper cooking utensils. Copper deficiency has been studied in animals in which it occurs naturally (dogs, pigs, chickens, lambs) and in experimental animals in the laboratory. Severe copper deficiency is responsible for several abnormalities: 1) swayback disease in lambs, 2) bone defects in dogs, pigs, and chickens, 3) decolorization of sheeps' wool and rat hair, 4) a reduction in the supply of copper enzymes, particularly cytochrome c oxidase, and perhaps most significantly, 5) a reduction in the synthesis of hemoglobin, causing anemia and a deficiency of proteins containing heme (the iron containing pigment precursor to hemoglobin) (1).

The recognition of anemia in the copper deficient animal was first reported in 1928 (3). This defect in iron metabolism in the copper deficient animal has been studied extensively by Cartwright, Wintrobe and their associates at the

University of Utah School of Medicine (4). Their work revealed that copper deficient pigs developed a hypochromic microcytic anemia. Lahey and his associates were able to determine the progression of metabolic events, resulting from the anemia (5). Blood analyses of five-day old pigs, maintained on a copper deficient diet, indicated an initial rapid decrease in serum copper, followed by a drop in serum iron, erythrocyte copper and finally a drastic reduction in red cell volume.

These observations led Cartwright's group to renew the ideas of a role for copper in hemoglobin biosynthesis. This role could be effected at one of three main events in hemoglobin biosynthesis; 1) the biosynthesis of protoporphrin or heme, 2) the utilization of iron, or 3) the biosynthesis of globin (6). Earlier efforts to find a copper-dependent step in heme biosynthesis were abandoned by Lee and his co-workers (7). Instead, they found that as anemia developed in the copper deficient pig, there was a 2-3 fold increase in the activity of heme biosynthetic enzymes. They concluded that copper deficiency anemia was not a result of defective heme biosynthesis and that copper was not a co-factor in any of these reactions. They also found no evidence for any impairment in globin biosynthesis. From their observations, it seemed most reasonable to assume that copper was essential for the proper utilization of iron (7).

One of the first major breakthroughs in the link between iron and copper metabolism was the result of a study made by Curzon and O'Reilly on a coupled iron ceruloplasmin oxidation system (8,9).

It had previously been demonstrated by Holmberg and Laurell that ceruloplasmin had oxidase activity "in vitro" towards many substrates, including p-phenylenediamine, quinol, cathechol, pyrogallol, dihydroxyphenylamine, adrenaline, and ascorbic acid (10). Curzon and O'Reilly further investigated this by studying the effects of metal ions in low concentration on the oxidase activity of ceruloplasmin. They found that ferrous iron greatly enhanced the activity of ceruloplasmin on a specific substrate, N, N-dimethyl-p-phenylenediamine (DPD). Furthermore, they were able to demonstrate that ceruloplasmin oxidized the ferrous ion to the ferric ion. Subsequently, they proposed a coupled iron-ceruloplasmin system, in which Fe (II) was oxidized by ceruloplasmin which in turn, increased the activity of ceruloplasmin towards its substrate DPD (8).

$$\mathrm{DPD} + \mathrm{Fe}^{+++} \underset{\text{ceruloplasmin}}{\rightleftharpoons} \overset{*}{\mathrm{DPD}} + \mathrm{Fe}^{++}$$

$\overset{*}{\mathrm{DPD}}$ = oxidized N, N-dimethyl-p-phenylenediamine dihydrochloride

Curzon and O'Reilly's data, which revealed the catalytic oxidation of Fe(II) by ceruloplasmin, led to further experiments by Osaki et. al, in which they attempted to determine a biological role for ceruloplasmin in iron metabolism (11).

At this time, it was generally believed that iron entered the bloodstream from the intestine mostly in the ferrous form (12). Once 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 protein molecule, forming a red Fe (III) complex. When Fe (II) is added to apotransferrin, (the iron free protein) 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 (13). Once saturated, the transferrin molecule is thought to be the protein which exclusively supplies iron to the marrow. Iron in the marrow is subsequently utilized by the developing red blood cell in the synthesis of hemoglobin (14).

With this knowledge, and the data from Curzon and O'Reilly's experiments, Osaki's group investigated Fe(II) oxidation under conditions which might be expected to prevail in human serum. These experiments demonstrated that ceruloplasmin catalyzed the oxidation of Fe(II) to Fe(III) and thereby promoted the incorporation of Fe(III) into apotransferrin (11).

In his experiments, Osaki used two methods to study the Fe(II) oxidation (11). One was a spectrophotometric method which measured the rate of Fe(III)-transferrin formation and the other method measured the rate of Fe(II) oxidation by an oxygen electrode. Osaki made a comparative study of the non-enzymic and enzymic rates of Fe(II) oxidation over a wide range of concentrations with respect to both Fe(II) and O_2. It was found that the ceruloplasmin catalyzed reaction was 10 to 20 times faster than the non-enzymic oxidation under physiological conditions. Furthermore, the non-enzymic oxidation of Fe(II) was estimated to be insufficient to account for a rate of Fe(III)-transferrin formation necessary to provide an adequate iron supply for hemoglobin biosynthesis. On the other hand, the enzymic oxidation by ceruloplasmin was found to be approximately 11 times more active than the non-enzymic oxidation and this rate would be sufficient to meet the actual requirement for the conversion of Fe(II) to Fe(III). From this information, Osaki proposed that the rate of formation of Fe(III) from Fe(II) in plasma and the incorporation of Fe(III) into transferrin could play a significant role in the overall turnover of iron. The fact that ceruloplasmin was capable of increasing this rate under physiological conditions suggested a possible biological role for ceruloplasmin in promoting the rate of iron saturation of transferrin and in stimulating iron utilization (11).

In view of the results obtained from these experiments, Osaki's group proposed that the enzymic activity of ceruloplasmin warranted its classification as a Ferro-O_2-oxidoreductase or serum ferroxidase. Although the name ceruloplasmin will always bear historical significance, the term ferroxidase, is a more useful and appropriate designation.

A group of researchers at the University of Utah School of Medicine studied the effects of ceruloplasmin and plasma iron in copper deficient swine in order to evaluate Osaki's hypothesis in an in vivo system (15,16,17).

It had previously been established that copper deficiency in swine produced an anemia characterized by defective movement of iron from reticuloendothelial cells, hepatic cells, and gastrointestinal mucosa to the plasma (18). In their studies, the Utah group was able to demonstrate that this defect could be reversed promptly by the intravenous administration of ceruloplasmin (15).

The animals used in this study were maintained on a copper and iron deficient diet for a period of 70 days. The administered dose of ceruloplasmin was based on an estimated plasma value. When the ceruloplasmin was administered to the copper-deficient animals, an immediate increase in plasma iron concentration was observed. This effect could not have been due simply to a partial correction of the copper deficient state, because when inorganic copper, in an amount equivalent to that contained in the injected ceruloplasmin, was administered, only a minimal transient increase in plasma iron concentration was observed (15).

The results of this study were important for two reasons: 1) they demonstrated "in vivo" that ceruloplasmin could reverse the symptoms of copper deficiency anemia by increasing the plasma iron concentration; 2) they demonstrated that the most probable source of iron which entered the plasma after the ceruloplasmin administration was the reticuloendothelial and hepatic cells. Since the animals were maintained on an iron deficient as well as a copper deficient diet, it was unlikely that the gut provided the source of iron induced by the ceruloplasmin injection (15).

As a result of this initial experiment, the Utah group sought additional "in vivo" evidence for the function of ceruloplasmin as a ferroxidase in promoting the rate of transferrin formation (16).

These workers assumed that if ceruloplasmin functioned physiologically in promoting the rate of transferrin formation, and if the abnormalities in iron metabolism in copper deficient swine were due to a deficiency of ceruloplasmin ferroxidase activity, then the following physiological events might be observed in such animals (16): 1) a deficiency of ceruloplasmin; 2) the deficiency of ceruloplasmin should precede the restriction in the flow of iron into the plasma as manifested by the development of hypoferremia; 3) an increase in ceruloplasmin in the circulation should precede the increase in plasma iron which follows the administration of copper; 4) the rate of transferrin formation "in vivo" from intravenously injected ferrous iron should be slower than that from intravenously administered ferric iron; 5) a rapid increase in plasma iron should occur following intravenous administration of ceruloplasmin.

The results confirmed their predictions. In each pig, a severe degree of hypoceruloplasminemia was observed before the plasma iron decreased. Hypoferremia did not occur until the ceruloplasmin activity decreased to less than 1% of the normal level. (Ceruloplasmin activity was determined by its ability to oxidize p-phenylenediamine, another enzymatic activity of ceruloplasmin.) (19) The administration of ceruloplasmin to the copper deficient pigs was followed by a prompt and appreciable increase in plasma iron. This effect could not be explained by the copper contained in ceruloplasmin. When inorganic copper was administered, some increase in plasma iron was observed; however, it was necessary to inject 100-150 ug/kg of inorganic copper in order to approximate the effect of 0.6-1.2 ug/kg of ceruloplasmin copper. Furthermore, the plasma iron response to this high dose of copper was delayed as compared with the response to ceruloplasmin, and the delay was associated with an increase in plasma ceruloplasmin of an order that would in itself stimulate iron outflow. Thus, the copper appeared to exert its effect on plasma iron by making ceruloplasmin synthesis possible (16).

The binding of iron by transferrin after an intravenous injection of ferrous and ferric iron was compared in the control and copper deficient pigs in order to test the relative efficiency of the spontaneous and enzymatic reactions in an in vivo system. In each case, an equal amount of iron was injected, and the increase in plasma iron was determined 10 minutes after the injection. When ferric iron was injected, there was no difference between the plasma iron values in the control and copper deficient animals. In contrast, when ferrous iron was injected, the hypoceruloplasminemic pigs were unable to retain ferrous iron in the plasma. Failure to retain iron in plasma implied that the iron did not become bound to transferrin, presumably because it was not oxidized to the ferric state (16).

The Utah group concluded from their studies that ceruloplasmin appeared to be essential for an optimal rate of Fe–III transferrin formation from ferrous iron and apotransferrin. The non-enzymic rate of iron oxidation was not adequate to insure complete iron-binding in this system. Thus these observations constituted an in vivo demonstration of the abnormality in iron binding in ceruloplasmin deficiency as predicted by Osaki et al. on the basis of their in vivo investigations (11).

These results provided such strong evidence supporting the hypothesis of Osaki et al. that it prompted Osaki's group to study further the mobilization of stored iron in an in vivo system (20).

Animals suffering from copper deficiency anemia exhibit an iron overload in storage organs such as the liver or spleen (18). In contrast, animals suffering from iron deficiency anemia have no iron in storage (21). Thus in copper deficiency the storage organs may contain iron but cannot mobilize and utilize it for hemoglobin synthesis (18). Osaki based his in vivo study on this knowledge.

In this experiment, excised mammaliam livers from normal as well as copper deficient specimens were flushed with a recyclable perfusion medium containing only the plasma protein apotransferrin. Several compounds including ceruloplasmin, $CuSO_4$, HCO_3^-, citrate, apotransferrin, glucose, fructose, and serum albumin (±Cu(II))were tested for their ability to mobilize iron from the liver into the perfusate. The appearance of iron was detected by Fe (III) transferrin formation and an independent iron analysis (20). Of the compounds tested only ceruloplasmin produced a rapid efflux of iron into the perfusate. (The ceruloplasmin concentration could be reduced to 0.2uM or approximately 10% of the normal human value without limiting iron mobilization.) Thus, even in copper deficient animals a mobilizable pool of iron was present. Once the pool was depleted, a reduction in iron mobilization occurred. When the same liver was perfused again for twice as long as the original flushing time, the same amount of enzyme infused resulted in twice the amount of iron released. Since varying concentrations of the enzyme produced no further increase in iron efflux it appeared that the extent of iron mobilization was proportional to the flushing time. These observations suggested that a pool of mobilizable iron was formed during the flushing time and that the total amount of iron mobilization depended on the size of this pool (20).

From these observations, Osaki et. al. were able to propose a physiological role of ceruloplasmin in iron mobilization. Figure (I) is a schematic representation of their proposal consistent with their data (20).

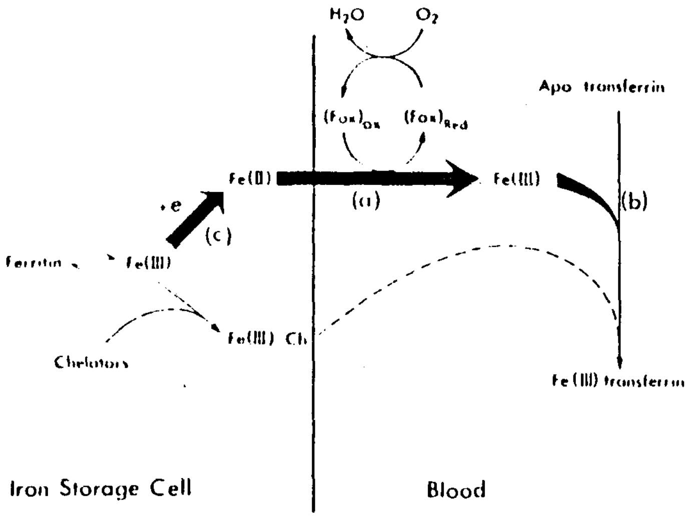


Figure 1

When the ceruloplasmin concentration in the plasma is normal a minimal mobilization pool of iron is expected. Step c is probably rate limiting, thus no appreciable pool is formed unless ferroxidase, step a, is eliminated. The binding between Fe(III) and apotransferrin represented by step b, does not itself facilitate iron mobilization. Thus, apotransferrin apparently serves only as a carrier of Fe(III) in the circulatory system.

In the copper deficient state the rate of iron efflux from the cell, (e.g. reticuloendothelial) begins to slow down when the ferroxidase concentration becomes rate limiting (step a). Consequently, more iron (in storage as well as in the mobilizable pool) would accumulate in various organs such as the intestinal mucosal cells, parenchymal cells of the liver, and reticuloendothelial system.

Osaki concluded that ceruloplasmin, due to its iron oxidase activity, generates a steep concentration gradient of Fe(II) between the iron storage cells and the capillary system, thus promoting efficient and maximum iron efflux and thereby promoting the rate of iron saturation of apotransferrin and stimulating iron utilization (20).

During the period when much of the experimental work on ceruloplasmin was accomplished, it became evident that a non-ceruloplasmin ferroxidase also exsisted in serum.

Ceruloplasmin can be isolated from the Cohn IV-I fraction of whole human serum. One of its characteristic properties is its sensitivity to sodium azide. Its ferroxidase activity is nearly 100% inhibited by 1mM sodium azide. The first clue that another non-ceruloplasmin ferroxidase might exist, was the presence of a residual non-azide sensitive ferroxidase activity in human serum and also in the Cohn IV-I fraction (22).

The existence of such an enzyme would account for and perhaps clarify some of the biological characteristics associated with Wilson's disease. This disorder is characterized by low plasma ceruloplasmin and an accumulation of copper in the liver and the brain. Due to the role of ceruloplasmin in iron mobilization one might expect to see disturbances in iron metabolism in this disease. However, most patients with this disorder have been found to have low normal or normal levels of iron transport. Another characteristic associated with Wilson's disease, is the diminished p-phenylenediamine oxidase activity, (another enzymatic activity of ceruloplasmin) which would be consistent with the low levels of ceruloplasmin. Paradoxically, the sera from patients with Wilson's disease display more ferroxidase activity than one would expect. Frieden and Osaki suggested the possiblity of an alternative ferroxidase protein, that would be capable of substituting for ceruloplasmin in Wilson's disease (23).

Such a protein was isolated and purified by R.W. Topham (24). It has been designated ferroxidase II and like ceruloplasmin it has significant ferroxidase activity; however, it also has several characteristics which distinguish it from ceruloplasmin as well as demonstrate how it could serve as a possible substitute in Wilson's disease.

Two of the more important characteristics that differentiate ceruloplasmin from ferroxidase II and that proved to be useful in several experimental studies were; 1) ferroxidase II was not inhibited by azide and 2) ferroxidase II had no p-phenylenediamine oxidase activity (24).

The differential sensitivity to azide was used as an initial probe to determine whether any ferroxidase II activity might be present in normal human serum and Wilson's disease serum. Its presence was confirmed in both sera.

A comparative study was made of the ferroxidase II activity and the activity of ceruloplasmin in the sera of normal humans and patients with Wilson's disease (24). In the Wilson's disease sera, both of the ferroxidase activities were lower; however, while the total ferroxidase activity was decreased by a factor of 17 to 18, that of ferroxidase II was decreased by a factor of only 3 to 4. Thus, in the Wilson's disease sera, ferroxidase II accounted for a larger percentage (approximately 30%) of the total ferroxidase activity, about a 5-fold increase over that observed in normal human serum (24).

This data helped resolve the apparent paradox in Wilson's disease concerning the normal levels of iron transport observed in patients with the disorder. Specifically, the ceruloplasmin level decreased dramatically in Wilson's disease sera, while ferroxidase II was less affected. The total ferroxidase activity of Wilson's disease sera was approximately 5 to 10% of that of normal sera. Osaki et. al. found that only about 10% of the total ferroxidase activity of normal serum was necessary to produce maximum iron mobilization response from the liver (20). Therefore, the total ferroxidase activity of Wilson's disease sera would be sufficient for normal or low normal iron metabolism. However, this would not be possible without the large contribution to the total activity from ferroxidase II. The presence of ferroxidase II also accounted for the fact that the p-phenylenediamine activity did not correlate with ferroxidase activity in Wilson's disease sera (24).

The kinetic parameters of ferroxidase II were studied and compared to those of ceruloplasmin (25). Both ferroxidases had low Km values for oxygen. The oxygen concentration of human blood has been estimated to be 53-120uM (26). The low Km value for O_2 indicated that ferroxidase II could catalyze the oxidation of Fe(II) at oxygen concentrations comparable to or even far below those of human blood. In addition, the pH optimum for ferroxidase II was found to be compatible with the pH of blood. The two ferroxidases have been shown to differ considerably in molecular weight and copper content (24). However, the molar activities and activities per copper atom are quite similar. Thus, kinetically, ferroxidase II, with a small Km value for oxygen, a pH optimum comparable to the pH of human blood, and a molar activity comparable to ceruloplasmin, would be capable of substituting for ceruloplasmin in normal human sera, and in Wilson's disease sera (25).

The physical properties of ferroxidase II have been extensively studied and documented (27,28,29,30). It is a unique and structurally complex enzyme containing lipids and copper, both of which are tightly associated with the protein and essential to its enzymic activity. It is ubiquitously distributed throughout the animal kingdom. Comprehensive comparative studies have shown that in human serum and in the sera of highly developed mammals ceruloplasmin is the major source of ferroxidase activity; whereas, in rodents and less highly developed animals ferroxidase II accounts for a larger proportion of the total ferroxidase activity. Thus less highly developed animals may be more dependent on ferroxidase II for metabolic mobilization of iron. This is more clearly demonstrated by the ferroxidase activity present in the serum of the New Zealand White Rabbit (Oryctolagus Cuniculus) (31). When ferroxidase II was isolated from whole rabbit serum it was found to contain three times the amount of copper per unit weight of protein and a specific activity three times larger than that of human ferroxidase II. Since ferroxidase II appears to play a more active role in less highly developed animals this evidence suggests there may have been an evolutionary transition from ferroxidase II to ceruloplasmin as the major source of serum ferroxidase activity.

INTRODUCTION

When ferroxidase II was isolated from whole rabbit serum, a rather interesting phenomena was revealed. As evident in the purification table (Table I), a seven-fold increase in the total ferroxidase activity was observed after the whole serum was passed over a sephadex G-200 column (31). In order to account for this observed increase in total activity, it was proposed that perhaps in whole serum there exists a potent inhibitor of ferroxidase II, that masks enzymic activity in the whole serum, but when separated from the enzyme by passage over a sephadex G-200 column, results in this large increase in total activity. Such an inhibitor could play a highly significant role in iron metabolism as it could serve as a naturally occuring modulator of the ferroxidase activity in the sera of less highly developed animals and thus regulate the release of iron from storage tissues.

The purpose of this study was to isolate and characterize the proposed inhibitor and to determine its contribution to the iron-ferroxidase metabolic pathway.

Table I. Purification of Rabbit Ferroxidase-II

Purification Step	Total Protein (mg)	Total Activity (ΔA_{460nm}/min)	Specific Activity $\frac{(\Delta A_{460nm}/min)}{mg\ protein}$	Fold Purification
Whole Serum	2860	7.24	0.003	1
Sephadex G-200	246	52.4	0.213	76
Sepharose-6B	115	43.7	0.381	136
DEAE-Sephadex	11.7	18.6	1.59	530

MATERIALS AND METHODS

MATERIALS AND METHODS

Sera. Fresh, frozen rabbit sera, non-sterile, non-hemolyzed was purchased from Pel-Freeze Biologicals, Inc., Rogers, Arkansas. Fresh human serum, HBs-Ag negative, was donated from the Richmond Metropolitan Blood Service Inc., Richmond, Virginia.

Human ceruloplasmin. Purified samples of ceruloplasmin were prepared using the method of Deutsch (32).

Human Ferroxidase-II. Purified samples of human ferroxidase II were prepared according to the method of Topham and Frieden (24).

Rabbit ferroxidase-II. Purified samples of rabbit ferroxidase-II were prepared according to the method of Chin, et al. (31).

Animals. Mature, female New Zealand White rabbits were used in these studies.

Diet. The low iron diet was purchased from ICN Nutritional Biochemicals, Cleveland Ohio. The animals receiving this diet, also received doubly deionized water.

Serum albumin. Solutions of bovine, rabbit, and human serum albumin (Sigma Chemical Co., St. Louis, Mo.) were prepared in distilled water to a concentration (30-60 mg/ml) equivalent to the range of concentrations of albumin in whole serum.

Chromatographic materials. Sephadex G-200-100 (Sigma Chemical Co., St. Louis, Mo.) was swollen and equilibrated with 0.03M acetate buffer pH 5.5, containing 0.15M NaCl and 0.02% NaN_3 as an antimicrobial agent. Sephadex G-75-120 (Sigma Chemical Company, St. Louis, Mo.) was swollen and equilibrated with 0.6N acetic acid, pH 3.0. Sephadex G-25-150 (Sigma Chemical Company, St. Louis, Mo.) was swollen and equilibrated with deionized, glass distilled water. DEAE* -Sephadex

---

* DEAE = Diethylaminoethyl

A-50-120 (Sigma Chemical Company, St. Louis, Mo.) was swollen and equilibrated with 0.05M acetate buffer, pH 5.5, containing 0.02% NaN_3. DEAE-Sepharose Cl-6B (Sigma Chemical Company, St. Louis, Mo.) was swollen and equilibrated with 0.0125 M sodium acetate buffer, pH 5.0 prior to use.

Dissociation and chelating reagent. Sodium dodecyl sulfate was purchased from Sigma Chemical Co., St. Louis, Mo. Disodium ethylene diamine tetra acetate was purchased from Fisher Scientific Co., Fair Lawn, N.J. Dithiothreitol was purchased from J.T. Baker Chemical Co., Phillipsburg, N.J.

Assays of Ferroxidase Activity and The Inhibition of Ferroxidase Activity. The enzymic oxidation and incorporation of iron into transferrin was measured spectrophotometrically at 460nm, where Fe (III)-transferrin exhibits maximal absorbance. This spectrophotometric assay of ferroxidase activity has been described in detail and validated in numerous previous reports (11, 20, 24, 26, 27, 31, 33 - 39). In assays containing no inhibitor, each cuvette (1.8ml capacity) contained 0.350 ml of a 0.6 M acetate buffer, pH 6.0; 0.250ml of a 2% (w/v) apotransferrin solution; 0.300 ml of a 4×1^{-4}M ferrous ammonium sulfate solution; 0.100 ml of a solution of either whole serum ferroxidase-II or ceruloplasmin; and 0.100 ml of a 0.05M acetate buffer, 0.15 NaCl, pH 5.5. In assays containing the inhibitor, the 0.100 ml of 0.05 M acetate buffer was replaced by 0.100 ml of a solution of the inhibitor which was prepared in the same buffer. The amount of inhibition was determined by obtaining the difference in the initial velocities of the ferroxidase reaction of assays with and without the inhibitor and was reported either as % inhibition or as Δ ($\Delta A_{460/min}$).

Column Monitoring. Protein elution from columns was monitored at 280nm with an Altex model 150 absorbance moniter, an Isco model UA-5 absorbance monitor with a type 6 optical unit or an Isco model 1840 absorbance monitor with a variable wavelength setting.

pH Measurements. All pH determinations were made with a Fisher Accument model 210 pH meter equipped with a Corning series 500 combination pH electrode or a Beckman Φ 60 pH meter.

Ultrafiltration. Protein solutions were concentrated in an Amicon ultrafiltration cell, model 52, with an Amicon PM 30 membrane.

Lyophilization. Protein solutions were concentrated by quick freezing on an FTS model SF-1 shell freezer, followed by lyophilization on an FTS 5 model FDX-1-54 freeze dryer.

Protein concentrations. Protein concentrations were determined by the method of Lowry et. al. (40) with bovine serum albumin as the standard.

Copper determinations. Copper analyses were performed using the method of Wharton and Rader. (41)

Lipid Analyses. Cholesterol analyses were performed according to the method of Zak (42). Phospholipid analyses were carried out using the method of Bartlett (43) and an average molecular weight of phospholipid (797ug/mole) was used for the calculation of phospholipid contents.

Albumin Determinations. Albumin concentrations were determined with Sigma Diagnostic kit no. 630 (Sigma Chemical Co., St. Louis, Mo.) which is based on the specific quantitative colormetric determination for serum albumin reported by Doumas and Briggs (44).

Carbohydrate Analyses. The carbohydrate content of protein samples was analyzed utilizing the anthrone reaction as described by Spiro (45).

Double Immunodiffusion. Immunodiffusion was performed using the Orjan Ouchterlong system as described by Gordon (46). Goat anti-human whole serum or goat anti-human albumin (Cappel Laboratories, Inc., Cochranville, Pa.) was placed in the center well of an immunodiffusion disc (Cappel Laboratories, Inc., Cochranville, Pa.) and purified human ferroxidase inhibitor or commercial albumin was placed in the surrounding wells. The disc was incubated at room temperature and examined after 2, 4, 8, and 24 hours for the presence of precipitation.

PAGE - Electrophoresis. In non SDS* electrophoresis, prepolymerized Biophore 7.5% or 4% polyacrylamide gels (Bio-Rad Laboratories, Richmond, CA.) were equilibrated with 0.188 M Tris-glycine buffer, pH 8.9, prior to use. A 40ul aliquot of Biotracking dye (Bio-Rad Laboratories, Richmond, CA.) containing 30% sucrose was added to 160ul of protein samples (1-4mg protein/ml) to obtain a final volume of 200ul. Aliquots (10-30ul) of these samples were applied to the tops of the gels. In SDS electrophoresis, identical gels were equilibrated with 0.25M Tris acetate, pH 6.6 containing 0.1% SDS. Samples were prepared for electrophoresis by incubating 200ul of each protein sample (1-4mg protein/ml) with 200ul of 1% SDS containing 0.04 dithiothreitol and 0.001 M EDTA** for 2 hours at 37°C. A 40ul aliquot of Biotracking dye was added to 150ul of these SDS-treated samples and aliquots of (20-60ul) of these samples were applied to the tops of the gels.

In both non-SDS and SDS electrophoresis, samples were run into the separating gels at 60 V. The electrophoretic runs were completed at 100-150 V. After electrophoresis, the gels were fixed with a solution of isopropanol; water; acetic acid (40:50:10, V/V/V), stained with coomassie blue, and destained with 7% acetic acid. The stained gels were photographed or scanned for protein at 280 nm with an ISCO Model 1310 gel-scanning attachment for the ISCO Model UA-5 absorbance monitor.

Analytical Electrofocusing. Analytical electrofocusing was performed in thin layers of polyacrylamide gels exactly as described by Winters, et. al. (47) utilizing an LKB 2117 Multiphor electrofocusing unit. A final pH range of 3.0-6.0 was utilized. Samples of 15ul (0.5-1.0mg protein/ml) of purified human ferroxidase inhibitor and commercial albumin were applied at various locations on each polyacrylamide electrofocusing plate. Plates were fixed with 5% sulphosalicylic acid -10% TCA, stained with coomassie blue, and destained with ethanol: acetic acid (3:1 V/V).

*SDS = Sodium dodecyl sulfate

**EDTA = Ethylene diamine tetraacetic acid

Estimation of Molecular Weight. Globular proteins of known molecular weight were passed through a sephadex G-200 column and the elution volumes (Ve) measured. The void volume (Vo) was determined by passage of blue dextran over the column. The ratios of the elution volume to the void volume (Ve/Vo) were then graphed against the logarithm of the molecular weight of the standards and a linear relationship was obtained. The molecular weight of the inhibitor was then determined by comparing its elution volume/void volume ratio to that of the standards.

Crossed linked species of hemoglobin of various molecular weights were electrophoresed on polyacrylamide gels equilibrated with SDS. Treatment of a protein molecule with the detergent SDS, dissociates the protein into subunits and completely unfolds each polypeptide chain to form a long rod like SDS - polypeptide complex. In this complex the polypeptide chain is coated with a layer of SDS molecules in such a way that their hydrocarbon chains are tightly associated through hydrophobic interactions with the polypeptide chain and the charged sulfate groups of the detergent are exposed to the aqueous medium. Such complexes contain a constant ratio of SDS to protein (Approximately 1.4:1 by weight) and differ only in mass. When an SDS treated protein is subjected to electrophoresis in a polyacrylamide gel equilibrated with SDS, its rate of migration is determined primarily by its mass. The electric field simply serves as the driving force for the molecular sieving (48). In this study, the mobility of each species of hemoglobin was measured and graphed against the logarithm of its molecular weight and a linear relationship was obtained. The mobility of the inhibitor on SDS gels was then determined and compared to that of the standards.

Peptic Digestion and Isolation of the Aspartic Acid Fragment of Bovine Serum Albumin. Solutions of bovine serum albumin were digested with pepsin (Sigma Chemical Co., St. Louis, Missouri) and the aspartic acid fragment was isolated according to the procedure described by Peters and Hawn (49). Specifically, a solution of 5% BSA was adjusted to pH 3.0 with 88% formic acid and allowed to stand at 25°C for 30 min. Pepsin, in 0.01 N HCL was added in a ratio of 1:300 pepsin to albumin (w/w) and digestion was carried out for 33 minutes at 25°C. Concentrated ammonium hydroxide was added to bring the pH to 7, and the solution was diluted to 1% protein and cooled to 0°C. Forty per cent trichloracetic acid (J.T. Baker Co., Phillipsburg, New Jersey) was added to the cooled mixture to give a 1.75% solution. The solution was centrifuged and solid trichloracetic acid was added to bring the supernatant to 10%. This solution was centrifuged and the precipitate washed with 10% trichloracetic acid and extracted with ether. The aqueous protein layer was lyophillized and reconstituted with 0.6N acetic acid, pH 3.0, and passed through a sephadex G-75 column equilibrated with 0.6N acetic acid, pH 3.0.

N-terminal Amino Acid Analysis. The N-terminal amino acid of the aspartic acid fragment was determined by thin layer chromatographic separation of DNS-CL (1-dimethyaminonapthalene-5-sulfonyl chloride) amino acids according to the method of Morse and Horecker, (50) using standards of dansyl-aspartic acid and dansyl phenylalanine. (Sigma Chemical Co., St. Louis, Missouri).

RESULTS

Results

Isolation and Purification of the Rabbit Ferroxidase Inhibitor

Purification Procedure. The ferroxidase inhibitor was purified from whole rabbit serum by a combination of gel filtration and ion exchange chromatography. Specifically, 30 ml of whole serum was applied to a column (2.5x 90 cm) of sephadex G-200. Three major bands of protein were eluted from this column. (Fig. 2). It had previously been established that the third major protein band contained a potent inhibitor of ferroxidase II (31). The fractions comprising this band were concentrated to 10-12 ml by ultrafiltration and applied to a second column (2.5x 90 cm) of sephadex G-200. One major band of protein was eluted from this column. (Fig. 3). Individual fractions were tested for inhibitory activity against the active enzyme and the inhibitor appeared to be associated with the latter portion of the major protein band. These fractions were combined and concentrated by ultrafiltration to 5-7 ml. This material represented no significant purification of the inhibitor; however, passage over this column facilitated the purification achieved with the ion exchange chromatography and was therefore maintained in the purification scheme.

The concentrated material obtained from the second sephadex G-200 column was applied to an anion exchange column (1.6 x 15 cm) of DEAE Sephadex, equilibrated with 0.5 M sodium acetate, pH 5.5 containing 0.15 M NaCl and 0.02% NaN_3. Two major bands of protein were eluted from this column. (Fig. 4). At this stage of the purification procedure, the individual fractions were too dilute to assay individually for inhibition; however, when the two peaks were individually concentrated and assayed for inhibitory

FIGURE 2

Elution Profile of Protein from G-200 Sephadex

A sample of 30 ml. of whole rabbit serum was fractionated on a column (2.5 x 90 cm) packed with spehadex G-200. The elution buffer was 0.05 M acetate, pH 5.5, containing 0.15 M NaCl and 0.02% NaN_3. Protein elution was monitored at 280 nm and fractions of 8 ml. were collected. Ferroxidase activity and the inhibition of ferroxidase activity were measured as described in "Materials and Methods".

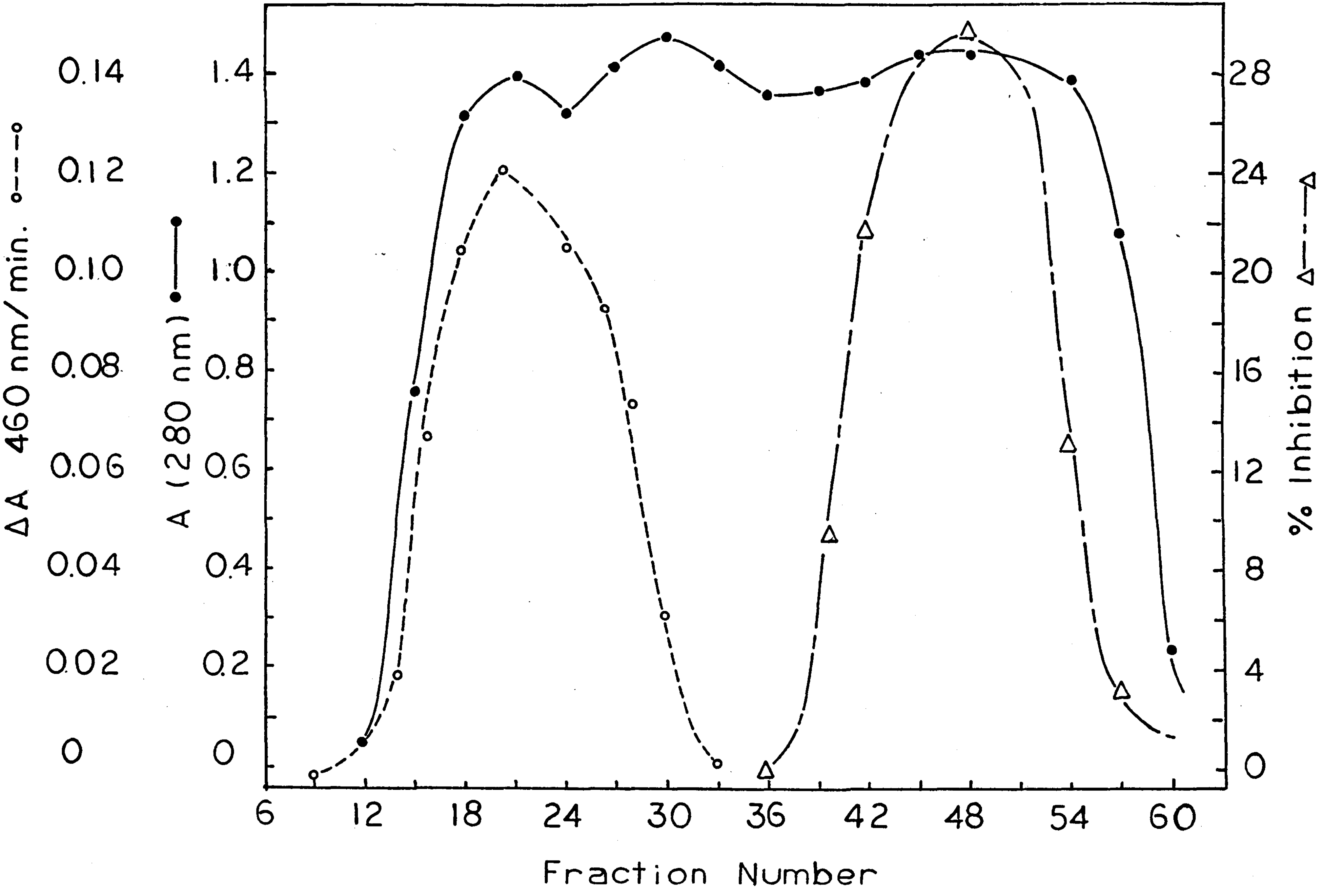

ΔA 460 nm/min. ○---○
0
0.02
0.04
0.06
0.08
0.10
0.12
0.14
A (280 nm) ●——●
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
% Inhibition △—-—△
0
4
8
12
16
20
24
28
6
12
18
24
30
36
42
48
54
60
Fraction Number

FIGURE 3

Elution Profile of Protein From Sephadex G-200

A sample of 10 - 12 ml. of concentrated protein was fractionated on a column (2.5 x 90 cm) packed with sephadex G-200. The elution buffer was 0.05 M acetate, pH 5.5, containing 0.15 M NaCl and 0.02% NaN_3. Protein elution was monitored at 280 nm and fractions of 8 ml. were collected. Inhibition of ferroxidase activity was measured as described in "Materials and Methods".

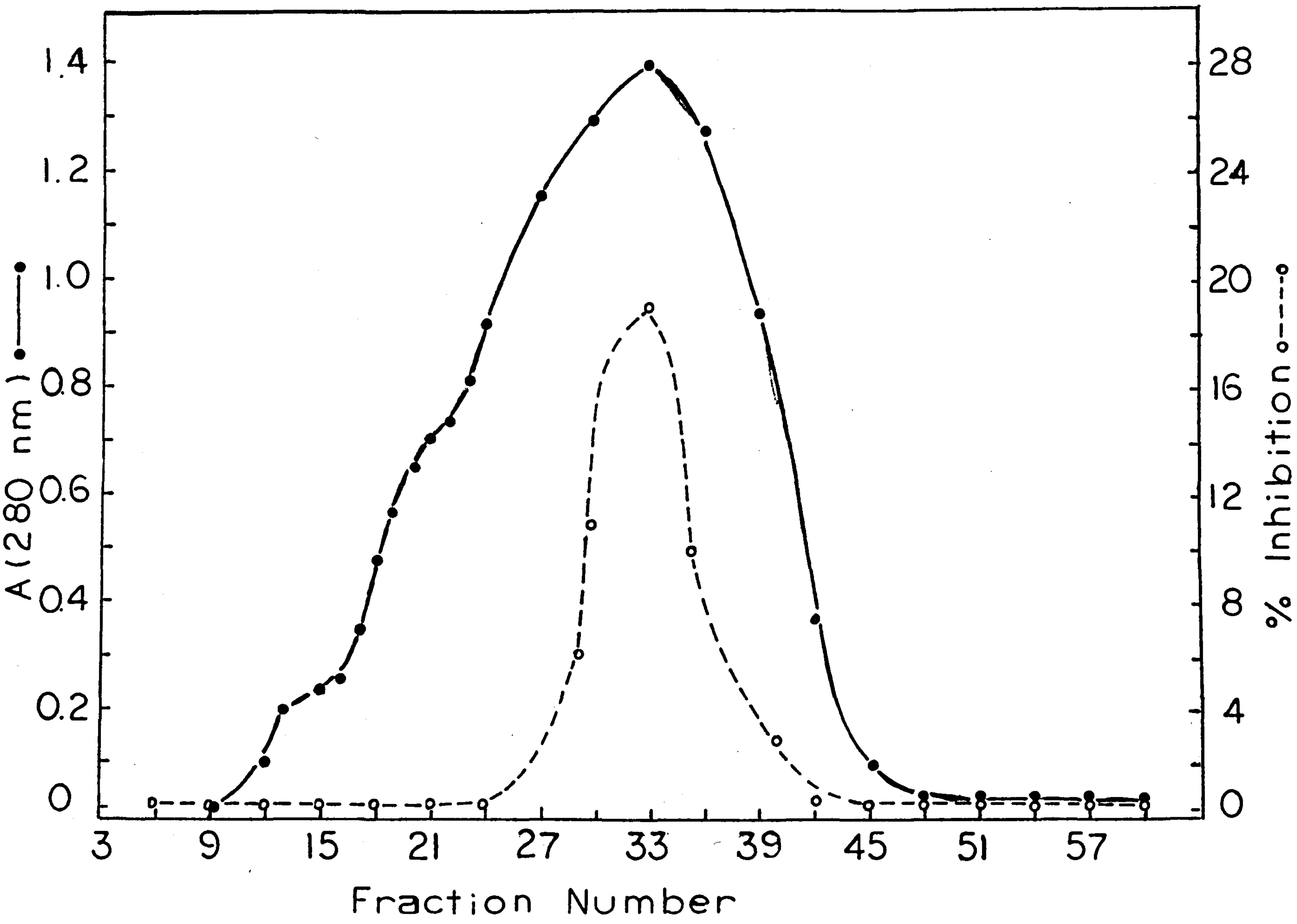

A (280 nm) ●—●
% Inhibition ○--○
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
0
4
8
12
16
20
24
28
3
9
15
21
27
33
39
45
51
57
Fraction Number

FIGURE 4

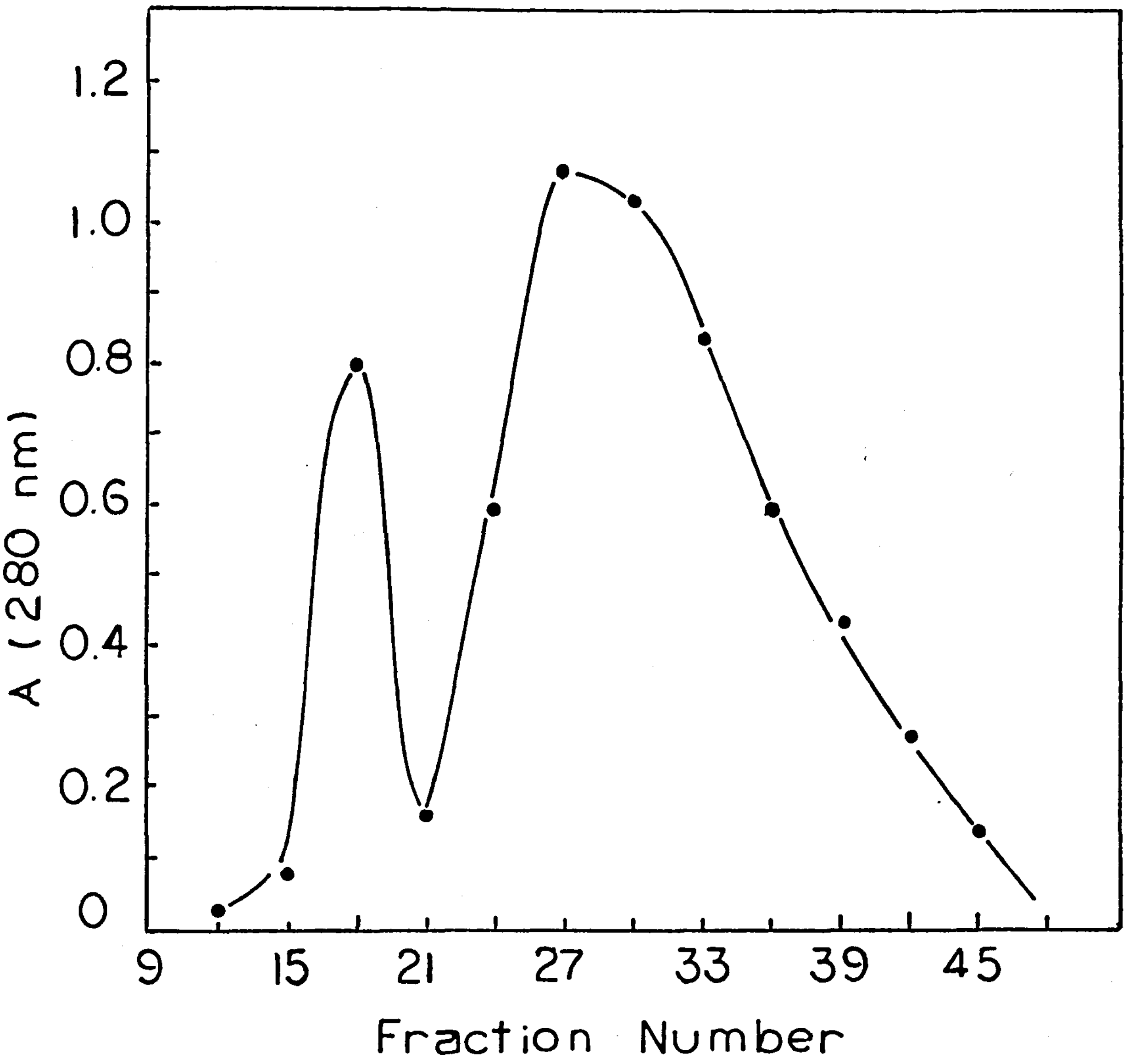
Elution Profile of Protein From DEAE Sephadex A - 50 - 120

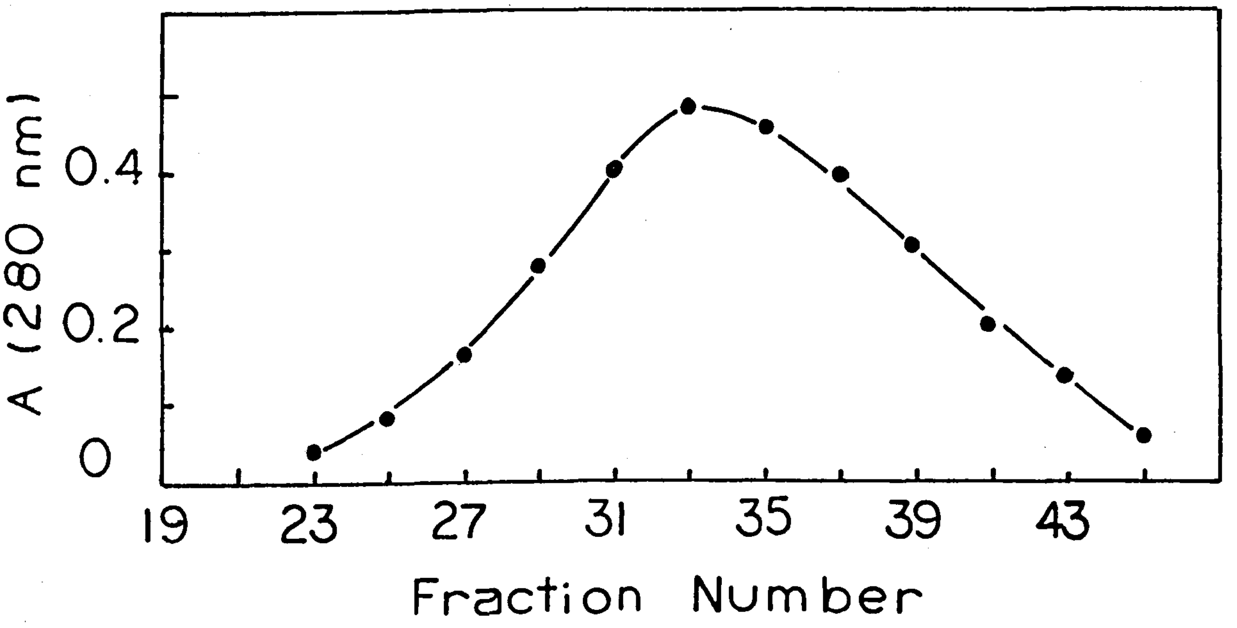
A sample of 5 - 7 ml. of concentrated protein was fractionated on a column (1.6 x 15 cm) packed with DEAE Sephadex A - 50 - 120. The elution buffer was 0.05 M acetate pH 5.5, containing 0.15 M NaCl and 0.02% NaN_3. Protein elution was monitored at 280 nm and fractions of 8 ml. were collected.

FIGURE 5

Elution Profile of Protein From DEAE Sephadex A - 50 - 120

A sample of 3 ml. of concentrated protein was fractionated on a column (1.6 x 15 cm) packed with DEAE Sephadex A - 50 - 120. The elution buffer was 0.05 M acetate pH 6.0, containing 0.15 M NaCl and 0.02% NaN_3. Protein elution was monitored at 280 nm and fractions of 8 ml. were collected.


A (280 nm)
1.2
1.0
0.8
0.6
0.4
0.2
0
9
15
21
27
33
39
45
Fraction Number


A (280 nm)
0.4
0.2
0
19
23
27
31
35
39
43
Fraction Number

activity, the inhibitor appeared to be associated with the second major protein band. The fractions comprising this band were concentrated by ultrafiltration to a volume of approximately 3 ml. This material represented a 3-4 fold purification as compared with the sample obtained from the first spehadex G-200 column.

The final purification step entailed applying the concentrated material obtained after DEAE ion exchange chromatography to a column (1.6 x 15 cm) of Deae sephadex equilibrated with 0.05 M sodium acetate, pH 6.0, containing 0.15 M NaCl and 0.02% NaN_3. A single symmetrical peak was eluted from this column. (Fig. 5). The fractions were concentrated by ultrafiltration to a volume of 2 ml. This material represented a 4-5 fold purification as compared with the sample obtained from the first sephadex G-200 column; however, it could represent a much greater fold purification when compared to whole rabbit serum. A significant amount of purification was probably accomplished upon separating the enzyme from the inhibitor after passage over the first sephadex G-200 column; however, since the enzyme and the inhibitor were both present in whole serum, it was impossible to measure the initial purification step. (Table II).

Homogeneity of the Purified Inhibitor

The active material obtained from the final purification step was subjected to polyacrylamide gel electrophoresis and a single band of protein was obtained. In addition, the final material was treated with SDS, a detergent that disrupts the non-covalent bonding in a protein molecule; dithiothreitel, which breaks any existant di-sulfide bonds; and EDTA, a chelating agent which binds any metals present. Treatment with this combination of reagents results in the dissociation of a protein molecule into its component subunits

TABLE II. PURIFICATION OF RABBIT FERROXIDASE-II INHIBITOR

Purification Step	Total Protein (mg)	Specific Inhibition (%/mg protein)	Fold Purification	% Recovery
G-200$_1$	761	3.00	1.00	100
G-200$_2$	529	3.10	1.03	70
DEAE pH 5.5	118	10.7	3.56	55
DEAE pH 6.0	38.2	14.4	4.80	24

(polypeptide chains). As a result of this treatment, electrophoresis indicated no dissociation of the final material into smaller molecular weight subunits. Since the active material migrated the same distance in both non-SDS and SDS gels with no further dissociation, it would appear that the complete purification procedure yielded a homogenous protein in the form of a single polypeptide chain. (Fig. 6)

Characterization of the Rabbit Ferroxidase Inhibitor

Estimation of the Molecular Weight

The molecular weight of the purified rabbit ferroxidase inhibitor was estimated using a column of sephadex G-200 calibrated with standard proteins of known molecular weight as prescribed in methods. The average molecular weight obtained through this method was 70,000 (Fig. 7).

In a second study, cross linked species of hemoglobin of various molecular weights were electrophoresed on polyacrylamide gels equilibrated with SDS as described in methods. A comparision of the mobility of the purified inhibitor with that of the standards indicated that the ferroxidase inhibitor had a molecular weight of approximately 60,000 (Fig. 7). As a result of these two studies, it would appear that the molecular weight of the ferroxidase inhibitor is between 60,000 and 70,000.

Comparision of the Lipid and Copper Contents of Ferroxidase II and the Ferroxidase Inhibitor

It has been established that lipids, primarily in the form of phosphotidyl choline and cholesterol, as well as copper, are tightly associated with ferroxidase II and essential to its emzyme activity (27,28,30).

FIGURE 6

Purity of the Ferroxidase Inhibitor as Determined by Polyacrylamide Gel Electrophoresis

In order to determine the purity of the ferroxidase inhibitor, samples of the protein were electrophoresed on 4% and 7.5% polyacrylamide gels as described in "Materials and Methods". A: 4% gel, B: 7.5% gel.

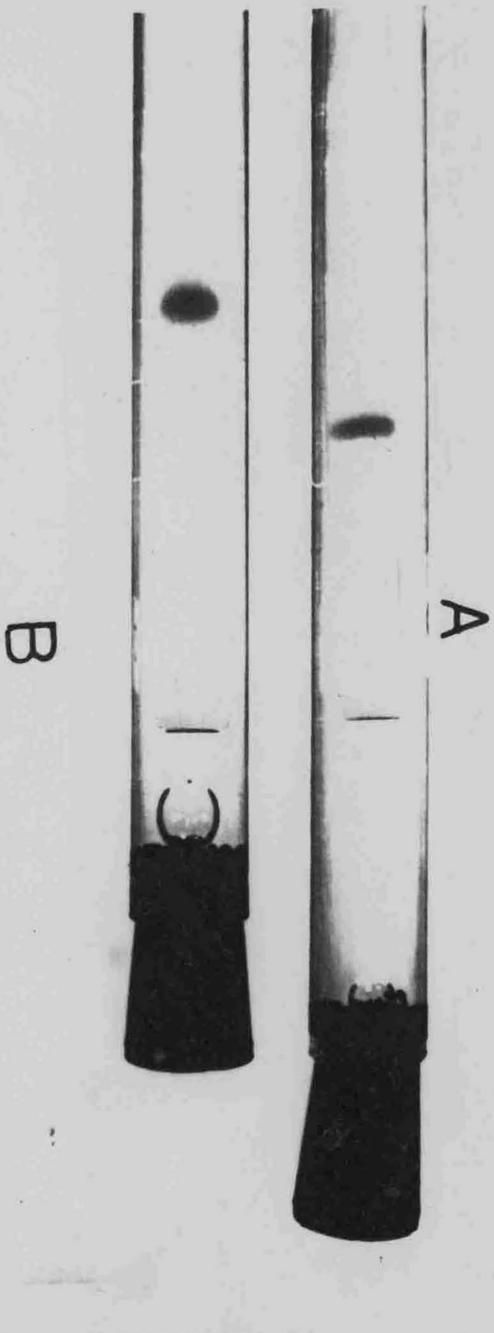
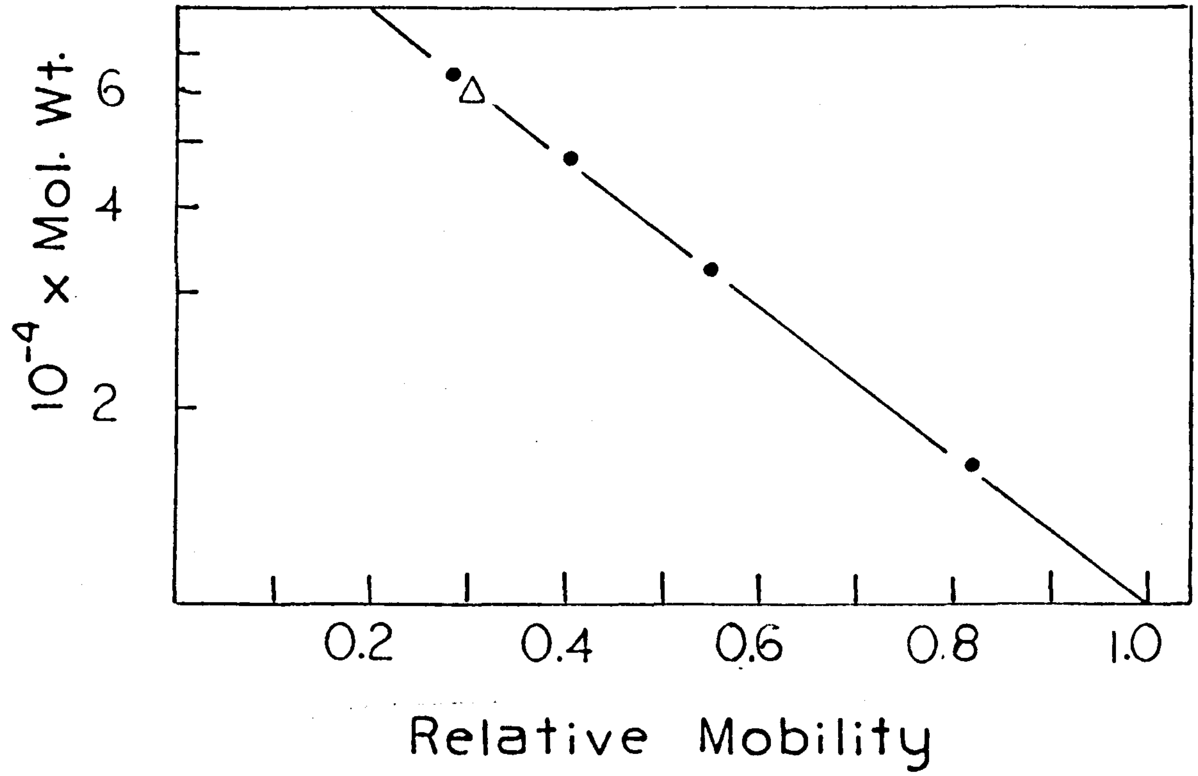
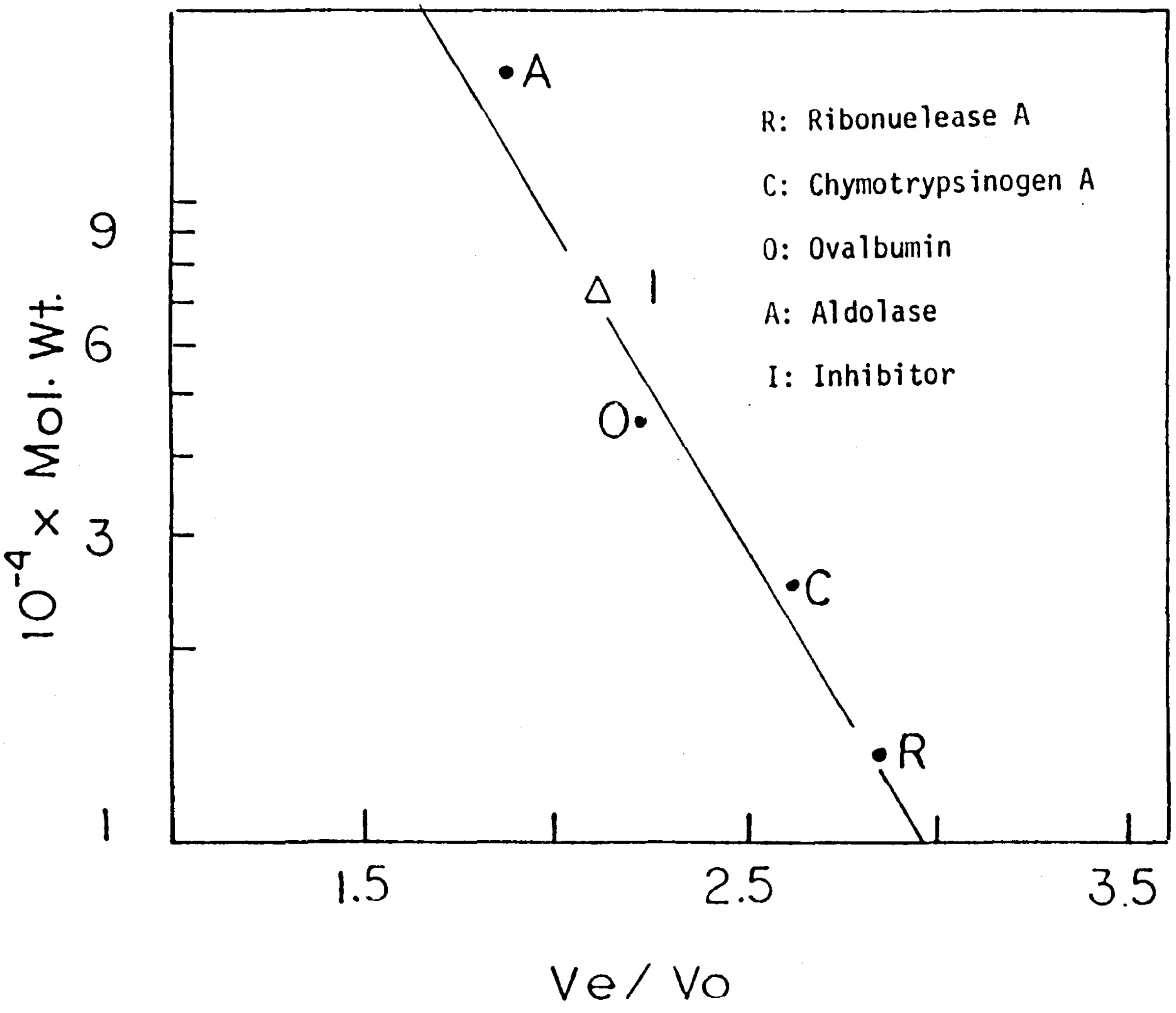

A
B

FIGURE 7

Determination of the Molecular Weight of the Purified Rabbit Ferroxidase Inhibitor

The molecular weight of the purified ferroxidase inhibitor was determined by SDS - electrophoresis and gel - filtration as described in "Materials and Methods". A: SDS - Electrophoresis, B: Gel - Filtration


10^{-4} x Mol. Wt.
6
4
2
0.2
0.4
0.6
0.8
1.0
Relative Mobility


10^{-4} x Mol. Wt.
9
6
3
1
1.5
2.5
3.5
Ve/ Vo
A
I
O
C
R
R: Ribonuelease A
C: Chymotrypsinogen A
O: Ovalbumin
A: Aldolase
I: Inhibitor

In order to investigate the possibility that the inhibitor might interact with ferroxidase II through hydrophobic interactions with the lipid components, the purified sample was examined for lipid content. In comparision to the enzyme, the inhibitor contained no significant amounts of phospholipid or cholesterol. Examination for the presence of copper again revealed no significant amounts when compared to that present in ferroxidase II. (Table III).

Range of Inhibition of the Purified Ferroxidase Inhibitor

The purified ferroxidase inhibitor was tested to see if it inhibited ceruloplasmin (ferroxidase I) as well as ferroxidase II, in order to determine if it was a more comprehensive inhibitor of serum ferroxidase activity (51). Samples of purified ceruloplasmin and ferroxidase II, containing comparable amounts of ferroxidase activity, were tested with equivalent amounts of the inhibitor. Seventy four per cent inhibition was observed with ceruloplasmin compared to 53% inhibition with ferroxidase II (51).

In Vivo Studies of the Rabbit Ferroxidase Inhibitor

Several *in vivo* studies have revealed that both ceruloplasmin and ferroxidase II are capable of mobilizing iron from tissue stores (20,51). Since the inhibitor has been shown to be effective against both serum ferroxidases, it could play a physiological role in iron metabolism by regulating iron efflux from these tissue stores. In order to investigate such a physiological role for the inhibitor, two experiments were designed to determine if there was any correlation between the levels of the inhibitor and the amount of ferroxidase activity in the serum of normal animals and iron deficient animals (51).

TABLE III. CHEMICAL COMPARISON OF RABBIT FERROXIDASE-II AND RABBIT FERROXIDASE-II INHIBITOR.

Sample	$\frac{\text{Phospholipid}}{\text{Protein}}$ (mg/mg protein)	$\frac{\text{Cholesterol}}{\text{Protein}}$ (mg/mg protein)	$\frac{\text{Copper}}{\text{Protein}}$ (n moles/mg protein)
Ferroxidase-II	0.178	0.149	32
Ferrixidase-II Inhibitor	0.005	0.022	0

In the first study, two New Zealand white rabbits were maintained first on a diet of normal iron content for four weeks, then on a diet of low iron content for four weeks, followed by a return to a diet of normal iron content for four weeks. During the period when the animals were maintained on a low iron diet, the total ferroxidase activity greatly increased while the total amount of the ferroxidase inhibitor dramatically decreased. Both the total ferroxidase activity and the amount of ferroxidase inhibitor returned to normal values when the animals were refed a diet of normal iron content (51) (Table IV).

To further substantiate this data, a third animal was maintained on a diet of normal iron content, and repetitively bled to accelerate the mobilization of iron from tissue stores. Blood samples were obtained from the animal on alternate days for a period of nine consecutive weeks. During this period, a steady decline in the total amount of the ferroxidase inhibitor occurred in the first five weeks of bleeding, with a corresponding steady increase in the total ferroxidase activity. When the repetitive bleeding was ceased for four weeks, both the total ferroxidase activity and the total amount of the ferroxidase inhibitor returned to their original values (51). (Fig. 8).

As a result of these experiments two important observations were made: 1) The content of the ferroxidase inhibitor in serum decreased when iron mobilization was accelerated from tissue stores by either dietary manipulation or repetitive bleeding 2) An inverse relationship was observed between the total serum content of the inhibitor and the total serum ferroxidase activity. Thus, this data suggested that the inhibitor could play a physiological role in iron metabolism by modulating the total

TABLE IV

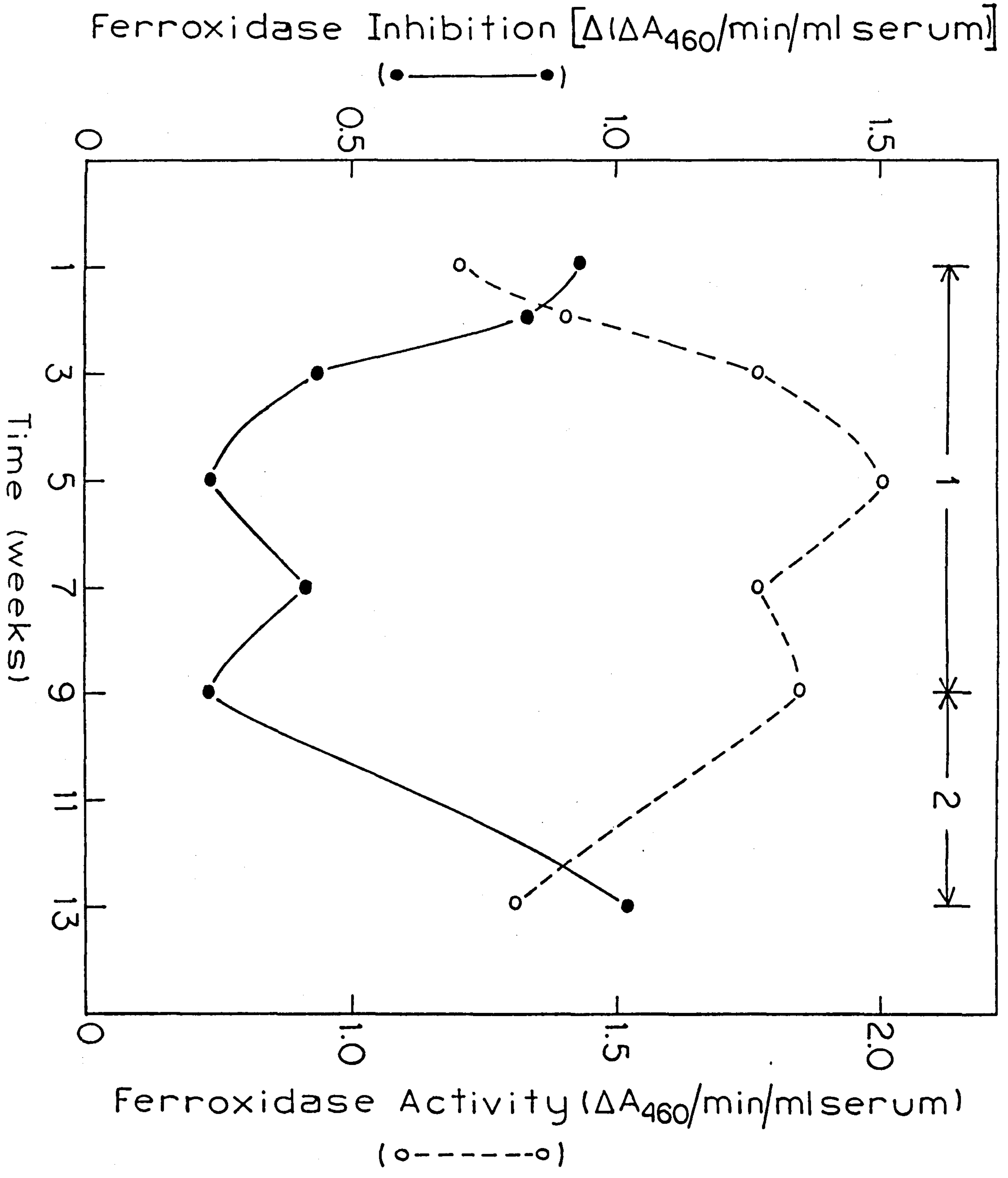
EFFECT OF DIET ON THE SERUM CONTENT OF FERROXIDASE ACTIVITY AND THE FERROXIDASE INHIBITOR

Rabbit	Diet	Total Ferroxidase Activity	Total Ferroxidase Inhibition
		(ΔA_{460}/min/ml serum)	Δ(ΔA_{460}/min/ml serum)
1	Normal Diet, 4 weeks	0.925	0.977
	Low Iron Diet, 4 weeks	2.80	0.298
	Refed Normal Diet, 4 weeks	0.913	0.924
2	Normal Diet, 4 weeks	1.17	1.14
	Low Iron Diet, 4 weeks	2.13	0.437
	Refed Normal Diet, 4 weeks	1.12	1.28

FIGURE 8

Effect of Repetitive Bleeding on the Serum Content of Ferroxidase Activity and the Ferroxidase Inhibition

The ferroxidase activity and the inhibition of ferroxidase activity were measured as described in "Materials and Methods".
1 = period of repetitive bleeding; 2 = period of no bleeding.


Ferroxidase Inhibition [Δ(ΔA460/min/ml serum)]
(●——●)
0
0.5
1.0
1.5
Time (weeks)
1
3
5
7
9
11
13
1
2
0
1.0
1.5
2.0
Ferroxidase Activity (ΔA460/min/ml serum)
(o------o)

ferroxidase activity and thereby regulating the efflux of iron from storage tissues.

Isolation and Purification of the Human Ferroxidase Inhibitor

Evidence for the Presence of a Human Ferroxidase Inhibitor

Due to the successful purification of the rabbit ferroxidase inhibitor and the physiological implications obtained from the "in vivo" studies, it seemed logical to investigate whether such an inhibitor also existed in whole human serum.

Whole human serum was processed in a manner identical to that of whole rabbit serum (31). After the initial passage over a sephadex G-200 column, a 7-fold increase in the total ferroxidase II activity was observed (Table V). The elution pattern obtained from this column was similar to that obtained with whole rabbit serum, and the third protein band inhibited ferroxidase II. (Fig. 9).

Purification of the Human Ferroxidase Inhibitor

The procedure for the purification of the rabbit ferroxidase II inhibitor was modified considerably for the most effective purification of the human ferroxidase inhibitor. Both procedures combined gel filtration with ion exchange chromatography; however, the purification of the human ferroxidase inhibitor required fewer gel filtration steps. In addition, DEAE sepharose was used as the ion exchange resin rather than DEAE sephadex. A column packed with Deae sephadex was **effective** for only one sample application because the sephadex resin has a tendency to shrink when exposed to buffers of increasing ionic strength. In contrast, DEAE sepharose can be used with repeated applications of the sample without shrinkage of the column bed,

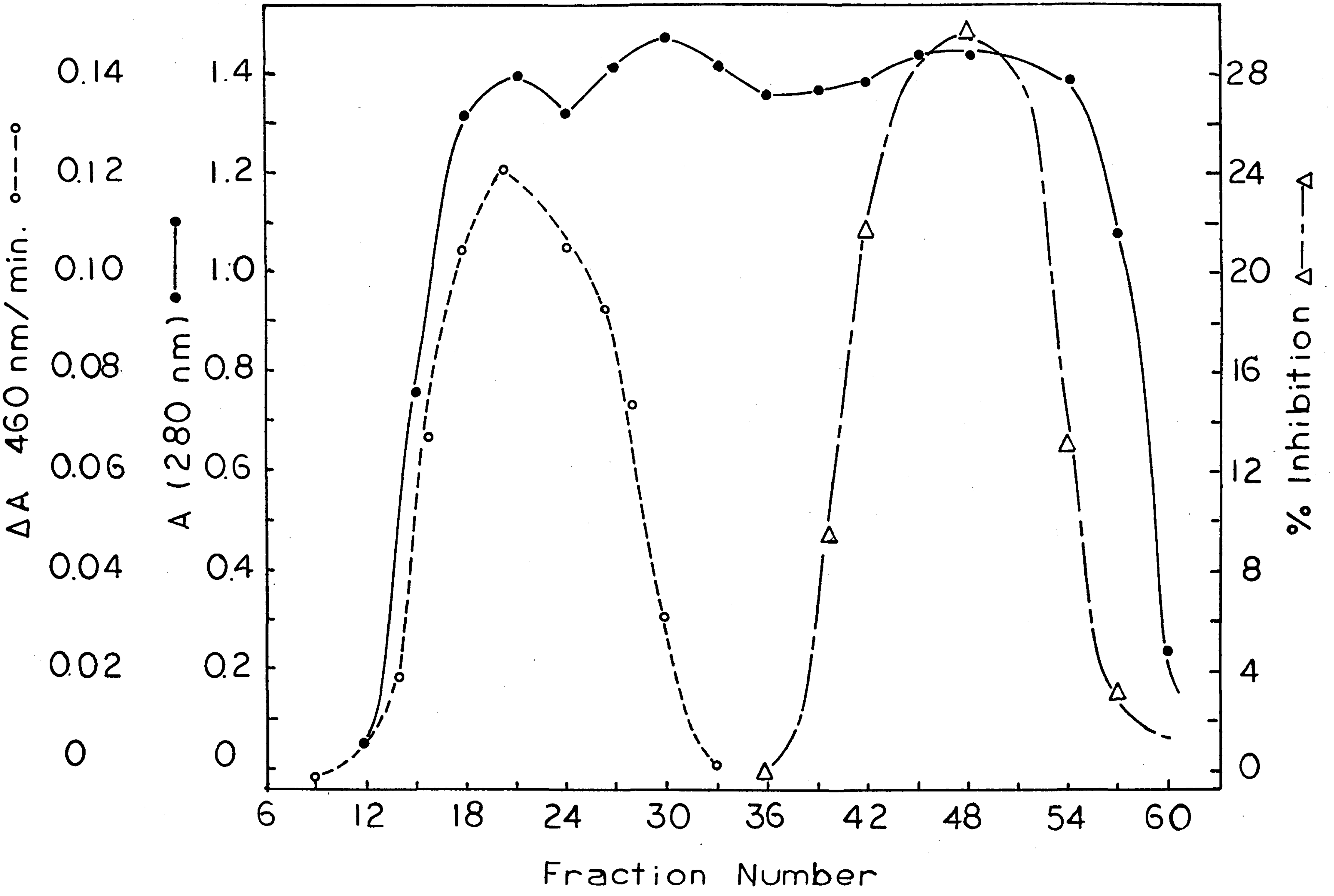
Table V Comparison of the Total Activity of Ferroxidase II in Whole Human Serum and after Elution from Sephadex G-200

Sample	Total Protein (mg)	Specific Activity $\left(\frac{\Delta A_{460nm/min}}{\text{mg protein}}\right)$	Total Activity $(\Delta A_{460nm/min})$
Whole Serum			
Ferroxidase II	1956	.0036	7.0
After Sephadex G-200			
Ferroxidase II	184	.2494	45.9

FIGURE 9

Elution Profile of Protein From G-200 Sephadex

A sample of 30 ml. of whole human serum was fractionated on a column (2.5 x 90 cm) packed with sephadex G-200. The elution buffer was 0.05 M acetate, pH 5.5, containing 0.15M NaCl and 0.02% NaN_3. Protein elution was monitored at 280 nm and fractions of 8 ml. were collected. Ferroxidase activity and the inhibition of ferroxidase activity were measured as described in "Materials and Methods".


0.14
0.12
0.10
0.08
0.06
0.04
0.02
0
ΔA 460 nm/min. ○---○
A (280 nm) ●—●
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
28
24
20
16
12
8
4
0
% Inhibition △-·-△
6
12
18
24
30
36
42
48
54
60
Fraction Number

when buffers of varying ionic strength are passed through the column. The method of concentration was also modified. Concentration by ultrafiltration resulted in loss of inhibitory activity; therefore, lyophilization was used as the method of concentration throughout the entire purification procedure.

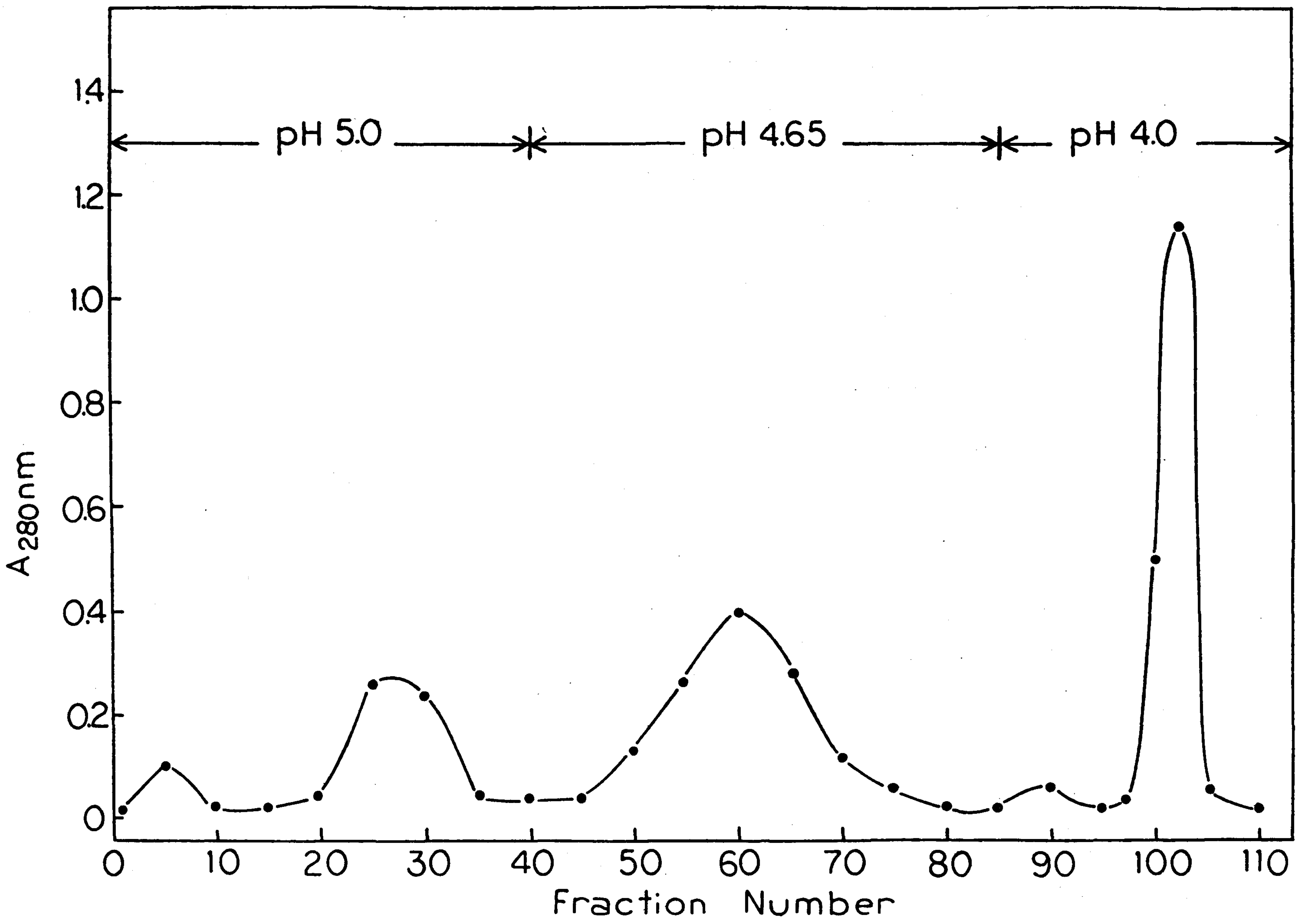
Specifically, 30ml of whole serum was applied to a column (2.5 x 90cm) of sephadex G-200 and protein eluted with 0.05 M sodium acetate buffer, pH 5.3 containing 0.15 M NaCl and 0.02% NaN_3. Three major bands of protein eluted from the column (Fig. 9). The fractions comprising each protein band were pooled and quickly frozen in a shell freezer containing a methanol bath at 54°C and concentrated by lyophilization. The lyophilized fractions were reconstituted with deionized glass distilled water to a volume of 5ml. Each reconstituted sample was tested for ferroxidase II activity and inhibitory power against the active enzyme. The first protein band eluted from the column contained the ferroxidase II activity whereas the third protein band contained the ferroxidase inhibitor.

The lyophilized sample containing the ferroxidase inhibitor, was desalted on a column (2.5 x 25cm) of sephadex G-25 and eluted with deionized glass distilled water. This desalted sample was applied to a column (2.6 x 10cm) of DEAE sepharose CL-6B and the column was developed sequentially with 0.0125M sodium acetate buffer, pH 5.0; 0.0125M sodium acetate buffer, pH 4.65; and 0.05M sodium acetate buffer, pH 4.0. Some protein was eluted from the column with each buffer (Fig. 10). The fractions comprising the individual protein bands were combined and lyophilized. The lyophilized samples were reconstituted with 2-3ml of deionized glass distilled water tested for inhibitory activity. The inhibitory activity was contained in the protein band eluted with the pH 4.65 acetate buffer. This material represented a 10-fold purification of the ferroxidase inhibitor with a recovery of 78% of the in-

FIGURE 10

Elution Profile of Protein from DEAE - Sepharose CL - 6B

A sample of 5 ml. of concentrated protein was fractionated on a column (2.6 x 10 cm) packed with DEAE Sepharose CL - 6B. The elution buffers were 0.0125 M sodium acetate, pH 5.0; 0.0125 M sodium acetate, pH 4.65; and 0.05M sodium acetate, pH 4.0. Protein elution was monitored at 280 nm and fractions of 8 ml. were collected.


pH 5.0
pH 4.65
pH 4.0
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
A_{280nm}
0
10
20
30
40
50
60
70
80
90
100
110
Fraction Number

hibitory activity as compared to whole human serum (Table VI). As in the case of the rabbit inhibitor, the calculation of the total inhibition contained in whole serum was complicated by the presence of both ferroxidase II and the inhibitor in whole serum. It was assumed that the total inhibition recovered after sephadex G-200 gel filtration represented essentially 100% of that contained in whole serum, and that the total inhibition calculated for the sephadex G-200 purification step was equivalent to the total inhibition present in whole serum. If some inhibition had been lost during gel filtration, the specific inhibitory activity of the whole serum would have been higher than the value presented in the purification table; therefore, the values in Table VI represent the minimal specific inhibitory activity that could exist in whole serum.

Electrophoresis in 4% and 7.5% polyacrylamide gels indicated that the inhibitor preparation contained a single protein component. The small value obtained for the final fold purification indicated that the inhibitor might possibly be a major serum protein.

Characterization of the Purified Inhibitor

Estimation of the Molecular Weight

The molecular weight of the purified ferroxidase inhibitor was estimated by SDS polyacrylamide gel electrophoresis and gel filtration, as described in methods (Fig. 11). The average molecular weight obtained by SDS electrophoresis was 64,000, and the average molecular weight obtained by gel filtration was 67,000. These values were in good agreement with those obtained with the rabbit inhibitor (i.e. SDS electrophoresis-60,000 and gel filtration-70,000) and were virtually identical to the molecular weight reported for human serum albumin (52). Furthermore, the human ferroxidase

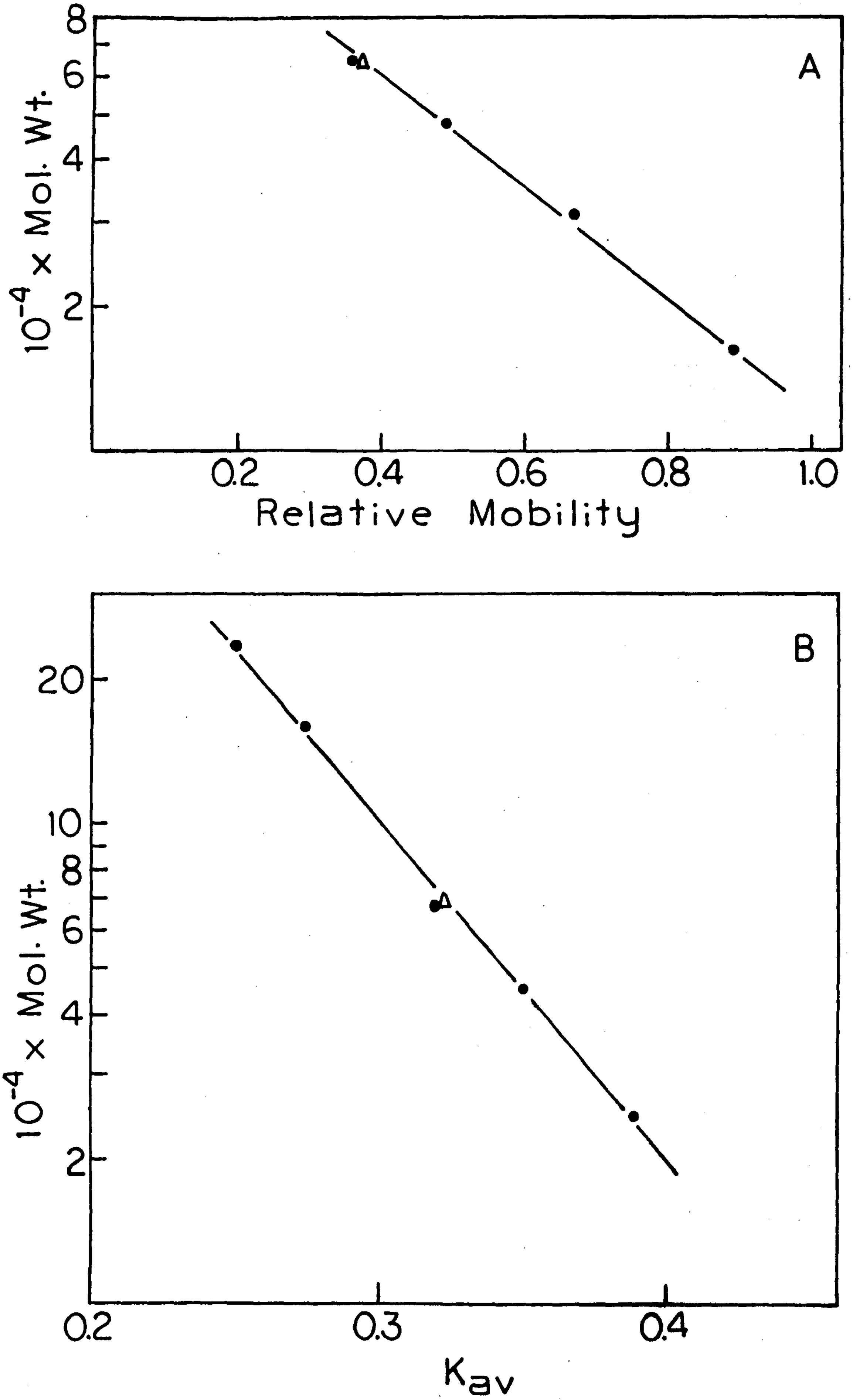
TABLE VI Purification of the Human Serum Ferroxidase Inhibitor

Purification Step	Total Protein	Total Inhibitory Activity	Specific Inhibitory Activity	Fold Purification	Percent Recovery
	(mg)	$\Delta(\Delta A_{460nm})$/min	$\Delta(\Delta A_{460nm})$/min/mg protein		
Whole Human Serum	2217	1.80	8×10^{-4}	1	100
Sephadex G-200	642	1.80	28×10^{-4}	3.5	100
DEAE Sepharose CL-6B	167	1.40	83×10^{-4}	10.4	78

FIGURE 11

Determination of the Molecular Weight of the Purified Human Ferroxidase Inhibitor

The molecular weight of the purified ferroxidase inhibitor was determined by SDS - electrophoresis and gel - filtration as described in "Materials and Methods". A: SDS - electrophoresis, B: Gel - Filtration.


A
8
6
4
2
10^{-4} × Mol. Wt.
0.2
0.4
0.6
0.8
1.0
Relative Mobility
B
20
10
8
6
4
2
10^{-4} × Mol. Wt.
0.2
0.3
0.4
K_{av}

inhibitor was eluted from DEAE sepharose with a buffer of identical ionic strength and pH as that used to elute human albumin from DEAE sepharose during its purification from whole serum. Lastly, at the pH at which electrophoresis was conducted, the ferroxidase inhibitor appeared to be a highly anionic protein, which is also characteristic of albumin. Therefore, several comparative studies were conducted with the purified ferroxidase inhibitor and authentic human serum albumin to determine whether the serum ferroxidase inhibitor was serum albumin.

Electrophoretic Comparisons

The electrophoretic mobilities of the purified ferroxidase inhibitor and authentic human albumin were compared in 7.5% and 4% non-SDS and SDS polyacrylamide gels. The mobilities of the ferroxidase inhibitor and the authentic albumin were identical in all four electrophoretic systems. Samples of the purified ferroxidase inhibitor and authentic human albumin were also subjected to analytical electrofocusing in thin layers of polyacrylamide gel with a final pH range of 3.4 - 6.0. Both samples focused at identical positions in the gel and specifically in the vicinity of pH 4.9, the isoelectric point reported for albumin. (Fig. 12, 12A)

Colorimetric Determination of Albumin

A specific quantitative procedure based on the affinity of albumin for bromcresol green was used to determine the concentration of albumin in the purified inhibitor samples (44). A value of 0.97 mg albumin/mg protein was obtained for the purified sample and authentic albumin; which indicated that with respect to the total protein concentration, at least 97% of the inhibitor sample was albumin.

FIGURE 12

Comparison of the Electrophoretic Mobility of the Purified Ferroxidase Inhibitor and Authentic Human Albumin in Non - SDS and SDS Systems

Samples of the purified ferroxidase inhibitor were electrophoresed in non - SDS and SDS systems as described in "Materials and Methods".

A: Ferroxidase Inhibitor on 7.5%
Non - SDS Gel

B. Authentic Human Albumin on 7.5%
Non - SDS Gel

C: Ferroxidase Inhibitor on 4%
Non - SDS Gel

D: Authentic Human Albumin on 4%
Non - SDS Gel

E: Ferroxidase Inhibitor on 7.5%
SDS - Gel

F: Authentic Human Albumin on 7.5%
SDS - Gel

G: Ferroxidase Inhibitor on 4%
SDS - Gel

H: Authentic Human Albumin on 4%
SDS - Gel

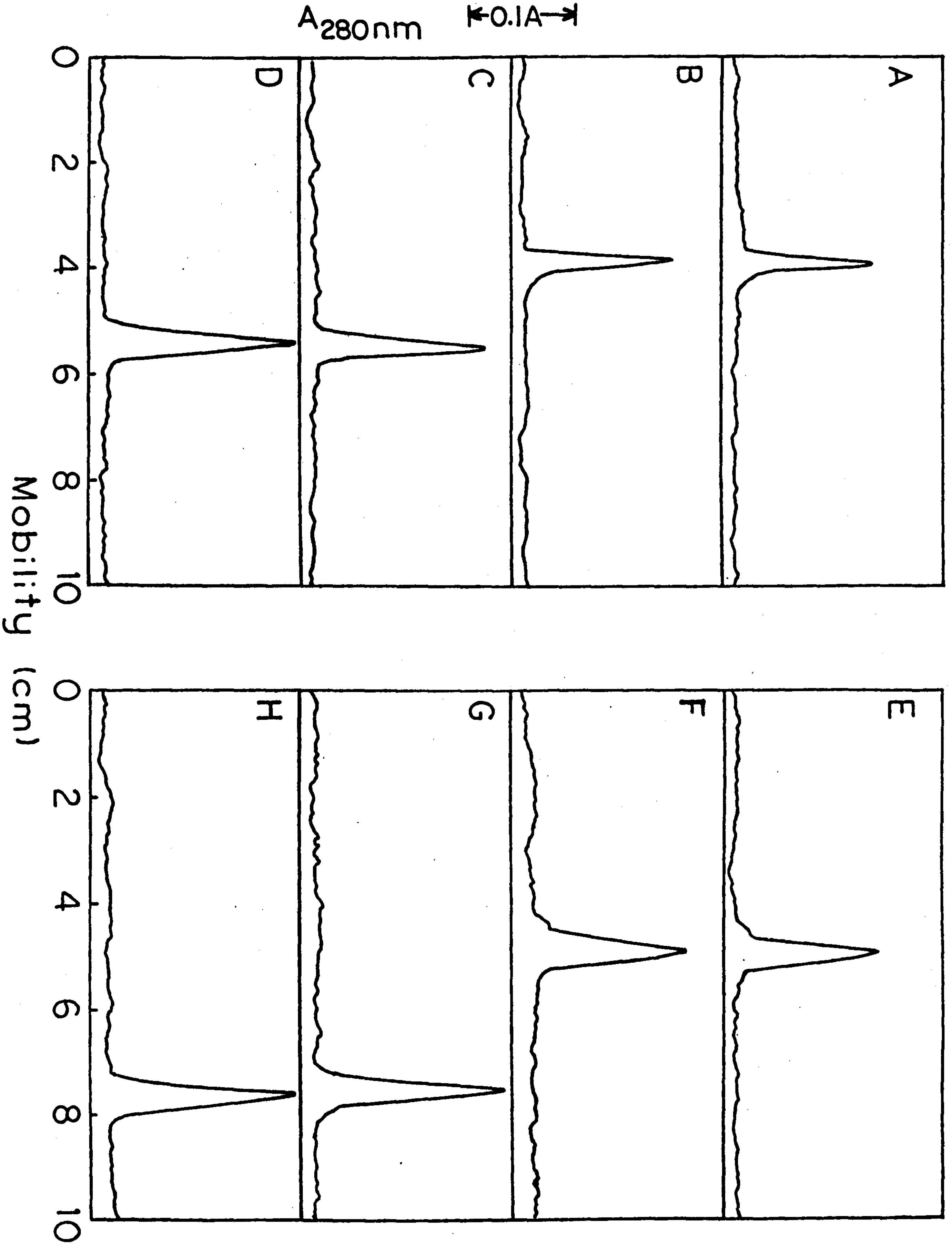
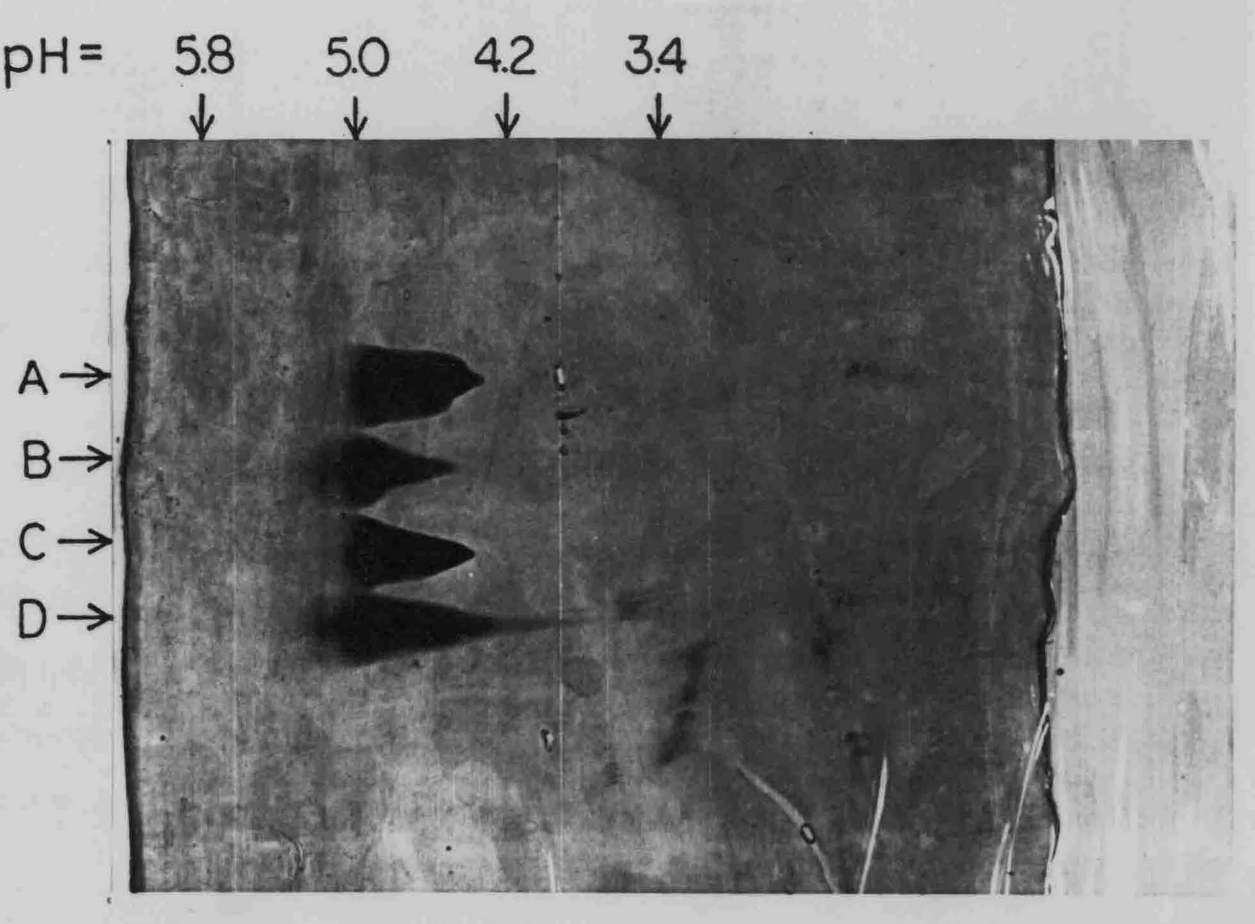

A280nm
0.1A
A
B
C
D
E
F
G
H
0
2
4
6
8
10
Mobility (cm)

FIGURE 12A

Comparison of the Electrofocusing Behavior of the Purified Ferroxidase Inhibitor and Authentic Human Albumin

Samples of the purified ferroxidase inhibitor and authentic human albumin were subjected to analytical electrofocusing as described in "Materials and Methods".

A: Ferroxidase Inhibitor
B: Authentic Human Albumin
C: Ferroxidase Inhibitor
D: Authentic Human Albumin


pH =
5.8
5.0
4.2
3.4
A
B
C
D

Carbohydrate Analysis

Serum albumin is a unique major plasma protein, in that it alone contains no carbohydrate (53). Assays of crystalline albumin show less than one sugar residue per molecule, and absence of carbohydrate is a classic criterion for purity of albumin (54). The purified ferroxidase inhibitor and authentic human albumin were analyzed for carbohydrate and both samples contained less than one sugar residue per molecule.

Immunodiffusion Studies

The purified ferroxidase inhibitor and samples of authentic human albumin were reacted with anti-human albumin and anti-human whole serum in a double immunodiffusion system as described in methods. Within 24 hours, a single line of precipitation was visible in all four systems, indicating that an antigen-antibody complex had formed (Fig. 13).

Cross Reactivities

A rather convincing piece of evidence supporting the identification of the purified ferroxidase inhibitor as serum albumin was obtained by testing and comparing the inhibitory potency of samples of authentic albumin with samples of the purified inhibitor. Both the inhibitor and the authentic albumin inhibited ferroxidase I (ceruloplasmin) and ferroxidase II with virtually the same potency (Table VII).

Mechanism of Inhibitor

Studies to elucidate the site and mode of action of the ferroxidase inhibitor were initiated. Extensive research has shown that human as well as other serum albumins have unique copper binding properties associated

FIGURE 13

Comparison of the Reaction of the Purified Ferroxidase Inhibitor and Authentic Human Albumin with Anti - Human Albumin and Anti - Human Whole Serum in an Immunodiffusion System

Samples of the purified ferroxidase inhibitor and authentic human albumin were incubated with anti - human albumin and anti - human whole serum in an immunodiffusion system as described in "Materials and Methods".

A: Ferroxidase Inhibitor vs. Anti - Human Albumin
B: Ferroxidase Inhibitor vs. Anti - Human Whole Serum
C: Authentic Human Albumin vs. Anti - Human Albumin
D: Authentic Human Albumin vs. Anti - Human Whole Serum

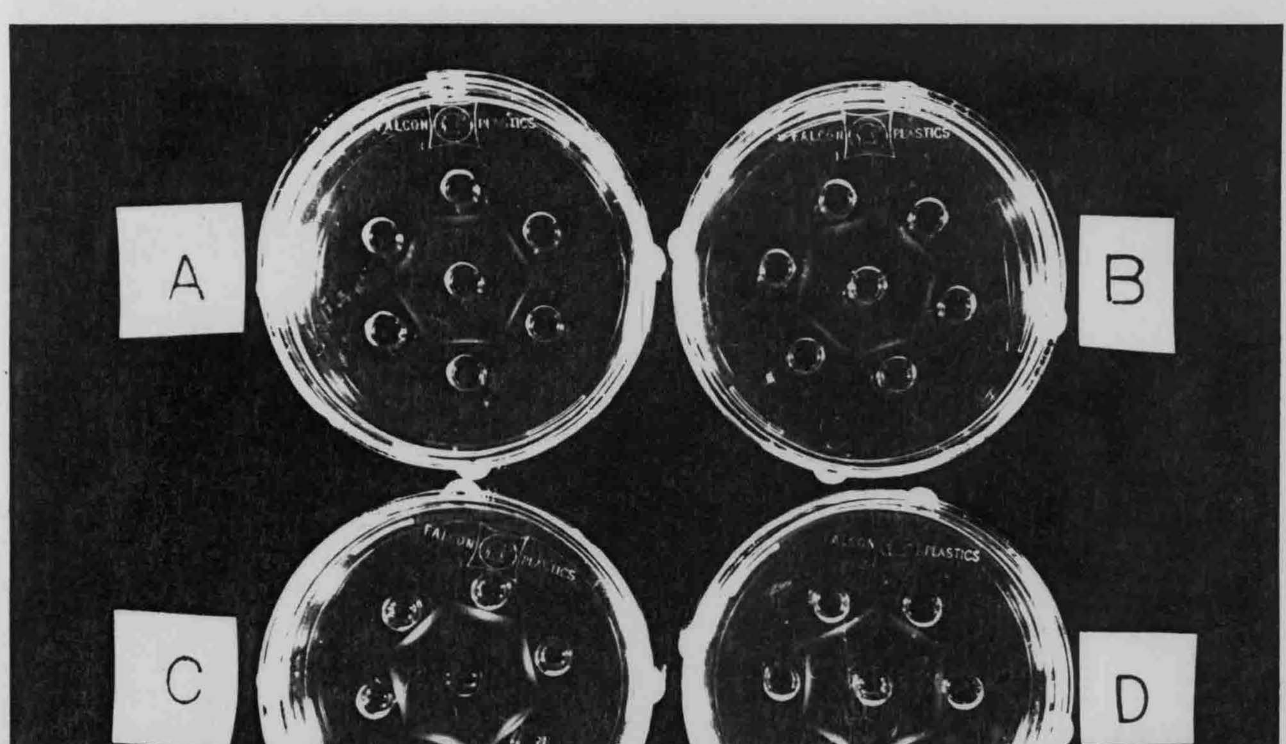

A
B
C
D
FALCON PLASTICS

TABLE VII Comparison of the Inhibitory Potencies of the Purified Ferroxidase Inhibitor and Authentic Human Albumin

Ferroxidase Sample	Inhibitor Sample*	Percent Inhibition
Human	Purified Ferroxidase Inhibitor	46
Ferroxidase-I	Authentic Human Albumin	42
Human	Purified Ferroxidase Inhibitor	51
Ferroxidase-II	Authentic Human Albumin	56

*All inhibitor samples were equivalent in protein concentration with the N-terminal end of the molecule (52). It has been demonstrated that albumin binds Cu(II), forming a square planar chelate eith the α amino nitrogen of the N-terminal aspartate residue, the first two peptide nitrogen atoms, and the 1-nitrogen of the imidazole ring of the histidyl residue at position 3 (52). Since copper is essential to the enzymic activity of both serum ferroxidases, it seemed logical to investigate whether this copper binding site on the albumin molecule did indeed interfere with the activity of the ferroxidases.

Albumin has been successfully fragmented and the copper binding site isolated and indentified (49,55-59). In the current study, preparations of the purified inhibitor were subjected to peptic digestion according to the methods of Peters and Hawn (49) (see methods). After digestion, the protein solution was treated with trichloracetic acid in a concentration range 1.75%(w/v)-10%(w/v), in order to precipitate out the desired fragment. The precipitate was then suspended in water and the trichloracetic acid extracted with ether. The aqueous protein layer was lyophillized and then reconstituted with 0.6N acetic acid, pH 3.0, to a volume of 25ml. This material was passed through a column (2.5 x 115cm) of spehadex G-75 and protein eluted with 0.6N acetic acid, pH 3.0. Protein elution is usually monitored at 280nm where absorption is due entirely to the presence of aromatic amino acids. However, proteins also absorb ultraviolet light between 210nm and 250nm due to absorption of aromatic and other residues and absorption due to some types of hydrogen bonds and other interactions concerned in conformation and helix content (60). In the experiment conducted by Peters and Hawn (49), the peptide fragment containing the copper binding site had very little absorption at 280 nm due to a low content of aromatic residues. In the current study, the desired peptide fragment was detectable at 230nm, therefore protein elution was monitored at 230nm. Four major bands of peptide material eluted from this column (Fig. 14). The fractions comprising each peptide band, were pooled and lyophilized. Each sample was reconstituted with 2 - 3ml of 0.6N acetic acid, pH 3.0 and tested for inhibitory activity against the enzyme. The inhibitory activity was associated with the fourth peptide band (Table VIII). This material was then applied to a column of spehadex G-25 equilibrated with 0.6N acetic acid, pH 3.0. The inhibitory activity was associated with the third protein band. (Fig. 15)

The fragmentation of albumin by pepsin yields two peptide chains termed the aspartic acid and phenylalanine fragments, because they include the two terminal sites of the albumin molecule. It is the aspartic acid fragment that contains the copper binding site (49,56-58). In order to determine whether the desired albumin fragment had been successfully isolated and recovered an N-terminal amino acid analysis was done on the peptide fragment obtained from the previously described experimental method. This was accomplished by thin layer chromatographic separation of DNS-CL (1-dimethyl-aminonapthalene-5-sulfonyl chloride) amino acids. (See methods). Dansyl derivatives of aspartic acid and phenylalanine were run as standards along with the dansyl derivative of the N-terminal residue of the isolated peptide fragment. When the thin layer plate was developed, it was observed that the peptide fragments migrated identically with the dansyl aspartic acid standard. It was concluded that the copper binding site of the albumin molecule had been successfully isolated, and due to its inhibitory activity could possibly be the site which interferes with the enzymic activity of the serum ferroxidase.

FIGURE 14

Elution Profile of Protein From G - 75 Sephadex

A sample of 25 ml. of concentrated protein was fractionated on a column (2.5 x 115 cm) packed with sephadex G - 75. The elution buffer was 0.6 N acetic acid, pH 3.0. Protein elution was monitered at 230 nm and fractions of 8 ml. were collected.

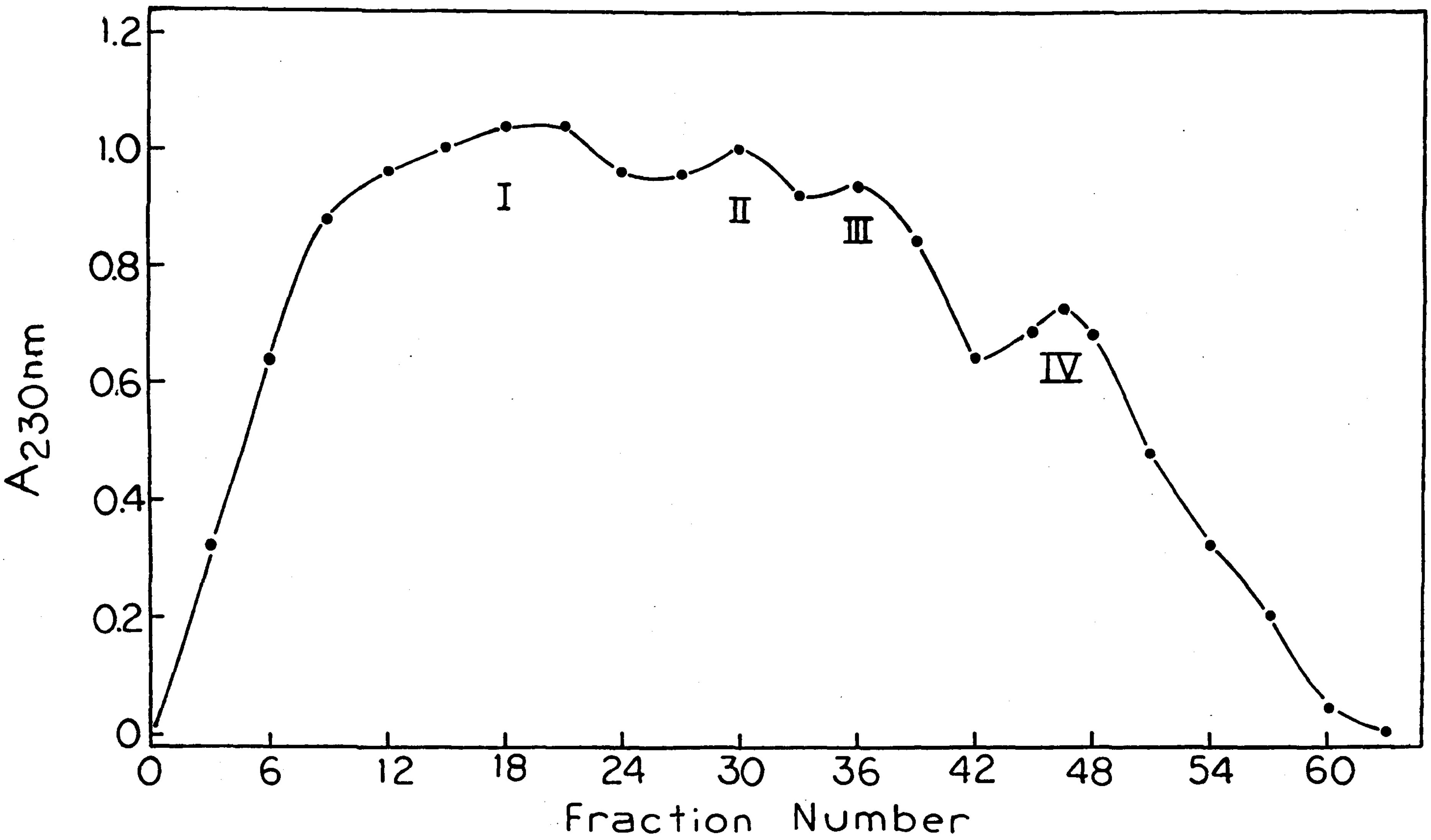

1.2
1.0
0.8
0.6
0.4
0.2
0
A230nm
I
II
III
IV
0
6
12
18
24
30
36
42
48
54
60
Fraction Number

FIGURE 15

Elution Profile of Protein From G - 25 Sephadex

A sample of 3 ml. of concentrated protein was fractionated on a column (1.5 x 52 cm) packed with sephadex G - 25. The elution buffer was 0.6 N acetic acid, pH 3.0. Protein was monitored at 230 nm and fractions of 8 ml. collected.

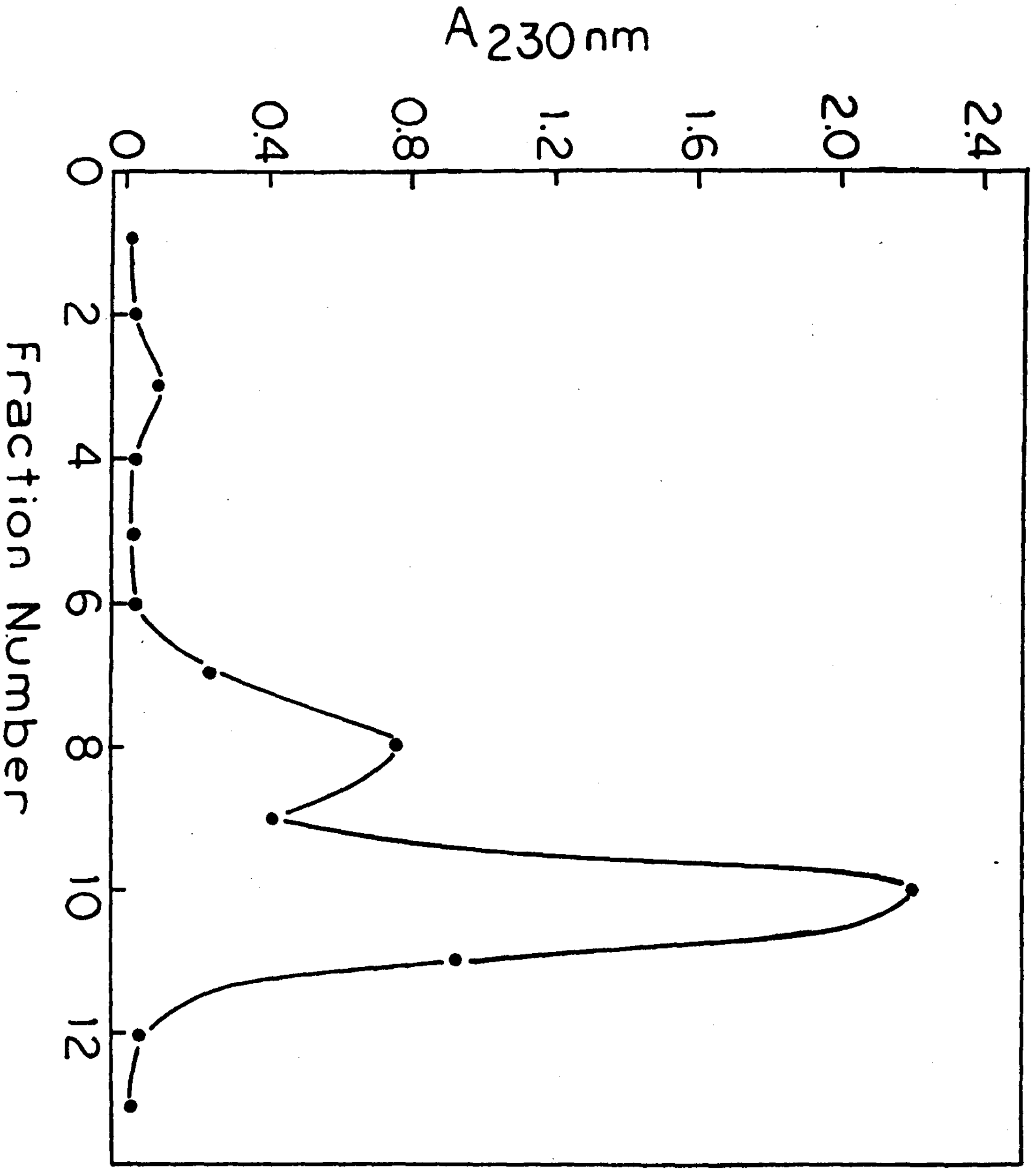

A230 nm
0
0.4
0.8
1.2
1.6
2.0
2.4
0
2
4
6
8
10
12
Fraction Number

Table VIII Comparison of the Inhibitory Activity of the Pepsin Generated Peptide Fragments Eluted from Sephadex G-75

Protein Band	Total Protein (mg)	Specific Inhibitory Activity (ΔA_{460nm})/min/mg protein	Total Inhibitory Activity (ΔA_{460nm})/min
Peak 1	197.4	—	—
Peak 2	65.9	.0026	.1713
Peak 3	73.9	.0023	.1700
Peak 4	18.7	.0644	1.2043

DISCUSSION

DISCUSSION

Numerous "in vivo" and "in vitro" studies have established that the serum ferroxidases, ceruloplasmin (ferroxidase I) and ferroxidase II, play a physiological role in promoting the oxidation and incorporation of iron into transferrin; thereby facilitating the mobilization of iron from tissue stores (11,15,16,20,13,16,34,35,51). Specifically, the serum ferroxidases catalyze the oxidation of Fe(II) to Fe(III) as iron fluxes from the liver to the bloodstream, thus providing a site for transferrin to sequester the ferric ion (11, 24).

The copper tightly bound to both serum ferroxidases has been shown to be essential to their enzymic activity (11,15,16,20,24,34-36,51,59,61). Previous studies have shown that copper deficiency in laboratory animals produces an anemia that emulates iron deficiency anemia; furthermore, the symptoms have been shown to be due to an inability to transfer iron from the cells to the plasma (5,18). This suggests that the serum ferroxidases could provide, at least in part, the much sought missing link between copper and iron metabolism (6,62).

Ferroxidase II has been purified from a number of sources including whole rabbit and whole human serum. In each case, a seven fold increase in the total ferroxidase activity was observed after the first purification step. This data suggested that perhaps there exists a potent inhibitor of ferroxidase II, that masks enzymic activity in whole serum, but when separated from the enzyme during purification results in this large increase in the total activity.

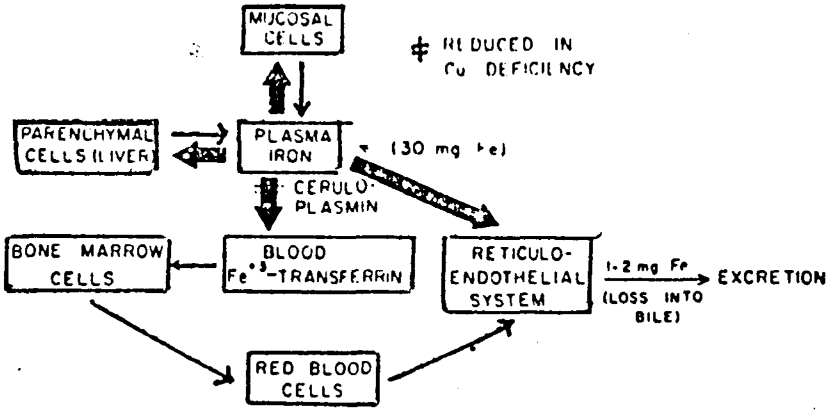
Such an inhibitor was isolated and purified to homogeneity and was found to be effective against ceruloplasmin (ferroxidase I) as well as ferroxidase II. Characterization studies strongly suggested that the purified inhibitor was serum albumin. This evidence would account for the small fold purification required to obtain a homogenous preparation of the ferroxidase inhibitor and the good recovery of inhibitory activity; since albumin is a relatively stable protein and accounts for such a large fraction of the total protein in whole serum (53-65 % of the total protein) (63).

The results of the characterization studies revealed that the purified inhibitor resembled serum albumin in terms of molecular weight, electrophoretic mobility, electrofocusing results, a specific colorimetric test, absence of carbohydrate, and immunodiffusion studies. Of particular interest was the effectiveness of commercial albumin as a potent inhibitor of serum ferroxidase activity. The authentic albumin inhibited both ceruloplasmin (ferroxidase I) and ferroxidase II with about the same degree of potency as the ferroxidase inhibitor. This is very meaningful from a physiological viewpoint. In human serum, ceruloplasmin accounts for greater than 90% of the total serum ferroxidase activity; however, in less highly developed animals, ferroxidase II contributes a larger fraction of the total serum ferroxidase activity (28,31). Thus, even though the relative contribution of the two serum enzymes to the total serum ferroxidase activity may vary from one animal species to another, the ferroxidase inhibitor, due to its potent inhibitory activity against both ferroxidase enzymes,would still be capable of modulating the total serum ferroxidase activity; and hence, modulating the mobilization of iron from tissue stores. This strongly suggests a physiological role for the ferroxidase inhibitor in the overall scheme of iron metabolism. This role is depicted in(figure 16) which is a schematic summary of iron metabolism. The serum ferroxidases facilitate iron mobilization by oxidizing Fe(II) to Fe(III) so that it can be incorporated into transferrin thus creating a large concentration gradient for Fe(II) across the plasma membrane of the liver cell. By inhibiting the enzymatic activity of the serum ferroxidases, the ferroxidase inhibitor would affect iron metabolism at the level of Fe(III) transferrin formation. The iron in the Fe(III)-transferrin is contributed directly

FIGURE 16

A Schematic Summary of Iron Metabolism

A schematic summary of iron metabolism depicting a possible physiological role for the ferroxidase inhibitor.


MUCOSAL CELLS
REDUCED IN Cu DEFICIENCY
PARENCHYMAL CELLS (LIVER)
PLASMA IRON
(30 mg Fe)
CERULOPLASMIN
BONE MARROW CELLS
BLOOD Fe+3-TRANSFERRIN
RETICULO-ENDOTHELIAL SYSTEM
1-2 mg Fe
EXCRETION
(LOSS INTO BILE)
RED BLOOD CELLS

to the developing reticulocyte in the bone marrow. When the mature red cell finishes its life cycle it is scavenged by the reticuloendothelial cells, principally in the liver and the spleen. The iron is recovered and released into the plasma where it can be absorbed by the mucosal cells of the intestine or recycled into the marrow by incorporatin into transferrin.

The results of several in vivo studies further support the suggestion that the ferroxidase inhibitor could play an active role in the regulation of iron mobilization. One would expect that animals receiving low iron diets would be more dependent upon the mobilization of iron from tissue stores for the maintenance of the plasma iron pool than animals receiving diets of normal iron content. Thus, the serum of animals receiving low iron diets should contain lower levels of the ferroxidase inhibitor and a greater total ferroxidase activity than the serum of the same animals when fed diets of normal iron content. Such a correlation between the serum content of the inhibitor and the total ferroxidase activity was observed in the dietary studies with rabbits. This correlation was further substantiated by the changes in the serum content of the ferroxidase inhibitor and the total ferroxidase activity observed in the rabbit that was repetitively bled. Thus both studies suggest that the inhibitor could be involved in the regulation of iron mobilization from tissue stores.

The finding that the ferroxidase inhibitor is effective against both ceruloplasmin (ferroxidase I) and ferroxidase II is also important from a mechanistic viewpoint. The serum ferroxidases differ markedly in physical properties and chemical composition. Ceruloplasmin is a cupro-glyco protein whereas ferroxidase II is a cupro-lippo protein (24,25,28). Although the two differ markedly in structure, they both contain tightly bound Cu(II) ions which are essential to their ferroxidase activity and their ability to facilitate iron mobilization from tissue stores (11,15,16,20,24,25,26,28,34,35,36,51,61). The assumption that the bound Cu(II) of the ferroxidases might be the functional site with which the inhibitor interacts correlates well with the idendification of the ferroxidase inhibitor as serum albumin. The unique copper binding properties of albumin associated with the amino terminal end of the molecule could possibly interfere with the binding of the substrate (iron) to the ferroxidase, oxidation of the iron by the ferroxidase, or sequestering of the oxidized iron from the ferroxidase by transferrin.

The pepsin generated aspartic acid fragment of the albumin molecule has been shown to contain the specific copper binding site (56). The finding that this fragment, and only this fragment, potently inhibits the serum ferroxidase activity, further supports the suggestion that the bound Cu(II) of the ferroxidases could be the site with which albumin interacts.

It is not unreasonable to speculate that albumin could play a functional role in iron metabolism. Albumin has several diverse functions that contribute to homeostasis through the mechanisms of hemodynamics, transport and nutrition (52). The plasma proteins are large colloidal molecules and are nondiffusable through capillary and glomerular walls as most other blood solutes. They are thus entrapped in the vascular system and exert a "colloidal osmotic pressure", which serves to maintain a normal blood volume, and a normal water content in the interstitial fluid and the tissues. Albumin exerts 80% of the colloidal osmotic pressure. If the albumin falls to low levels, water will leave the blood vessels and enter the extracellular fluid and the tissues, thus producing edema (63).

Another important function of albumin is its participation in normal fat metabolism; the main metabolic abnormality of analbuminemic persons (those who lack or have extremely low levels of albumin) is a gross disturbance in lipid transport. Albumin accepts, the free fatty acids released by lipoprotein lipase, keeps them in solution, and transports them between liver and peripheral tissues in the course of their active metabolism (64).

Albumin also serves to stabilize the plasma concentrations of calcium, tryptophan, and hormones including cortisol, testosterone, and estrogens. Tryptophan binding varies inversely with the load of fatty acids. This causes the concentration of free tryptophan to rise when fatty acid levels rise and may have important consequences in the levels of tryptophan in the brain and the production of serotonin (52).

Albumin is one of the few extracellular proteins having a free thiol group which serves both to detoxify Hg^{2+} and to transport poorly soluble disulfides through the mechanism of thiol-disulfide exchange. Albumin also binds bilirubin, (a by-product of red cell degeneration) which is particularly important in the newborn infant where the concentration of free bilirubin determines its passage into the hydrophobic tissues of the brain with concomitant risk of damage through "kernicterus" (52).

The nutritive function of albumin arises from its availability to cells as a source of amino acids through pinocytosis. Possibly 10% of protein metabolism may be carried on in this manner. Albumin formed by the liver postprandially when the level of amino acids in the portal blood is high, is utilized by cells when circulating free amino acids are in short supply (32).

The multifunctionality of albumin could be linked to what is termed its "microheterogeneity". This description was first used in reference to the changes the albumin molecule underwent when exposed to a low pH. In the pH range from 2 to 4, the molecule expands, becoming longer and more asymmetric without a change in molecular weight (65,66,67). The helical content decreases and the molecule unfolds so that interior parts become accessible (68,69). Microheterogeneity appears to be present even in the albumin of a single donor; polymorphism of a donor population is not its cause. Removal of tightly bound fatty acids decreases but does not eliminate this microheterogeneity (70,71). Possible causes are (1) intramolecular disulfide bond interchange, (2) differences in amide content or location, or (3) modifications of circulating molecules ("molecular aging") such as acetylation of -amino groups by aspirin. There is no reason to suspect that the plasma of a typical individual contains albumin species differing in amino acid sequence. Rather it seems that albumin, once freed of globulin by crystallization or chromatography, is a single protein that can become polydisperse by uptake of ligands or perhaps by intramolecular isomerization (52).

It is quite possible that the inhibitory activity of the albumin molecule is somehow related to one molecular form of microheterogeneous albumin rather than related to the entire serum pool of albumin. Evidence to support this is apparent from some of the data obtained from the "in vivo" experiments. When a rabbit was placed on a low iron diet it was observed that the levels of the inhibitor dropped dramatically, whereas the total protein level remained fairly constant. Since albumin represents such a large fraction of the total protein in serum, one would expect the levels of the inhibitor and the total protein to correlate better. This discrepancy suggested that the actual inhibitory activity of albumin may be due to a small specific substance that is bound to the albumin molecule, or to one molecular form of the microheterogeneous protein. Additional experimental work will have to be completed before this evidence is clearly understood. More exact information concerning the copper binding site in the molecule and kinetic data determining the type of inhibition should help to elucidate the mechanism by which the inhibitor interacts with the serum ferroxidases.

LIBRARY
UNIVERSITY OF RICHMOND
VIRGINIA

BIBLIOGRAPHY

BIBLIOGRAPHY

1. Frieden, Earl, Scientific American, May, 1968, 103.

2. Holmberg, C. G., Laurell, C. B., Acta Chem. Scand. (1948) 2, 550.

3. Hart, E. B., Steenbock, H., Waddell, J., Elvehjem, C. A., J. Biol. Chem. (1928) 77, 797.

4. Cartwright, G. E., Gubler, C. J., Bush, J. A., Wintrobe, M. M., Blood (1956) 11, 143.

5. Lahey, M. E., Gubler, C. J., Chase, J. S., Cartwright, G. E., Wintrobe, M. M., Blood (1952) 7, 1053.

6. Frieden, Earl, Advances in Chemistry #100 (1971) 292.

7. Lee, G. R., Cartwright, G. E., Wintrobe, M. M., Proc. Soc. Exptl. Biol. Med. (1968) 127, 977.

8. Curzon, G., O'Reilly, S., Biochemical and Biophysical Research Communications Vol. 2, No. 4 (April 1960).

9. Curzon, G., Biochem. J. (1961) 79, 656.

10. Holmberg, C. G., Laurell, C. B., (1951) Acta Chem. Scand., 5, 476.

11. Osaki, S., Johnson, D. A., Frieden E., Journal Biological Chemistry, (1966) 241, 2746.

12. Aasa, B., Malmstrom, B. G., Saltman, P., Vanngaro, T., Biochim. Biophys. Acta. 75, 208 (1963).

13. Koechlin, B. A., J. Am. Chem. Soc., 74, 2649 (1952).

14. Katz, J. H., Jandl, J. H. in F. Gross (Editor), Iron Metabolism, Springer-Verlag, Berlin, 1964, p. 103.

15. Ragan, H. A., Nacht, S., Lee, G. R., Bishop, C. R., Cartwright, G. E., American Journal of Physiology (1960) 1320.

16. Roesser, H. P., Lee, G. R., Nacht, S., Cartwright, G. E., Journal of Clinical Investigation (1970) 49, 2408.

17. Lee, G. R., Roeser, H. P., Nacht, S. Cartwright, G. E., J. Clin. Invest. (1970) Abstr. 177.

18. Lee, G. R., Nacht, S., Lukens, J. N., Cartwright, G. E., J. Clin. Invest. (1968) 47, 2058.

19. Ravin, H. A., J. Lab. Clin. Med. (1961) 58, 161.

20. Osaki, S., Johnson, D. A., Frieden, E. J. Biol. Chem. (1971) 246, 3018.

21. Undritz, E., in F. Gross (Editor), Iron Metabolism, Springer-Verlag, Berlin, 1964, p. 406.

22. Frieden, E., Osaki, S., Second Rochester Conference on Toxicity, Rochester, New York (1969).

23. Ibid.

24. Topham, R. W., Frieden, E., J. Biol. Chem., (1970) 245, 6698.

25. Topham, R. W., Johnson, D. A., Archives of Biochemistry and Biophysics (1974) 160, 647.

26. Osaki, S., J. Biol. Chem. (1966) 241, 5053.

27. Sung, Christine SM, Topham, R. W., Biochemical and Biophysical Research Communications (1973) 53, 824.

28. Topham, R. W., Sung, Christine SM, Morgan, F., Prince, D., Jones, S., Archives of Biochemistry and Biophysics, (1975) 167, 129.

29. Lykins, L., Akey, C., Christian, E., Duval, G., Topham, R. W., Biochemistry, (1977) 16, 693.

30. Topham, R. W., Sung, Christine SM, Federations Proceedings, Fedn. Am. Socs. Exp. Biol. 30, (1973) 1292.

31. Chin, J., Calisch, M. P., Topham, R. W., International Journal Biochemistry 9 (1978) 775.

32. Deutsch, H. F. (1960) Arch. Biochem. Biophys. 89, (1960) 225.

33. Osaki, S., Johnson, D. A., J. Biol. Chem. (1969) 244, 5557.

34. Osaki, S., Johnson, D. A., Topham, R. W., Frieden, E., Fed. Proc. (1970) 29, 695.

35. Williams, D. M., Lee, G. R., Cartwright, G. E., Am. J. Physiol. (1974) 227, 1094.

36. Osaki, S., Walaas, O., J. Biol. Chem. (1967) 242, 2653.

37. Frieden E., Osaki, S., The effects of Metals on Cells, Subcellular Elements, and Macromolecules (edited by Maniloff, J., Coleman, J. R., Miller, M. W.) Thomas, Springfield, Ill. (1970) 39.

38. Johnson, D. A., Osaki, S., Frieden, E., Clin. Chim. (1967) 13, 142.

39. Topham, R. W., Johnson, D. A., Arch. Biochem. Biophys. (1974) 160, 647.

40. Lowry, O. H., Rosenbrough, J. J., Farr, A. L., Randall, R. J., J. Biol. Chem. (1951) 193, 265.

41. Wharten, D. C., Rader, M., Analyt. Biochem., (1970) 33, 226.

42. Zak, B., Am. J. Clin, Path. (1957) 27, 583.

43. Bartlett, G. R., J. Biol. Chem. (1959) 234, 446.

44. Doumas, B. T., Biggs, H. G.: Determination of Serum Albumin in Copper GR. (ed): Standard Methods of Clinical Chemistry, vol. 7, New York, Academic Press, 1972, p. 175.

45. Spiro, R. G., Methods in Enzymology, vol. VIII. New York, Academic Press, 1966, p. 3.

46. Gordon, B. I., Essentials of Immunology (ed. 2). Philadelphia, F.A. Davis Co., 1974, p. 54.

47. Winters, A., Ek, K., Anderson, V. B., Analytical Electrofocusing in Thin Layers of Polyacrylamide Gels. Application note 250, Bromma Sweden, LKB-Produckter AB, 1977.

48. Lehninger, Albert, Biochemistry, Second Edition, Worth Publishers Inc., New York, New York (1975).

49. Peters, T. Jr., Hawn, Cynthia, J. Biol. Chem. (1967) 242, 1566.

50. Morse, D., Horecker, B. L., Annals Biochem. (1966) 14, 429.

51. Topham, R. W., Woodruff, James H., Neatrour, G. Peyton, Calisch, Melissa P., Russo, Richard B., Jackson, Mark R., Biochemical and Biophysical Communications, (1980) vol. 96, 70.4, 1532.

52. Peters, T. Jr., Serum Albumin, in Putnam, F. W. (ed): The Plasma Proteins, vol. 1. New York Academic Press, 1975, p. 133.

53. Eylar, E. H., J. Theor. Biol. (1965) 10:89.

54. Cohn, E. J., Hughes, W. L. Jr., Weare, J. H., J. Amer. Chem. Soc. (1947) 69, 1753.

55. Weber, Grejorio, Young, Lorna B., J. Biol. Chem. (1964) 239, 1415.

56. Peters, T. Jr., Blumenstock, F. A., J. Biol. Chem. (1967) 242, 1574.

57. Shearer, W. T., Bradshaw, R. A., Gurd, F.R.N., Peters, T. Jr., J. Biol. Chem. (1967) 242, 5451.

58. Bradshaw, R. A., Shearer, W. T., Gurd, F.R.N., J. Biol. Chem. (1968) 243, 3817.

59. Lau, Show-Jy, Sarkar, B., J. Biol. Chem. (1971) 246, 5938.

60. White, A., Handler, P., Smith, E. L., Principles of Biochemistry, 4th Edition, McGraw Hill, New York, New York. (1968) p. 154.

61. Osaki, S., J. Biol. Chem. (1966) 241, 5053.

62. Frieden, E., Osaki, S. (1970) The effects of Metals on Cells, Subcellular Elements, and Macromolecules (edited by Maniloff, J., Coleman, J. R., Miller, M. W.) Thomas, Springfield, Il. 39-72.

63. Tietz, N. W., Fundamentals of Clinical Chemistry, W. B. Saunders Co., Philadelphia, Pa., 1970, p. 229.

64. Whayne, T. F., Jr., Felts, J. M., Clin. Biochem. (1972) 5, 109.

65. Harrington, W. F., Johnson, P., Ottewill, R. H., Biochem. J. (1956) 62, 569.

66. Foster, J. F. (1960). In "The Plasma Proteins" (F. W. Putnam, ed.). vol 1 pp. 179-233., Academic Press, New York.

67. Champaqne, M., J. Polym. Sci. (1957) 23, 863.

68. Brenon, E. S., Hallaway, B. E.,Lumry, R. W., J. Biol. Chem. (1963) 239, 122.

69. Wallevik, K. J. Biol. Chem. (1973 b) 248, 2650.

70. McMenamy, R. H., Arch. Biochem. Biophys. (1967) 122, 635.

71. Wong, K. P., Foster, J. F., Biochemistry (1969) 8, 4096.