THE DEVELOPMENT OF IRON EXCHANGE SYSTEM FOR THE TREATMENT OF CHRONIC IRON OVERLOAD

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ABSTRACT

Iron overload is a common finding in patients with refractory anemias who require long-term transfusion therapy. Since there is no physiological excretory system for iron, excess iron from transfused erythrocytes accumulates in the liver, the heart and endocrine glands and causes serious dysfunction of these organs and early death. The only way to remove excess iron is by the administration of chelating agents which are, however, only partially effective. Therefore, new approaches for therapy of iron overload are needed.

In the present study, a new system for iron removal from the plasma iron binding protein, transferrin, was investigated. A decreased level of plasma iron will lead to an increase in the level of apotransferrin, iron-free transferrin, which should mobilize iron from stores in tissues. Therefore, theoretically, we should be able to mobilize and "deplete" tissue iron in patients with transfusional iron overload if we were to remove iron from transferrin. This system should enable us to investigate the factors involved in iron mobilization from tissues by transferrin and may lead to the development of a more effective therapy for secondary iron overload.

We have demonstrated that apolactoferrin, prepared in our laboratory, can take up iron from transferrin (separated by a dialysis membrane) in the presence of citrate, nitrilotriacetate or pyrophosphate, thus forming apotransferrin. Some exchange of iron from transferrin to apolactoferrin can be demonstrated at pH 7.4 but the rate of iron transfer is significantly stimulated when the pH of the buffer is decreased to 6.5. The rate of transfer of iron from transferrin to apolactoferrin may be limited by diffusion when a

dialyzing membrane is used to separate the proteins. We have also shown that the transfer of iron from transferrin to apolactoferrin is much faster if apolactoferrin is bound to activated sepharose as compared to dialysis.

Finally, we have demonstrated that apotransferrin can be prepared by the reductive release of iron from iron-saturated transferrin. This process requires the use of reducing agents like thioglycolate and dithionite. The reduced iron (Fe^{2+}) can then be sequestered by bathophenanthroline sulfonate (BPS). The by-products, Fe^{2+} BPS and oxidized forms of the reducing agents are then removed by encapsulated activated charcoal.

RESUME

On constate fréquemment une surcharge en fer chez les sujets souffrant d'anémie réfractaire qui nécessite une thérapie transfusionnelle à long terme. Etant donné qu'il n'existe pas de système excréteur du fer dans l'organisme, l'excédent de fer provenant des érythrocytes transfusés s'accumulent dans le foie, le coeur et les glandes endocrines, provoquant un grave dérèglement de ces organes et souvent une mort précoce. L'unique moyen de se débarrasser du fer excédentaire consiste à administrer des chélates qui ne sont toutefois que partiellement efficaces. C'est pourquoi il est nécessaire de trouver de nouvelles méthodes de traitement de la surcharge en fer.

Dans la présente étude, nous avons étudié un nouveau système d'élimination du fer de la transferrine, protéine du plasma sanguin qui fixe le fer. Une réduction de la concentration de fer dans le plasma se traduira par une plus grande concentration d'apotransferrine, transferrine sans fer, qui devrait mobiliser le fer provenant des réserves tissulaires. C'est ainsi que théoriquement, nous devrions être en mesure de mobiliser et d'"épuiser" les réserves tissulaires de fer chez les sujets atteints de surcharge en fer transfusionnelle si nous arrivions à éliminer le fer de la transferrine. Ce système devrait nous permettre d'étudier les facteurs qui entrent en jeu dans la mobilisation du fer des tissus par la transferrine et nous permettre de mettre au point une thérapie plus efficace des surcharges en fer secondaires.

Nous avons démontré que l'apolactoferrine, préparée dans nos laboratoires, est capable de fixer le fer de la transferrine (séparée par une membrane à dialyse) en présence de citrate, de nitrilotriacétate ou de pyro-

phosphate, formant ainsi l'apotransferrine. On peut prouver qu'il y a échange de fer de la transferrine à l'apolactoferrine à pH 7.4, mais le taux de transport du fer augmente sensiblement lorsque le pH du tampon est abaissé à 6.5. Le taux de transport du fer de la transferrine à l'apolactoferrine peut être limité par diffusion lorsqu'on se sert d'une membrane à dialyse pour séparer les protéines.

Nous avons également démontré que le transport de fer de la transferrine à l'apolactoferrine est beaucoup plus rapide si l'apolactoferrine est liée à une sépharose activée plutôt que soumise à une dialyse.

Enfin, nous avons démontré que l'apotransferrine peut se préparer par la libération réductive de fer de la transferrine saturée de fer. Ce procédé nécessite l'emploi d'agents de réduction comme le thioglycolate et la dithionite. Le fer (Fe^{2+}) ainsi réduit peut alors être séquestré par le sulfonate bathophenanthroline (SBP). On élimine alors les dérivés, Fe^{2+}-SBP ainsi que les formes oxydées des agents réducteurs au moyen de charbon activé encapsulé.

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LIST OF ABBREVIATIONS

apoLf	Iron-free lactoferrin
apoTF	Iron-free transferrin
BPS	Bathophenanthroline sulfonate
C	Celsius (degree centrigrade)
cm	Centimetre
cpm	Counts per minute
dl	Decilitre
DHB	Dihydroxybenzoic acid
EDDHPA	Ethylenediamine di - (O-hydroxyphenyl acetic acid)
g	Gram
Hb	Hemoglobin
HEPES	4 - (2-hydroxyethyl) - 1 - piperazineethane sulfonic acid
hr	hour
Lf	Iron-saturated lactoferrin
m	Metre
ug	Microgram
ul	Microlitre
uM	Micromilar (10^{-6}M)
mA	Milliampere
mC	Millicurie
mg	Milligram
ml	Millilitre
mm Hg	Millimetre of mercury pressure
mu	Millimicron
mM	Millimolar (10^{-3}M)

min	Minute
M	Molar or moles per litre
nM	Nanomolar (10^{-9}M)
NTA	Nitrilotriacetate
OD	Optical Density
pH	- Log H^+ concentration
RBC	Red blood cell
rpm	Revolutions per minute
Tf	Iron-saturated transferrin
Tris	2 - amino - 2 - hydroxymethyl propane - 1, 2 - diol

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INTRODUCTION

A. General Introduction

Iron is an essential element in metabolism and with the possible exception of lactic acid bacteria, iron is required by all organisms. As part of the hemoglobin molecule, iron is the constituent of red blood cells which binds oxygen and transports it from the lungs to all tissues of the body. Within living cells iron in various forms is also involved in the production of energy from foodstuffs. Furthermore, iron in its non-heme form is also required for some specific enzymes such as ribonucleotide reductase.

At physiological pH and oxygen tension, the stable form of iron is Fe^{3+}. However, Fe^{3+} is virtually insoluble and therefore cannot be transported in an ionic form in the circulation. The concentration of free hydrated Fe^{3+} in solution cannot exceed $10^{-17}M$ (1). Organisms have developed specific iron sequestering molecules to maintain iron in soluble form (2). In vertebrates, a complex protein has been evolved to manage the internal transport of iron. This protein, plasma transferrin, is responsible for shuttling iron between sites of absorption, storage and utilization.

In the body under normal conditions, iron absorption and exchange are regulated so as to meet the general needs of the organism. The daily obligatory loss of iron is about 1 mg and this loss is usually balanced by intestinal absorption of iron. Several mechanisms have been proposed on the regulation of iron absorption (3,4). Iron absorption can be divided into phases of mucosal uptake and mucosal transfer (5). The initial mucosal uptake

of iron appears to be a passive process and perhaps diffusion-controlled (6). After the initial uptake, iron is believed to be transported by a transferrin-like protein to the antiluminal membrane of the enterocyte (7,8). Iron is then transferred to plasma transferrin. It has been suggested that iron absorption is determined by changes in the amount of transport protein present in the brush-border of the intestinal mucosa (3,9). However, it has been demonstrated that these proteins are not the regulators of intestinal iron absorption (7,9,10). It is also suggested that iron absorption is regulated by a "mucosal block". According to this theory, the accumulation of excess iron in the mucosal epithelia cells blocks iron absorption (130,131).

The uptake of iron from transferrin by cells, such as reticulocytes and hepatocytes depends on specific surface receptors (11-14). It has been shown that the uptake of iron by reticulocytes and hepatocytes may be controlled at least in part by the number of transferrin receptors on the plasma membrane (15). The mechanism of iron release from transferrin to cells is still controversial. Two hypotheses have been suggested. One holds that after binding to its specific receptor, transferrin is internalized by endocytosis before its iron is made available to cells (16,17). Several techniques have been used to demonstrate endocytosis of transferrin and these methods include electron microscope autoradiography, horseradish peroxidase and fluorescein isothiocynate as tracers for transferrin (17-19). Tracers conjugated to transferrin have been shown to enter reticulocytes and nucleated erythroid cells. On the other hand, unconjugated tracers could not enter these cells. Endocytosis of transferrin is also supported by the observation that inhibitors of microtubule function block transferrin and iron uptake by reticulocytes (20). The other hypothesis of iron release from transferrin to cells suggest

that iron is released from transferrin at or near the cell membrane and is transported within the cell to an intracellular labile or transit pool. From this pool iron is made available for heme synthesis or iron containing enzymes or when in excess for ferritin synthesis (11).

The major site for iron utilization in the body is the bone marrow where iron is required for the biosynthesis of hemoglobin (21). The liver is also important in iron metabolism. In the liver, iron is stored as well as utilized for the synthesis of iron containing enzymes. Furthermore, the liver is the major site for plasma transferrin synthesis which is increased in response to iron deficit and inhibited by excess iron (22). Iron is stored as ferritin and hemosiderin which are present in the reticuloendothelial system and the parenchymal cells of the liver, spleen and the bone marrow. Increased cellular levels of iron stimulate ferritin synthesis (23).

B. Iron Binding Proteins

I. Transferrin

a) Structure and Properties: Transferrin is a glycoprotein consisting of a single polypeptide chain of molecular weight of about 80,000. Human transferrin contains about 676 amino acids and about 6% carbohydrate by weight (24). This carbohydrate is linked to the protein chain in two identical and symmetrical branched heterosaccharide chains which are joined to the protein by N-glycosidic linkage to asparagine residues in the C-terminal half of the transferrin molecule (24,25). The polypeptide chain of transferrin is made up of two compact regions each of which contains an iron binding site (26-29). The N- and C-terminal halves of transferrin molecule contain about 40%

homology. Each of the two halves is believed to have originated from a common single ancestral protein as a result of gene duplication and fusion during the course of evolution (24,26,30). One molecule of transferrin is capable of binding two atoms of iron (Fe^{3+}) (31,34). The binding of each Fe^{3+} also requires the binding of an anion which under physiological conditions is bicarbonate. The bound anion is labile and can exchange slowly with unbound bicarbonate in solution (32,35,36). The two iron binding sites show differences in their physical chemistry. For instance, the two sites differ in their accessibility to iron present in various chemical forms and in their spectroscopic properties (15). Fletcher and Huehns have proposed functional differences in the iron binding sites (37). However, the two iron binding sites of human transferrin have been shown to have no functional differences (38). Iron saturated transferrin has a different conformation from that of apotransferrin being more compact (39-41).

b) Function: The primary role of transferrin is the transport of iron among sites of absorption, storage and utilization. Transferrin is a true carrier molecule which is not degraded during its interaction with iron requiring cells. Transferrin can undergo many cycles or iron transport during its 8 day lifetime (15). The interaction of transferrin with its target tissue is a time, temperature and energy-dependent process which requires receptors on the plasma membrane (42-45). The receptors for transferrin are saturable, specific and bind transferrin reversibly (46). Transferrin receptors have been shown to be involved in iron transport by the cells of the liver, placenta, erythroid and human lymphoblastoid cells (46-51). Transferrin may also have an important role in defence against infection which can be overcome by iron salts and by some microbial iron chelators (50).

The biological function of the carbohydrate component of transferrin molecule still remains unclear. It has been suggested that it may have a recognition function for receptors of non-erythroid cells, but there is no evidence for this (2).

II. Lactoferrin

a) Structure and Properties: Lactoferrin is found in colostrum, milk and other body secretions such as pancreatic juice and small intestinal fluids of several species including man and also in neutrophils (51-57). It is a glycoprotein of molecular weight of about 80,000 and it contains a single polypeptide chain (58-59). Lactoferrin also contains two carbohydrate groups (60). Like transferrin, there is some evidence of internal homology in the structure of lactoferrin (61,62). However, lactoferrin, at least in humans, differs from serum transferrin in its immunological properties, heterosaccharide chain sequences, conformation and localization of iron binding sites (63-65). Lactoferrin binds two Fe^{3+} and like transferrin, requires the concomitant binding of an anion which is probably bicarbonate under physiological conditions. The iron of lactoferrin iron complex dissociates only at pH below 4 (63). On the other hand, transferrin iron complex starts to dissociate its bound iron at pH 6. Finally, like transferrin iron complex, iron saturated lactoferrin has a maximum absorbance between 460 and 465 mu.

b) Function: Iron free lactoferrin in milk and other body secretions has been demonstrated to be bacteriostatic. Lactoferrin binds iron, thus making iron unavailable to microorganisms. This antimicrobial action of lactoferrin is easily reversed by the addition of excess iron (66,67). The lactoferrin contained in neutrophils is also thought to be bacteriostatic.

Neutrophil derived lactoferrin which is normally iron-free may be associated with the hypoferraemia of inflammation (68-72). Lactoferrin in neutrophils has also been suggested to be the granulocyte derived inhibitor of the production of colony-stimulating factor (55-57), because it seems to block production or release of colony-stimulating activity by monocytes.

C. Iron Overload

The body lacks an effective means of excreting iron. Virtually all of the iron taken in by absorption or by transfusion of red blood cells is retained in the body and stored as ferritin or hemosiderin in the reticuloendothelial cells or eventually in the parenchymal cells of tissues. Excess iron accumulates in hepatocytes, cardiac muscle , pancreas and endocrine glands. The accumulation of iron leads to cirrhosis of the liver, congestive heart failure, diabetes mellitus and darking of the skin (71,72). Thus excess iron in the body can lead to severe pathological changes and eventually death. The normal iron content of an adult is about 4 g. However, with iron overload, the total iron in the body can be as high as 90 g.

I. Toxicity of Excess Iron, Mechanisms Involved

a) Free radical intermediates and lipid peroxidation of membranes: Iron has been shown to generate hydroxyl radicals, .OH, which may be produced by the reaction between superoxide and hydrogen peroxide, a reaction which is catalyzed by iron. Hydroxyl radicals, which are strong oxidants, then attack virtually any organic molecule in their search for free electrons. In iron overload, membranes of target cells, particularly mitochondrial and lysosomal membranes have been shown to be damaged (73-75). The sequence of reactions

leading to such membrane damage is known as lipid peroxidation. Two possible mechanisms have been suggested as the cause of lipid peroxidation. First, hydroxyl radicals may induce lipid peroxidation (76,77). Secondly, peroxidation may also be caused by the involvement of membrane phospholipid bilayer in iron-catalyzed oxidative processes (78,80). It is believed that free cellular iron can initiate lipid peroxidation by the formation of perferryl ion (FeO^{2+}) or the formation of a ternary free radical complex between arachidonic acid, ferrous iron and oxygen, leading to peroxidation of the hydrocarbon chain (80).

b) Formation of secondary lysosomes: It is well established that in iron overload, some ferritin and hemosiderin accumulate within membrane bound vesicles or bodies (81). These bodies can be separated by ultracentrifugation and a number of lysosomal enzymes can be identified in this fraction (82,83).

c) Excessive deposition of collagen: Collagen has been shown to accumulate in the liver of very young infants with iron overload but without any evidence of cellular damage (81). It has been suggested that this deposition of collagen is caused by iron directly (84). Iron is believed to cause collagen accumulation by two mechanisms. First, the enzyme protocollagen proline hydrolase requires iron. This enzyme which is involved in hydroxylation of proline to hydroxyproline, is required for the conversion of soluble precursor collagen to mature and insoluble collagen (85). Surplus iron in the body is believed to enhance this conversion process. Secondly, it has been suggested that pericellular collagen is broken down by collagenase released by cells and that a reduction of collagenase would lead to the accumulation of collagen (86). It is believed that the accumulation of iron in lysosomes may somehow prevent the release of collagenase (85).

II. Clinical Forms Of Iron Overload

a) Idiopathic hemochromatosis: Idiopathic hemochromatosis has been shown to be HLA related. Patients with idiopathic hemochromatosis tend to have a significantly higher frequency of HLA-A3, HLA-B7 and HLA-B14 antigen than control population (87). Idiopathic hemochromatosis is believed to be the result of excess absorption of iron by the intestine. Several hypotheses have been put forward to explain the mechanisms involved. It has been suggested that increased affinity of the liver cells for iron may be the cause of idiopathic hemochromatosis (88). Secondly, it is also suggested that abnormal function of transferrin or excessive ferritin biosynthesis may be the cause of idiopathic hemochromatosis. All these hypotheses have been shown to be incorrect (89,90). However, the hypothesis about defective intestinal mucosa appears to have gained recent support. It has been shown that the intestinal mucosa of patients with idiopathic hemochromatosis tend to have high affinity for iron at low concentration (91). In idiopathic hemochromatosis, the iron distribution in the affected organs is predominantly parenchymal. It is generally believed that the accumulation of iron in parenchymal cells may produce tissue damage and the clinical manifestation of hemochromatosis.

b) Secondary hemochromatosis: Secondary hemochromatosis is found mostly in patients with aplastic anemia, beta-thalassaemia major and sickle cell disease. These patients have anemia, ineffective erythropoiesis, increased plasma iron turnover, increased intestinal absorption of iron and excessive iron accumulation. Furthermore, some of these patients need multiple blood transfusions and the iron from transfused red blood cells

cannot be excreted. For every half litre of blood infused, a patient gets about 250 mg of iron. Therefore, a patient with 100 to 200 transfusions will receive 25 to 50 g of iron (92). Repeated transfusions result in the accumulation of iron deposits in reticuloendothelial cells and eventually in parenchymal cells as well.

III. Treatment of Iron Overload

a) Phlebotomy: Repeated phlebotomies can lead to the removal of excess iron and subsequent clinical improvement in patients with idiopathic hemochromatosis (93). The usual procedure is the removal of 500 ml of blood per week. This will lead to depletion of iron in the body. The weekly phlebotomies are stopped when the hemoglobin concentration of the patient falls below 11g/100ml. The iron stores are then monitored by serum ferritin, serum iron concentration and transferrin saturation. Serum ferritin concentration below 10 ug/ml indicates that the patient is approaching iron deficiency. It should be mentioned that the laboratory diagnosis of idiopathic hemochromatosis is indicated by elevated serum iron, increased transferrin saturation, increased serum ferritin and increased parenchymal iron in liver biopsies. In order to prevent reaccumulation of iron in the body, phlebotomies are usually carried out at about 3-month intervals.

b) Desferrioxamine: Phlebotomy cannot be used in patients with secondary hemochromatosis, since these patients need continuous infusion of blood. The only way to treat secondary iron overload is to use iron chelating agents such as desferrioxamine (94-97). Desferrioxamine, introduced in 1960, is a hydroxamic acid compound produced by Streptomyces pilosus (98,99). It has a high affinity for iron (a stability constant of $10^{31}M^{-1}$) and binds iron

to form ferrioxamine which is excreted in urine and feces. However, there are problems with the use of desferrioxamine. First, the drug has to be given by continuous subcutaneous infusion (94). If the drug is given by mouth it is not absorbed by the intestine and with intravenous infusion, the drug is very quickly excreted. Furthermore, desferrioxamine can lead to increased incidence of cataract (100). Another drawback with the use of desferrioxamine is that the drug is very expensive costing about $8,000 per year. Finally, desferrioxamine is usually only partially effective for the treatment of secondary iron overload (101,105).

c) Other chelating agents:

Rhodotorulic acid.

This drug has been shown to be potentially useful (106). Like desferrioxamine, rhodotorulic acid is a naturally occuring hydroxamic acid derivative which must be administered parenterally to be effective. Rhodotorulic acid has been shown to be twice as potent as desferrioxamine on weight basis (107). Furthermore, although the toxicity of rhodotorulic acid appears to be minimal, human subjects have been shown to experience painful local reaction to rhodotorulic acid (108).

Two-three dihydroxybenzoic acid (2,3-DHB).

2,3-DHB which can be given orally has been shown to be effective in removing excess iron from the body (109). However, the drug is known to be toxic and it is not as effective as desferrioxamine (110).

D. Purpose of The Present Study

Neither desferrioxamine nor any other chelating agent is completely satisfactory and therefore there is a need for the development of other methods for the treatment of secondary iron overload. As discussed earlier, phlebotomy is the best treatment for idiopathic hemochromatosis. Loss of blood leads to increased erythropoiesis. Thus, high numbers of immature erythroid cells with transferrin receptors are produced. Iron saturated transferrin then binds to these receptors resulting in the removal of iron from transferrin. This leads to the formation of apotransferrin which in turn picks up more iron from storage sites and transports it to the bone marrow for the formation of hemoglobin in a highly expanded erythron. This results in the depletion of storage iron. Thus with subsequent phlebotomy more and more iron is removed from the body.

This study was undertaken to develop an analogue of phlebotomy as schematically shown in Figure 1. The plan was to develop a system in which iron was removed from plasma transferrin and subsequently from the circulation, leading to an increase in the level of apotransferrin which should mobilize iron from tissues.

At present there are no data available which would enable us to predict whether or not the system described above could remove iron from transferrin in blood at a rate comparable to normal iron turnover. However, simple calculations indicate that such a rate of iron exchange could be achieved. We propose to use a Gambro Lundia Plate dialyzer (priming volume 20 ml) inserted between the carotid artery and jugular vein in the rabbit. Mean blood flow in

the rabbit carotid is about 10 ml/min. One total blood exchange through the dialyzer would be accomplished within 20 min for a total blood volume in rabbit of about 200 ml. This amount of blood contains about 130 ug of iron bound to plasma transferrin. If all the plasma iron is exchanged to apolactoferrin during 20 min, 390 ug or 9.4 mg of iron would be removed from plasma per 1 or 24 hr respectively. This is a maximum estimate which cannot occur in vivo but it represents a 6.2 fold higher rate of iron removal from plasma than is the normal plasma iron turnover (1.5 mg Fe/day/rabbit, calculated on the basis of known plasma iron turnover in humans). Therefore, even if the removal of iron proceeds with 16% efficiency of the maximum estimate, the rate of iron exchange would be comparable to the normal plasma iron turnover.

MATERIALS AND METHODS

All chemicals used were reagent grade and were purchased from commercial suppliers. ^{59}Fe (2 mC/mg Fe) as ferric chloride in 0.1 N HCl was obtained from New England Nuclear and was measured in a Nuclear Chicago automatic gamma counter. Iron-free human transferrin was purchased from Boehringer. Colostrum was obtained from McGill Macdonald College Farm.

A. Isolation of Lactoferrin From Bovine Colostrum

Lactoferrin was isolated from colostrum by a modification of the method described by Parry and Brown (111). Colostrum was centrifuged for 30 min at 2800 rpm (IEC-Centra 7R). Fat which collected at the top of the supernatant was then removed. Centrifugation and removal of fat were repeated two more times. Casein was precipitated by heating the fat-free colostrum to 45° -

50°C with the pH adjusted to 4.5 - 4.6 with glacial acetic acid. After heating, the liquid was filtered through cheesecloth and this was followed by 2 centrifugations for 30 min at 2800 rpm. The precipited casein pellet was discarded. Lactoferrin in the supernatant was saturated with Fe using 5 mM Fe-citrate (10 ml/990 ml of the supernatant). The 5 mM Fe-citrate stock solution contained 20 fold excess citrate over iron. Bicarbonate was added slowly to the liquid to a final concentration of 0.1 M and pH 6.6 - 7.0. The solution was then dialyzed extensively against 0.2 M Tris-HCl buffer, pH 8.0. The lactoferrin was purified using a CM-50 Sephadex column equilibrated with 0.2. Tris-HCL, pH 8.0. Lactoferrin (reddish-brown) was adsorbed to the column which was then washed with 0.2 M Tris-HCl, 0.2 M sodium chloride, pH 8.0 until OD_{280} of the effluent was zero. The adsorbed lactoferrin was then eluted with 0.2 M Tris-HCl, 0.5 M sodium chloride, pH 8.0. The lactoferrin was dialyzed extensively against distilled water and freeze-dried.

B. Preparation of Apolactoferrin

The iron contained in a 1% lactoferrin solution was removed by dialysis against 0.1 M citric acid as described by Masson and Heremans (112).

C. Preparation of ^{59}Fe-labelled Transferrin

Sodium bicarbonate was added to apotransferrin in 10 mM Tris-HCl buffer, pH 7.4 to a final concentration of 0.6 M. ^{59}Fe-citrate which contained 20 fold excess of citrate was slowly mixed with the apotransferrin solution. The final pH was always between 7 and 8. As shown by Bates et al, transferrin-iron complexes form very rapidly in 1:20 iron:citrate solution with excess

bicarbonate (113). After 3 hr standing, unbound ^{59}Fe was removed by overnight dialysis against 3 changes of 10 mM Tris-HCl buffer, pH 7.0. The ^{59}Fe-saturated transferrin was stored at 4°C.

D. Removal of ^{59}Fe From ^{59}Fe-transferrin By Dialysis

It is well established that for the transfer of iron from transferrin to another iron binding protein, such as lactoferrin, a ligand is required (114). A ligand in this context is a low molecular weight iron binding compound with relatively low affinity for iron. Afer a search of the literature, the ligands citrate, nitrilotriacetate and pyrophosphate (synergistic anion) were selected because these have been shown to accelerate iron transfer from transferrin to iron acceptor proteins (115-117).

a) Transfer of ^{59}Fe in 10 mM Tris-HCl buffer: The experimental design was as shown diagrammatically in Figure 2. Two chambers were separated by a dialysis membrane (Cellulose; Sargent Welch S25275-00 K.A.). One chamber contained ^{59}Fe-transferrin (12.5 nM transferrin) while the other chamber contained apolactoferrin (12.5 nM) or just buffer. The proteins were dissolved in 10 mM Tris-HCl buffer at different pH's. Both chambers contained a ligand (citrate or nitrilotriacetate or pyrophosphate) and the final volume of the reaction mixture in each chamber was 1 ml. The iron exchange from ^{59}Fe-transferrin to apolactoferrin (test) or to a solution without apolactoferrin (control) was performed at room temperature. Samples were taken for gamma counts at different time intervals and then returned to the respective chambers. The experiment was repeated using different concentrations and ratios of transferrin and apolactoferrin.

b) Transfer of ^{59}Fe in whole blood: Iron was transferred from ^{59}Fe-transferrin in whole blood to apolactoferrin (dissolved in 0.9% sodium chloride) by using a Gambro pediatric plate dialyzer (Figure 3) having a large surface area (1.25 m^2). The dialyzer required the use of two pumps. A Sigmamotor pump (flow rate, 10 ml/min) was used on the blood compartment side which contained ^{59}Fe-transferrin (12.5 nmoles transferrin, ^{59}Fe- 300 cpm/ml) in whole blood in a final volume of 100 ml. On the dialysate side, a Varistaltic pump (flow rate, 100 ml/min) was used. The dialysate compartment contained 1250 nmoles of apolactoferrin, 10 mM Tris-HCl and 1 mM pyrophosphate all in 0.9% sodium chloride, pH 6.0. The total volume of the dialysate was 500 ml. The containers of the respective reaction mixtures were kept at 37°C in a water bath. The pH of the blood mixture was lowered by allowing the blood to drip through a chamber filled with carbon dioxide which was maintained at a constant pressure of 200 mm Hg. The blood pH was monitored by an in-line pH electrode. At indicated time intervals, samples were taken from the blood compartment for radioactivity determination. After counting, these samples were returned to the blood container.

E. Investigation of The Rate Limiting Factor In The Transfer of Iron From Transferrin To Apolactoferrin By Dialysis

Preliminary studies indicated that the rate of iron transfer by dialysis from iron-saturated transferrin to apolactoferrin was very slow. Therefore, experiments were performed to investigate if diffusion was a limiting factor in the transfer of iron from iron-saturated transferrin to apolactoferrin by dialysis. In these experiments, iron was transferred from iron-saturated transferrin to apolactoferrin by direct contact in solution. This was

achieved by mixing together the two proteins, ^{59}Fe-transferrin (62.5 nmoles transferrin), 625 nmoles apolactoferrin and 1 mM pyrophosphate all in 10 mM Tris-HCl buffer, pH 6.0. The total volume of the reaction mixture was 10 ml. The ^{59}Fe exchange was performed at room temperaure. At different time intervals, samples (1 ml) were taken and mixed with 1 ml of 10 mM Tris-HCl buffer, pH 11.5. The final pH of each new mixture (1 ml of the initial reaction mixture plus 1 ml of the 10 mM Tris-HCl buffer, pH 11.5) was 9.0. This final pH of 9 should have stopped all the transfer of ^{59}Fe from ^{59}Fe-transferrin to apolactoferrin.

After stopping the exchange of ^{59}Fe between ^{59}Fe-transferrin and apolactoferrin, the proteins were separated by cellulose acetate electrophoresis (118). The electrophoresis tank was filled with barbital buffer, pH 8.6 (103 g sodium diethylbarbiturate, 16.4 g diethylbarbituric acid, 6.2 g calcium lactate and 10 g sodium azide). The cellulose acetate strips (Sartorius, #11200, 57X145 BBG) were soaked in 100 ml barbital buffer for 15 min, blotted gently between sheets of filter paper and placed parallel to one another on the electrophoresis tank wicks across the bridges. The glass rod tensioners of the electrophoresis tank were then placed against the outside edges of the bridges to ensure contact between the strips and to hold and strips level taut. Twenty ul samples were applied to the mid-point of each cellulose acetate strip. The tank lid was immediately placed over the tank and the electrical leads of the tank were connected to a power supply. The current on the power supply was adjusted to give 1.5 mA/2 cm width of the cellulose acetate strip. After 90 min of electrophoresis the strips were removed from the tank and placed in 0.2 g Panceau S dye/100 ml of the barbital buffer. Excess dye was removed by gentle blotting of the cellulose acetate strips

between filter papers. As a control, standard ^{59}Fe-transferrin or ^{59}Fe-lactoferrin was applied to two separate cellulose acetate strips and the electrophoresis was carried out as described above. Thus the positions of ^{59}Fe-transferrin and ^{59}Fe-lactoferrin of the reaction mixture (test) could be located on the strips by comparison with the control strips. The two bands on the test cellulose acetate strips were then cut separately and the respective radioactivity on the bands were determined. This was followed by the calculation of the percentage of ^{59}Fe transferred from ^{59}Fe-transferrin to apolactoferrin.

F. Immobilization of Apolactoferrin

a) Binding of lactoferrin to activated sepharose: The binding of lactoferrin to activated sepharose was achieved by a modification of the method described by the manufacturer of the activated sepharose (Pharmacia Fine Chemicals, Uppasala, Sweden) (Figure 4). One gram of activated sepharose (4B) was washed 4 times with 50 ml portions on 10^{-3} M HCl. After each wash, the beads were allowed to settle and the supernatant was removed. The washing with 10^{-3}M HCl was followed by 2 washes with 50 ml portions of coupling buffer (0.1 M sodium bicarbonate, 1 M sodium chloride, pH 8.0). The final supernatant was removed after centrifugation for 2 min at 200 rpm. This was followed by the addition of 10 mg lactoferrin (in 2 ml of the above coupling buffer) to 2 ml of the swollen activated sepharose beads which was shaken overnight, at 4°C.

After the overnight coupling, the sepharose was centrifuged at 200 rpm for 2 min. The volume of the supernatant was measured and the supernatant was set aside for subsequent OD_{280} measurement. The lactoferrin-sepharose beads

were then washed with 4 ml of 0.1 M Tris-HCl buffer, pH 8 at room temperature for 2 hours. This was followed by centrifugation at 200 rpm for 2 min and the supernatant was set aside for subsequent OD_{280} measurement. The lactoferrin-sepharose beads were then washed with additional 0.1 M Tris-HCl buffer, pH 8 (4 ml). The washing was repeated using 4 ml of the following respective solutions, one at a time: solution #1 (0.1 M sodium acetate, 1 M sodium chloride, pH 8.0), solution #2 (0.1 M sodium acetate, 1 M sodium chloride, pH 4.0), solution #3 (10 mM sodium citrate, pH 4.0), solution #4 (0.1 M sodium bicarbonate, 1 M sodium chloride, pH 8.0), solution #5 (0.1 M sodium acetate, pH 8.0) and solution #6 (phosphate buffered saline). Again, the respective supernatant solutions were removed after centrifugation at 200 rpm for 2 min and the solutions were measured at OD_{280}. The washing of lactoferrin-sepharose beads with 10 mM sodium citrate, pH 4.0 leads to the removal of bound iron. Thus, apolactoferrin-sepharose beads were formed. The apolactoferrin-sepharose beads were stored in phosphate buffered saline, pH 7.4 at 4°C.

The amount of bound apolactoferrin per ml of the activated sepharose was determined by calculating the difference between the initial OD_{280} units given by the protein added to the activated sepharose and the total OD_{280} units of unbound lactoferrin in all the various supernatant solutions.

b) Investigation of iron binding capacity of apolactoferrin-sepharose: It is well established that iron can bind to apolactoferrin or apotransferrin in solution (Figure 4). The stoichiometry of the binding of iron to apolactoferrin or apotransferrin is 2 atoms of iron per 1 molecule of protein. It was important to demonstrate that apolactoferrin bound to sepharose could bind iron with the same stoichiometry as free apolactoferrin in solution.

This was measured by adding different quantities of ^{59}Fe citrate (1.7, 7.2, 16.8, 25.6, 34.0 nmoles ^{59}Fe) to 0.5 ml aliquots of apolactoferrin-sepharose beads (different samples). The amount of apolactoferrin bound to the activated sepharose per sample was 8.5 nmoles. Sodium bicarbonate was added to each test-tube to a final concentration of 0.6 M and the buffer used was 10 mM Tris-HCl, pH 7.4. The final volume of the reaction mixture per test-tube was brought to 2 ml with the above buffer. The samples were then incubated at 37°C for 3 hours on a shaker and centrifuged at 200 rpm for 2 min. The supernatants were removed and the ^{59}Fe bound to apolactoferrin-sepharose beads was determined by counting ^{59}Fe radioactivity before and after washing the beads twice with the above buffer containing 10 mM sodium citrate. As a control, non-activated sepharose samples were treated in the same way as described above.

c) Transfer of ^{59}Fe from ^{59}Fe-transferrin to apolactoferrin-sepharose beads: Equal portions (2 ml) of apolactoferrin-sepharose beads (48 nmoles apolactoferrin) in 10 mM Tris-HCl buffer, pH 6.0 were centrifuged for 2 min at 200 rpm and the supernatant was discarded. Then to the apolactoferrin-sepharose beads in each test-tube were added ^{59}Fe-transferrin (0.24 nmoles transferrin in a volume of 19.2 ul) and 1 mM pyrophosphate all in 10 mM Tris-HCl buffer, pH 6.0. The final volume of each reaction mixture per tube was 1 ml and the ratio of apolactoferrin to transferrin was 200:1. The tubes were incubated at 37°C for 1 or 3 hours after which ^{59}Fe transferred from transferrin to apolactoferrin was measured. The ^{59}Fe radioactivity was determined before and after washing the apolactoferrin-sepharose beads with the above buffer. As a control experiment, samples of non-activated sepharose with no bound apolactoferrin were treated as described.

G. The Reductive Release of Iron From Fe^{3+} Transferrin-CO^{2-}

a) Examination of the effect of different combinations of reducing agents (thioglycolate, dithionite) and pyrophosphate: Iron was reductively removed from Fe-transferrin in serum by a modification of the method described by Kojima and Bates (121). Various concentrations of thioglycolate, dithionite and pyrophosphate (as specified in the Results Section) were examined to investigate their affect on the release of Fe^{2+} in the presence of 1 mM bathophenanthroline sulfonate which was used to sequester Fe^{2+}. The concentration of the bathophenanthroline sulfonate always exceeded that of iron by a factor of 3 or greater, since bathophenanthroline sulfonate forms 3:1 bathophenanthroline sulfonate:iron complex (121). The Fe^{2+} bathophenanthroline sulfonate formed was measured at OD_{538} at different time intervals. The amount of Fe^{2+} removed by reduction at any given time was calculated from a standard curve of OD_{538} against known iron concentration (ugFe/ml).

b) Removal of $^{59}Fe^{2+}$-bathophenanthroline sulfonate by dialysis: During the reductive release of iron from Fe-transferrin, the by-products Fe^{2+}-bathophenanthroline sulfonate and oxidized forms of thioglycolate and dithionite are formed and must be removed for our planned in vivo experiments.

To remove these by-products, dialysis was tried with a Gambro plate dialyzer (cellulose membrane). One compartment of the dialyzing unit contained ^{59}Fe-transferrin (200 uM transferrin), 1 mM bathophenanthroline sulfonate and 10 mM dithionite all in 10 mM Tris-HCl buffer, pH 7.4 in a total volume (compartment A) of 100 ml and the pump on this side of the dialyzing

unit was Sigmamotor (flow rate 10 ml/min). The dialyzate compartment (B) contained 10 mM Tris-HCl buffer (500 ml) and the pump on this side of the dialyzing unit was a Varistaltic pump (flow rate 100 ml/min). At different time intervals, samples were taken from compartment A. These samples were returned to the compartment after each radioactivity determination. The removal of $^{59}Fe^{2+}$-bathophenanthroline sulfonate by dialysis was carried out for 3 hours. However, the dialysate was changed every 1 hour.

c) Removal of $^{59}Fe^{2+}$-bathophenanthroline sulfonate by encapsulated activated charcoal: The removal of $^{59}Fe^{2+}$-bathophenanthroline sulfonate by encapsulated charcoal was performed by adding 10 ml of $^{59}Fe^{2+}$-bathophenanthroline sulfonate solution (200 uM Fe) to either 5 or 10 g of encapsulated charcoal. The mixtures were rotated gently on a shaker and at different time intervals the shaker was turned off and the microcapsules were allowed to settle. Aliquots (1 ml) of the supernatant were taken for radioactivity determination. After the ^{59}Fe radioactivity determination, the aliquots were returned to the mixtures. The total radioactivity of $^{59}Fe^{2+}$-bathophenanthroline sulfonate in the supernatant at time zero was taken as 100%.

H. Iron Transfer From Transferrin To Various Iron Chelating Agents

The reaction mixture containd 45 uM transferrin saturated with iron, 4.5 mM of an iron chelating agent and 1 mM or 10 mM pyrophosphate. The final volume of each mixture was 0.2 ml. The buffer used was 0.1 M HEPES, pH 7.4. The mixtures were incubated at 37°C and at various time intervals, samples were taken out and quickly cooled to 4°C. To the cooled samples were added 1 ml bovine serum albumin and 1.5 ml of ice cold 95% ethanol. The samples were

mixed well and left on ice for 15 min and then centrifuged at 300 rpm for 15 min. The distribution of ^{59}Fe between supernatant and precipitate was measured by gamma count. The iron chelating agents examined were desferrioxamine, tropolone, catechol and ethylenediamine di-(O-hydroxyphenyl acetic acid).

We also had to demonstrate that ^{59}Fe complexed to the above chelating agents did not coprecipitate with transferrin and bovine serum albumin by 95% ethanol. Therefore, a separate experiment was performed to investigate the solubility of iron-chelating agent complex in ice cold 95% ethanol. The reaction mixture contained iron-citrate (50 uM Fe), 4.5 mM of a chelating agent and 1 mM or 10 mM pyrophosphate all in 0.1 M HEPES buffer, pH 7.4. The respective maximum absorbances of the various iron-chelating agent complexes were then determined on a spectrophotometer. This was followed by comparing the OD units (at maximum wavelength) of the various iron-chelating agent solutions, this time, diluted 1 in 2 with either 0.1 M HEPES buffer or ice cold 95% ethanol. The results demonstrated that ice cold 95% ethanol did not precipitate the various iron-chelating agent complexes.

<u>^{59}Fe Exchange To Ethylenediamine di-(O-hydroxyphenyl acetic acid) Using ^{59}Fe-transferrin In Whole Blood or 0.1 M HEPES.</u>

<u>Transfer of ^{59}Fe in whole blood</u>

700 ul of ^{59}Fe-transferrin (200 uM Fe. 150 uM transferrin, i.e. iron concentration was 10 times higher than normal plasma iron concentration) was added to 15.05 ml of blood (defibrinated, which was taken from normal non-anemic rabbit). The mixture was mixed well and kept on an ice bath. 450 ul of the above mixture was used in the reaction mixture for the transfer of ^{59}Fe to ethylenediamine di-(O-hydroxyphenyl acetic acid).

Transfer of ^{59}Fe in 0.1 M HEPES buffer, pH 7.4

200 ul of ^{59}Fe-transferrin solution (as described above) was added to 4.3 ml of 0.1 M HEPES buffer. The mixture was mixed well and kept on an ice bath. 450 ul of this ^{59}Fe-transferrin and HEPES buffer mixture was used in the reaction mixture for the transfer of ^{59}Fe to ethylenediamine di-(O-hydroxyphenyl acetic acid).

With the experiments involving whole blood, the reaction mixture per sample contained ^{59}Fe-transferrin in blood, different concentrations of ethylenediamine di-(O-hydroxyphenyl acetic acid) and pyrophosphate all in 0.9% sodium chloride solution (as specified in the Results Section). The samples were incubated for 10, 30 and 60 min at 37°C. The tubes were taken out at the various time intervals and were quickly put on ice bath and 1 g of activated encapsulated charcoal was added to the tubes. The supernatant was removed after centrifugation for 2 min at 200 rpm. The encapsulated activated charcoal was washed twice with 0.9% sodium chloride solution and the radioactivity in both supernatant and charcoal was determined. The whole procedure was repeated using ^{59}Fe-transferrin in 0.1 M HEPES buffer, pH 7.4, instead of ^{59}Fe-transferrin in blood. However, the same concentration of ethylenediamine di-(O-hydroxyphenyl acetic acid) (500 uM) was used and the encapsulated activated charcoal after centrifugation was washed with 0.1 M HEPES buffer. A prior study had indicated that activated encapsulated charcoal could bind and remove ^{59}Fe-ethylenediamine di-(O-hydroxyphenyl acetic acid) from solution.

RESULTS AND DISCUSSION

A. Removal of ^{59}Fe From ^{59}Fe-transferrin By Dialysis

These experiments were performed to investigate the optimal conditions for the exchange of iron between transferrin and apolactoferrin by dialysis. The conditions investigated were pH, the presence of low molecular weight ligands (citrate, nitrilotriacetate, pyrophosphate) and different ratios of transferrin to apolactoferrin.

The experiments were performed as described under Materials and Methods (Section D (a) page 27). As shown in Table 1, some exchange of iron from iron-saturated transferrin can be demonstrated at pH 7.5. But the rate of transfer of iron was stimulated when the pH of the buffer was decreased to 6.0. The finding that apolactoferrin can take up iron from iron-saturated transferrin was in agreement with a study by Van Snick and colleagues who demonstrated that at pH 7.3 about 50% of the iron bound to transferrin is taken up by apolactoferrin within one hour (69). Furthermore, the finding that the transfer of iron from iron-saturated transferrin was stimulated at pH 6.0 was in agreement with a study by Laurell who showed that transferrin iron dissociates from transferrin-iron complex at pH 4-6 (123). It should be mentioned that the binding of each iron atom by transferrin leads to the displacement of three hydrogen ions from the transferrin molecule and at the same time one bicarbonate anion binds. Thus at low pH, iron dissociates readily from iron-transferrin complex (124,125).

Our results also indicated the importance of a low molecular weight ligand in the transfer of iron from iron-transferrin to apolactoferrin. In the absence of a ligand, considerably less iron was transferred to apolactoferrin. Of the various ligands examined in this study, pyrophosphate gave the highest amount of iron transfer to apolactoferrin (Figure 5). The transfer of iron from iron-transferrin to an acceptor is believed to be impeded by a kinetic barrier which can be removed by a low molecular weight ligand (114). It has been suggested that the low molecular weight ligand overcome the kinetic barrier to iron transfer by substituting for bicarbonate in the iron-transferrin-bicarbonate complex. The iron-ligand complex is then separated from the transferrin and the iron is carried to the iron acceptor protein or compound by the ligand (114,126,127). Thus the iron-ligand complex acts as an intermediate in the transfer of iron, as shown in Reaction I.

Reaction I

The substitution and mediation of a low molecular weight ligand in the transfer of iron, a modified scheme (114).

1. $Fe^{3+}Tf - CO_3^{2-}$ + Ligand $\rightleftharpoons$ Fe^{3+} Tf - Ligand + CO_3^{2-}
2. $Fe^{3+}Tf$ - Ligand $\rightleftharpoons$ ApoTf + Fe-Ligand
3. Fe-Ligand + ApoLf $\rightleftharpoons$ Fe^{3+} Lf + Ligand

Our finding that pyrophosphate gave the highest amount of iron transferred from iron-transferrin to apolactoferrin confirmed published results in which iron was transferred from iron-saturated transferrin to desferrioxamine in direct contact in 0.1 M HEPES buffer, pH 7.4 and with or without various ligands. The transfer of iron was monitored at 37°C. Twenty-nine various low

molecular weight ligands including pyrophosphate were examined and pyrophosphate was shown to give the highest percentage of iron transfer, 94% of the initial iron was transferred to desferrioxamine in 30 min. On the other hand, in the absence of pyrophosphate, only 5% of the initial iron was transferred to desferrioxamine in 30 min (116).

Our results also indicated that the transfer of iron from iron-saturated transferrin to apolactoferrin depends on the ratio of transferrin to apolactoferrin. There was increased iron transfer with increased concentration of apolactoferrin (Table 1). This may be a simple mass law effect.

Finally, the results indicated that it was possible to transfer iron from iron-saturated transferrin to apolactoferrin by dialysis and thus form apotransferrin in the process. However, the rate of iron transfer was too slow to be of any practical use. The transfer of iron by dialysis took 24 hours to reach equilibrium (Figure 5). The rate of iron transfer observed in this experiment was too slow in comparison with other studies in which the transfer of iron from transferrin to an iron acceptor was performed in direct contact in solution. In such a study, iron transfer has been shown to reach equilibrium in 30 min (116). Our finding that the rate of iron transfer from iron-transferrin to apolactoferrin by dialysis was slow suggested that diffusion may be a limiting factor in the exchange of iron. However, it should be mentioned that the presence of apotransferrin might have slowed down the rate of iron transfer, assuming equilibrium across the dialysis membrane. There is also a possibility that some of the transferrin might have been denatured during prolonged incubation which may be expected to slow down the removal of iron.

Transfer of ^{59}Fe from ^{59}Fe-transferrin in whole blood to apolactoferrin separated by dialysis.

Previous results (see Table 1) indicated that it was possible to transfer iron from iron-saturated transferrin to apolactoferrin in 10 mM Tris-HCl buffer by dialysis.

The experiments under this section were performed to investigate the feasibility of transferring iron from iron-saturated transferrin in whole blood to apolactoferrin (in 0.9% sodium chloride) by dialysis. With iron-saturated transferrin in whole blood, it was felt that non-transferrin plasma proteins may somehow interfere with the transfer of iron to apolactoferrin by dialysis. [The experiments were performed as described under Materials and Methods, Section D(b) page 28].

The results indicated that it was also possible to transfer iron from iron-saturated transferrin in whole blood to apolactoferrin by dialysis (Table 2). However, in addition to the fact that the rate of iron transfer was slow, the percentage of iron transferred (22% of the initial ^{59}Fe in 3 hours) was considerably lower than the percentage (43% of the initial ^{59}Fe in 3 hours) transferred when iron-saturated transferrin was in 10 mM Tris-HCl buffer (Tables 1 and 2). The slower rate of iron transfer when iron-saturated transferrin was in whole blood suggested that some substances or compounds of whole blood may somehow bind some iron after its release from iron-saturated transferrin. Hence, considerably far less iron was available for transport across the dialysis membrane to the apolactoferrin. For example, rabbit reticulocytes, which may represent up to 1% erythrocytes, are known to take up iron

from transferrin and may decrease the amount of iron available for transport across the dialysis membrane.

B. Transfer of ^{59}Fe From ^{59}Fe-transferrin To Apolactoferrin By Direct Contact In Solution

To find out if diffusion was a limiting factor in the exchange of iron to apolactoferrin by dialysis, the transfer of iron was performed under conditions in which iron-saturated transferrin and apolactoferrin were in direct contact in solution. (These experiments were performed as described under Materials and Methods Section E page 28). After the iron exchange, the two proteins were separated by cellulose acetate electrophoresis and the distribution of ^{59}Fe between transferrin and lactoferrin was determined. A separate control electrophoresis run with a mixture of ^{59}Fe-transferrin and ^{59}Fe-lactoferrin applied to cellulose acetate strips indicated that there was no exchange of ^{59}Fe between the two proteins during the electrophoresis.

The results suggested that by the direct contact of ^{59}Fe-transferrin and apolactoferrin in solution, there was an immediate transfer of ^{59}Fe to apolactoferrin in solution. Ninety-two percent of the initial ^{59}Fe bound to transferrin was transferred to apolactoferrin in 30 min (Table 3) suggesting that the transfer of iron from iron-saturated transferrin to apolactoferrin by dialysis may be limited by diffusion. A comment should be made on our observation that the transfer of iron from ^{59}Fe-transferrin to apolactoferrin was not progressive with time. This may be due to the fact that at low pH (6.0), ^{59}Fe-transferrin (80% saturation) released most of its bound iron instantaneously when a more powerful iron-binding protein, apolactoferrin, was available as a sink for the released iron.

C. Investigation Of The Iron-binding Capacity Of Apolactoferrin Bound To Activated Sepharose

We have previously shown that iron can be transferred instantaneously from iron-saturated transferrin to apolactoferrin if the two proteins were in direct contact in solution (see Table 3). Apolactoferrin was bound to activated sepharose as described under Materials and Methods [Section F (a) page 30]. The reason for having apolactoferrin bound to activated sepharose was to pass ^{59}Fe-transferrin through a chamber containing apolactoferrin-sepharose and thus separate the formed ^{59}Fe-lactoferrin-sepharose from iron-free transferrin.

To demonstrate that apolactoferrin bound to activated sepharose can bind iron with the same stoichiometry as free apolactoferrin, the iron-binding capacity of apolactoferrin bound to activated sepharose was measured. Briefly, iron in the form of ^{59}Fe-citrate (in different concentrations) was incubated with apolactoferrin-sepharose (equal amounts but different samples) in the presence of 0.6 M sodium bicarbonate. After 3 hours of incubation at 37°C, radioactivity of ^{59}Fe was determined before and after washing the lactoferrin-sepharose beads with 10 mM Tris-HCl containing 10 mM sodium citrate. The results (Figure 6) showed that at low concentration of iron, few iron binding sites of apolactoferrin-sepharose were occupied and that with increasing concentration of iron, there was a rise in the iron bound to apolactoferrin-sepharose until saturation was reached. After saturation,

increased concentration of iron did not increase the amount of iron bound to apolactoferrin-sepharose, thus a hyperbolic curve was obtained (Figure 6, curve #3). However, the expected level of iron binding to apolactoferrin-sepharose was not achieved (Figure 6, curve #1). Some of the bound apolactoferrin may have been inactivated during the binding of lactoferrin to the activated sepharose. Furthermore, some iron binding sites of apolactoferrin bound to sepharose may have been hidden within the sepharose beads. The results also suggested that there was specific binding of iron to apolactoferrin-sepharose. Even after washing the lactoferrin-sepharose beads with 10 mM Tris-HCl buffer containing sodium citrate, ^{59}Fe remained bound (Figure 6, curves #2, #3). On the other hand, after the washing of the non-activated sepharose beads with the above buffer, almost all the ^{59}Fe on these beads came off (Figure 6, curves #4, #5).

Our finding that iron can bind apolactoferrin-sepharose complex stoichiometrically was in agreement with the well established observation that free apolactoferrin binds iron in solution stoichiometrically. Evidence for this stoichiometric binding of iron to free apolactoferrin in solution was given by a study by Masson and Heremens (112). In their study, different concentrations of iron in the form of ferrous ammonium sulfate (stabilized by ascorbic acid) were incubated with free apolactoferrin in 5 mM phosphate buffer, pH 7.6. The binding of iron to free apolactoferrin was then monitored by absorbance at 450 mu. The results showed that there was initial rise of the saturation curve followed by a nearly horizontal segment. This curve was similar to the hyperbolic curve obtained in our study. Our overall results (Figure 6) indicated that there was specific stoichiometric binding of iron to

apolactoferrin-sepharose complex and that iron can bind the complex with the same stoichiometry as iron binds free apolactoferrin in solution.

Transfer of ^{59}Fe from ^{59}Fe-transferrin to apolactoferrin-sepharose

The transfer of ^{59}Fe from transferrin to apolactoferrin-sepharose was measured as described [Section F(c) page 32]. Basically, ^{59}Fe-saturated transferrin was incubated at 37°C with apolactoferrin-sepharose in the presence of pyrophosphate for 1 or 3 hours. After the incubation, the ^{59}Fe transferred to apolactoferrin-sepharose was determined before and after washing the lactoferrin-sepharose with 10 mM Tris-HCl buffer. The results indicated that 93% of the initial ^{59}Fe was transferred from ^{59}Fe-transferrin to apolactoferrin-sepharose in 1 or 3 hours (Table 4). On the other hand, in the control experiment in which non-activated sepharose beads (sepharose without any bound apolactoferrin) was used, only 2% of the initial ^{59}Fe was transferred from ^{59}Fe-transferrin to the non-activated sepharose in 1 or 3 hours (Table 4).

These results suggested that it was possible to achieve a significant and rapid transfer of iron from iron-saturated transferrin to apolactoferrin bound to activated sepharose.

D. Reductive Release of Iron From Fe^{3+} Transferrin - CO_3^{2-}

There is no specific binding of ferrous iron to apotransferrin (119,120). Thus with the reduction of Fe-transferrin, ferrous iron is released and iron-free transferrin is formed. The reaction route that appears to be operative in the reductive release of iron from transferrin is shown in reaction II.

Reaction II

Reductive release of iron from Fe^{3+} transferrin - CO_3^{2-}:

1. Fe^{3+} Tf - CO^{2-} + Rd $\rightleftharpoons$ Fe^{2+} Tf - CO^{2-} + OX
2. Fe^{2+} Tf - CO^{2-} + $\rightleftharpoons$ Fe^{2+} + ApoTf-CO^{2-}
3. Fe^{2+} + nBPS $\rightleftharpoons$ Fe^{2+} $(BPS)_n$ measured at OD_{538}

Where Rd = Reducing agent

OX = Conjugate oxidized form of the reducing agent

BPS = Bathophenanthroline sulfonate (This is a chromogenic Fe^{2+} acceptor)

The following set of experiments was undertaken to investigate the reductive release of iron from iron-saturated transferrin by various reducing agents (dithionite, thioglycolate) and pyrophosphate which were used alone or in different combinations to examine their possible synergistic effects. Briefly, iron was reductively released from Fe-transferrin and the released iron was sequestered by excess bathophenanthroline sulfonate. The trapped iron in the form of Fe^{2+} bathophenanthroline sulfonate was then determined spectrophotometrically. The total serum iron was measured by a standard colorimetric method described by Boehringer Mannheim Diagnostica. Portions of standard iron-citrate (0.5 ml, concentration range; 0.2 to 10 ugFe/ml) were added to 1.5 ml portions of reagent mixture (20 ml of detergent solution containing 600 mg sodium dithionite). The samples were then mixed well and their absorbances at OD_{538} before and after adding 20 ul of 17 mmol/l bathophenanthroline sulfonate were measured. As a reagent blank, 0.5 ml of redistilled water was used in place of a standard iron-citrate solution. The absorbance at OD_{538} was given by the following equation:

(A_2 sample - A_1 sample) - (A_2 RB - A_1 RB) = Change in OD_{538}

Where A_1 sample = OD_{538} value of a given sample before the addition of bathophenanthroline sulfonate

A_2 sample = OD_{538} value of A_1 sample after the addition of bathophenanthroline sulfonate

A_1 RB = OD_{538} value of the reagent blank before the addition of bathophenanthroline sulfonate

A_2 RB = OD_{538} value of A_1 RB after the addition of bathophenanthroline sulfonate

A graph of change absorbance at OD_{538} values against their respective iron concentration (ugFe/ml) was then plotted.

The results indicated that 36% of the initial iron bound to transferrin was reductively released by 10 mM thioglycolate in 60 min. However, using a combination of 10 mM thioglycolate and 26 mM dithionite, 53% of the initial iron was reductively released in 60 min (Table 5A). With 26 mM dithionite as the reducing agent, 40% of the initial iron was reductively released from iron-saturated transferrin in 60 min. On the other hand, with a combination of 26 mM dithionite and 10 mM pyrophosphate, 76% of the initial iron was released from iron-saturated transferrin in 60 min (Table 5B). These results indicated that a combination of a reducing agent and pyrophosphate enhanced the rate of iron release from iron-saturated transferrin. This finding of the synergistic effect of the above agents was in agreement with a previous study by Kojima and Bates (121).

These results therefore suggested that the reduction of iron-saturated transferrin can be used to form apotransferrin in serum at physiological pH.

Removal of $^{59}Fe^{2+}$- bathophenanthroline sulfonate by dialysis after the reductive release of iron from $^{59}Fe^{3+}$- transferrin

For the reductive release of iron from iron-saturated transferrin, a reducing agent (dithionite or thioglycolate), pyrophosphate and excess bathophenanthroline sulfonate are required. The reaction sequence of the reductive release of iron leads to the formation of iron-free transferrin and the by-products, Fe^{2+}-bathophenanthroline sulfonate and oxidized forms of the reducing agents which must be separated from iron-free transferrin.

The removal of one of these compounds, $^{59}Fe^{2+}$- bathophenanthroline sulfonate by dialysis using Gambro plate dialyzer was investigated. The experiment was performed as described under Materials and Methods [Section G(b) page 33]. The removal of $^{59}Fe^{2+}$- bathophenanthroline sulfonate from one compartment of the Gambro dialyzer to a dialysate was monitored by measuring the radioactivity in samples from the $^{59}Fe^{2+}$- bathophenanthroline sulfonate containing compartment. Table 6 shows that only 26.8% of the initial $^{59}Fe^{2+}$- bathophenanthroline sulfonate was transferred to the dialysate in 3 hours. Therefore, other means of removing $^{59}Fe^{2+}$- bathophenanthroline sulfonate from the solution were investigated.

Removal of $^{59}Fe^{2+}$- bathophenanthroline sulfonate by encapsulated activated charcoal

The possibility of removing $^{59}Fe^{2+}$- bathophenanthroline sulfonate by encapsulated activated charcoal was examined. The encapsulated activated charcoal technology was developed at McGill University by Dr. Chang. Encapsulated activated charcoal are microcapsules of activated charcoal surrounded by

cellulose acetate membrane (122). A small volume of these microcapsules has a large surface area. The microcapsules allow low molecular weight compounds to cross its thin membrane and these small compounds are then trapped by the activated charcoal. On the other hand, large molecular weight compounds cannot cross the membrane of the microcapsules.

Initially, the $^{59}Fe^{2+}$- bathophenanthroline sulfate binding capacity of encapsulated activated charcoal was investigated. Different concentrations of $^{59}Fe^{2+}$ bathophenanthroline sulfonate (range 200 - 3000 nmoles ^{59}Fe) were added to encapsulated activated charcoal (0.5 g different samples). The various samples were then rotated gently on a shaker for 30 min at room temperature. After shaking, the encapsulated activated charcoal was allowed to settle and the amount of $^{59}Fe^{2+}$ bathophenanthroline sulfonate trapped by the charcoal were determined before and after washing the charcoal with 10 mM Tris-HCl buffer, pH 6.0. The results indicated that with increasing amounts of $^{59}Fe^{2+}$ bathophenanthroline sulfonate, there was corresponding increasing amounts of $^{59}Fe^{2+}$ bathophenanthroline sulfonate trapped by the encapsulated charcoal until a saturation level was reached (Figure 7). Figure 7 also shows that the washing of the encapsulated charcoal with the above buffer did not remove the trapped $^{59}Fe^{2+}$ bathophenanthroline sulfonate. The iron binding capacity was found to be 520 nmoles ^{59}Fe/0.5 g of encapsulated activated charcoal (Figure 7). The rate of removal of $^{59}Fe^{2+}$- bathophenanthroline sulfonate by encapsulated activated charcoal was determined as described under Materials and Methods. We demonstrated that 99% of the initial $^{59}Fe^{2+}$- bathophenanthroline sulfonate can be removed in 10 min by 5 g or 10 g of encapsulated activated charcoal (Table 7). Therefore, encapsulated activated charcoal proved to be effective in the removal of $^{59}Fe^{2+}$ bathophenanthroline sulfonate complex.

E. The Transfer of Iron From Fe-transferrin To Various Chelating Agents

This study was undertaken to investigate the transfer of ^{59}Fe from Fe-transferrin to iron chelating agents in the presence of pyrophosphate. It was believed that if such a transfer of iron was feasible, then the technique may be used to remove iron from transferrin in serum or whole blood and thus form iron-free transferrin. Encapsulated activated charcoal could then be used to remove pyrophosphate and iron bound to chelators from the blood. The iron chelating agents investigated were desferrioxamine, tropolone, catechol and ethylenediamine di-(O-hydroxyphenyl acetic acid) which all have high affinities for iron. Their respective stability constants for iron are: $10^{31}M^{-1}$ (desferrioxamine), $10^{32}M^{-1}$ (ethylenediamine di-(O-hydroxyphynel acetic acid), $10^{16}M^{-1}$ (tropolone) and $10^{12}M^{-1}$ (catechol). Although catechol does not have very high affinity for iron, it was selected because it is also a reducing agent.

At different time intervals, we determined the transfer of ^{59}Fe from ^{59}Fe-transferrin to the above chelating agents. At the end of the various ^{59}Fe exchanges, bovine serum albumin and ice cold ethanol were added to all the samples. Bovine serum albumin was used as a carrier and ethanol to precipitate the proteins. In a control experiment, we demonstrated that ^{59}Fe removed from transferrin, before the precipitation of transferrin, remained in the supernatant bound to a chelating agent.

From all the chelating agents tested, ethylenediamine di-(O-hydroxyphenyl acetic acid) showed the highest rate of ^{59}Fe removal from transferrin (Table

8). After 60 min, 82% of the initial ^{59}Fe was found to be bound to ethylenediamine di-(O-hydroxyphenyl acetic acid). The percentage of the initial ^{59}Fe transferred to tropolone, desferrioxamine and catechol were 23%, 13% and 2% respectively (Table 8). The results confirmed the reported high affinity of ethylenediamine di-(O-hydroxyphenyl acetic acid) for iron (128). However, the percentage of ^{59}Fe transferred to desferrioxamine was very low compared to reports in the literature and we do not have any explanation for this discrepancy. Based on these results, ethylenediamine di-(O-hydroxyphenyl acetic acid) was selected for further studies.

Iron exchange from transferrin to ethylenediamine di-(O-hydroxyphenyl acetic acid) in whole blood:

The experiments were performed as described under Materials and Methods (Section H page 35). The results showed that the highest ^{59}Fe exchange (73% in 60 min) was achieved with 100 uM ethylenediamine di-(O-hydroxyphenyl acetic acid) and 10 mM pyrophosphate in the reaction mixture (Table 9). However, the rate of iron exchange was considerably lower than that of the situation in which iron-saturated transferrin, ethylenediamine di-(O-hydroxyphenyl acetic acid) and pyrophosphate were all in 0.1 m HEPES buffer (the rate was 97.3% in 60 min). The reason as to why less iron was transferred to ethylenediamine di-(O-hydroxyphenyl acetic acid) in whole blood may be due to the fact that other cations from the whole blood may become bound to ethylenediamine di-(O-hydroxyphenyl acetic acid) and thus decrease its potential to bind iron. Furthermore, the plasma proteins may somehow interfere with the transfer of iron to ethylenediamine di-(O-hydroxyphenyl acetic acid) in an unknown way.

These results suggested that it was possible to transfer iron from iron-saturated transferrin to ethylenediamine di-(O-hydroxyphenyl acetic acid) in whole blood even though the rate of iron exchange was lower than that obtained with the reaction mixture in 0.1 M HEPES buffer, pH 7.4.

CONCLUSION AND PLAN FOR FURTHER STUDIES

The findings in the present study suggest three possible ways of removing iron from transferrin to form iron-free transferrin.

First, in the presence of a low molecular weight ligand and low pH, iron can be transferred from transferrin to apolactoferrin separated by a dialysing membrane. However, the transfer of iron is much faster if apolactoferrin is immobilized on activated sepharose and the iron exchange is performed in the presence of pyrophosphate at pH 6.0 - 7.4. Further study is required to investigate the feasibility of removing iron from transferrin in whole blood using the apolactoferrin-sepharose complex. Hemoperfusion with small diametre sepharose beads is known to cause extensive hemolysis. On the other hand, hemoperfusion with large diametre (1000 - 10,000 microns) sepharose beads has been shown to be feasible (129). The diametre of the sepharose beads used in our study was 60 - 140 microns. Enlarged sepharose beads have been shown to be hemocompatible. Furthermore, platelet loss is less and hemolysis is also only slight in hemoperfusion with large diametre sepharose (129).

The second finding in the present study indicates that apotransferrin can also be formed by reductive removal of iron from transferrin. This finding confirms reports in the literature (121). The removal of iron by reduction

requires the use of reducing agents (dithionite, thioglycolate), pyrophosphate and an iron sequestering compound (bathophenanthroline sulfonate). The by-products of the reductive release of iron, Fe^{2+} bathophenanthroline sulfonate, oxidized forms of the reducing agents and pyrophosphate (after acting as a synergistic anion in the iron exchange) are all easily removed by encapsulated activated charcoal.

Finally, apotransferrin can also be formed by transferring iron from transferrin to various chelating agents. Of the chelating agents examined, ethylenediamine di-(O-hydroxyphenyl acetic acid) was shown to be the agent which gave the highest rate of iron exchange from transferrin.

In the present study, we have investigated the optimal conditions for the formation of apotransferrin in vitro. The plan for the future is to adapt these conditions in order to perform in vivo studies of the mobilization of iron from storage sites by apotransferrin. We hope that learning more about the physiological factors involved in iron mobilization from storage sites by apotransferrin may lead to the development of a more effective therapy for secondary iron overload.

Figure 1

How exchange system might serve as an analogue of phlebotomy

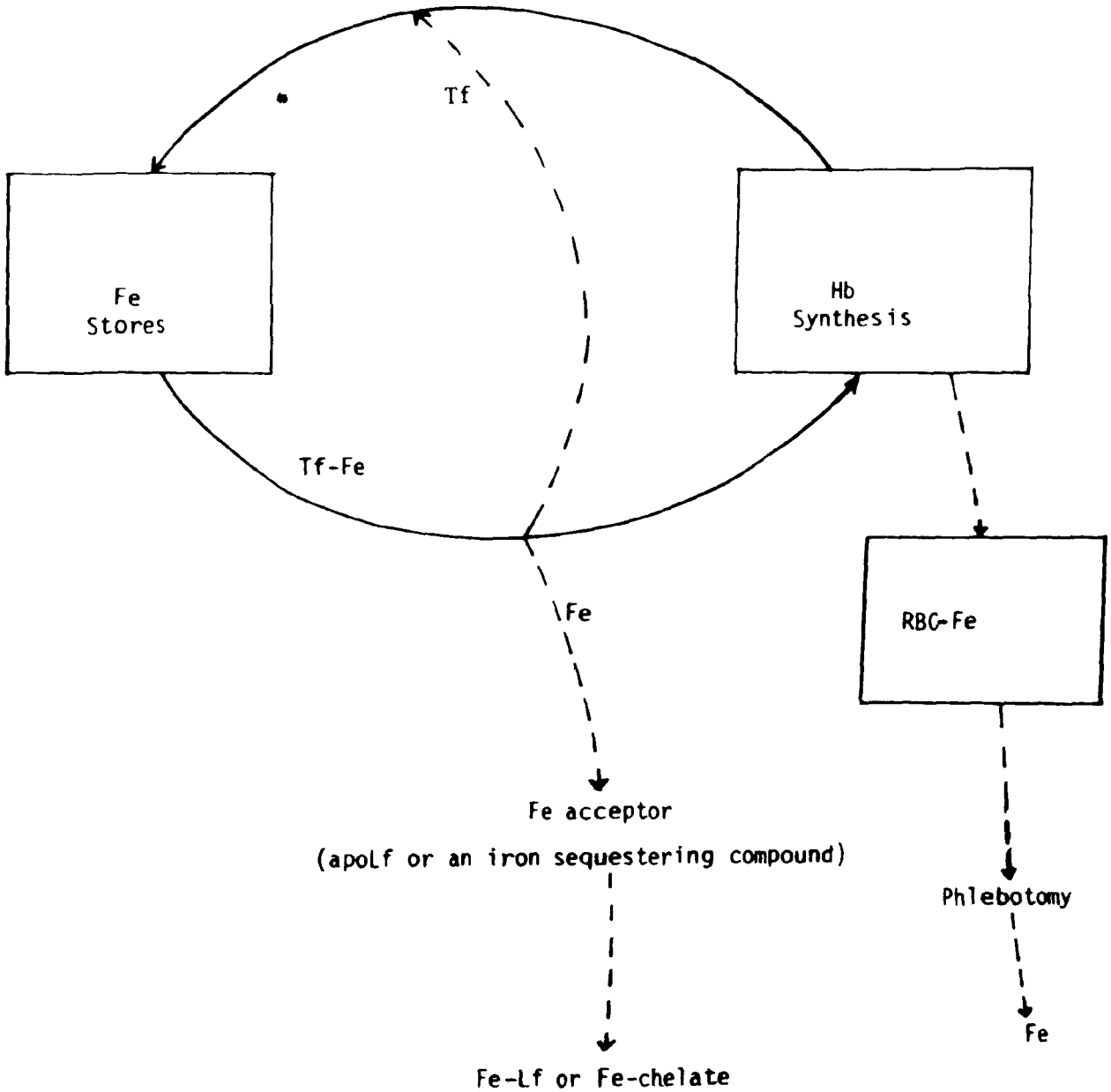


Figure 2

A Design of Dialysis Experiments.

Transfer of ^{59}Fe from ^{59}Fe-transferrin to apolactoferrin in 10 mM Tris-HCl Buffer.

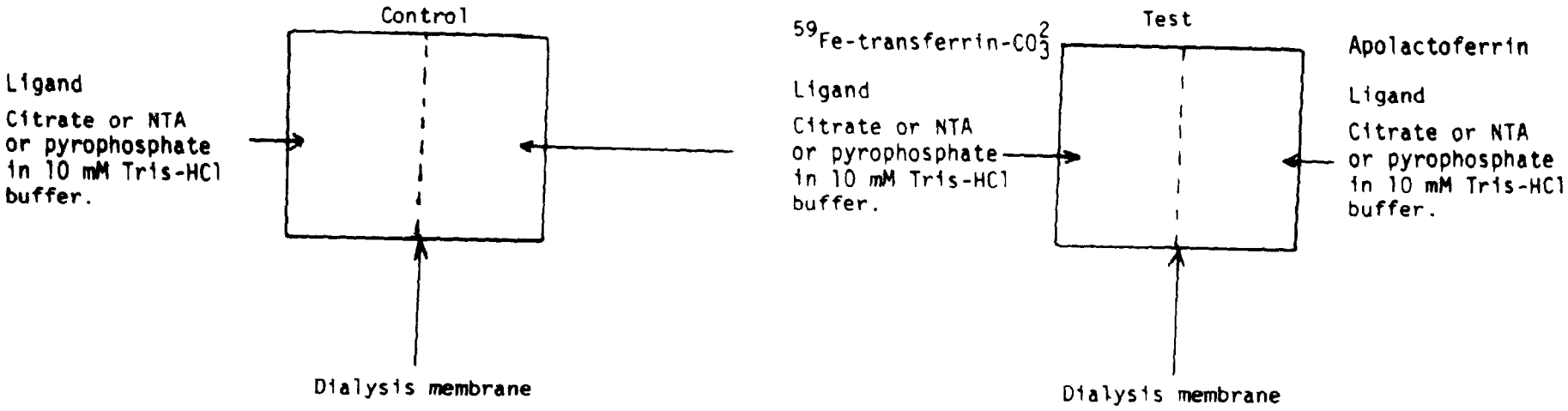
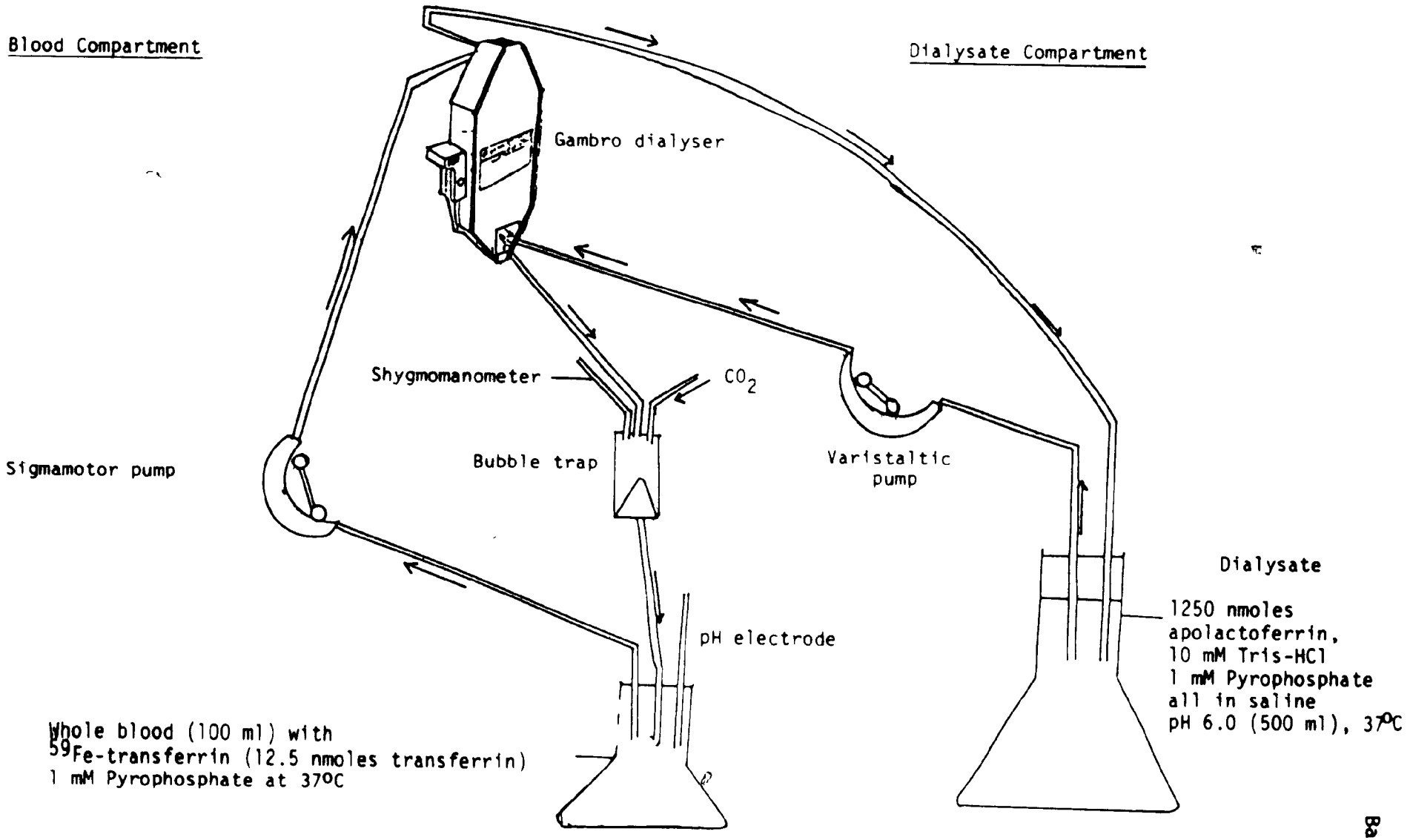


Figure 3

A Design of Dialysis Experiment

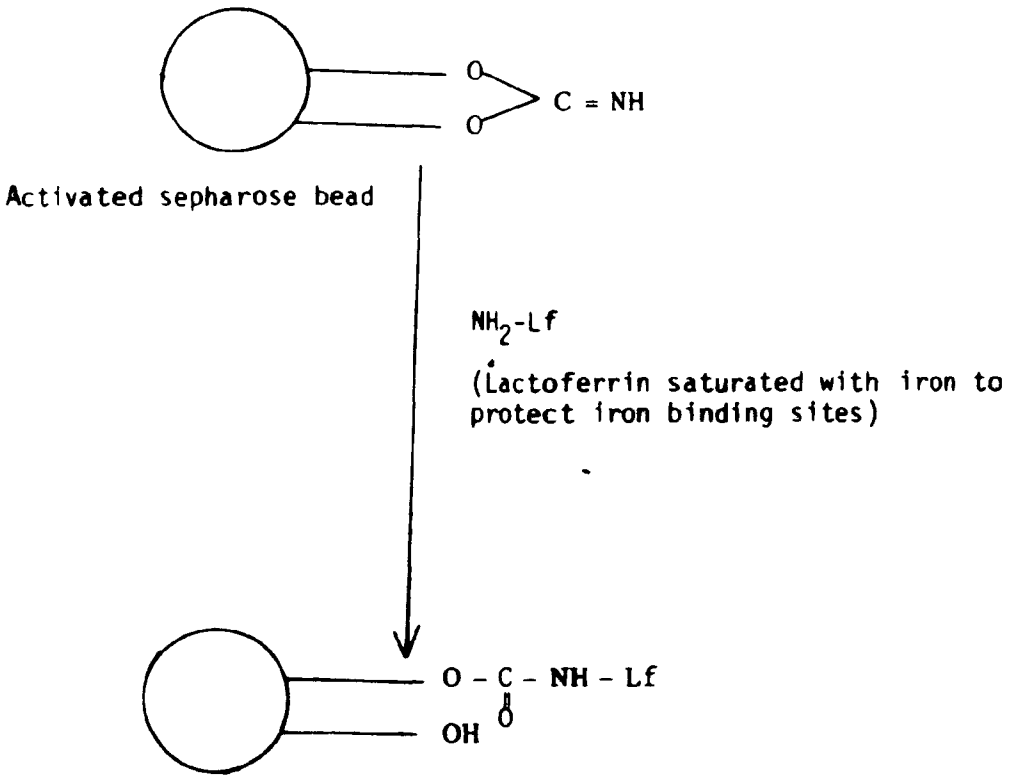
Transfer of ^{59}Fe from ^{59}Fe-transferrin in whole blood to apolactoferrin using Gambro plate dialyser.



Baffour, R. 55

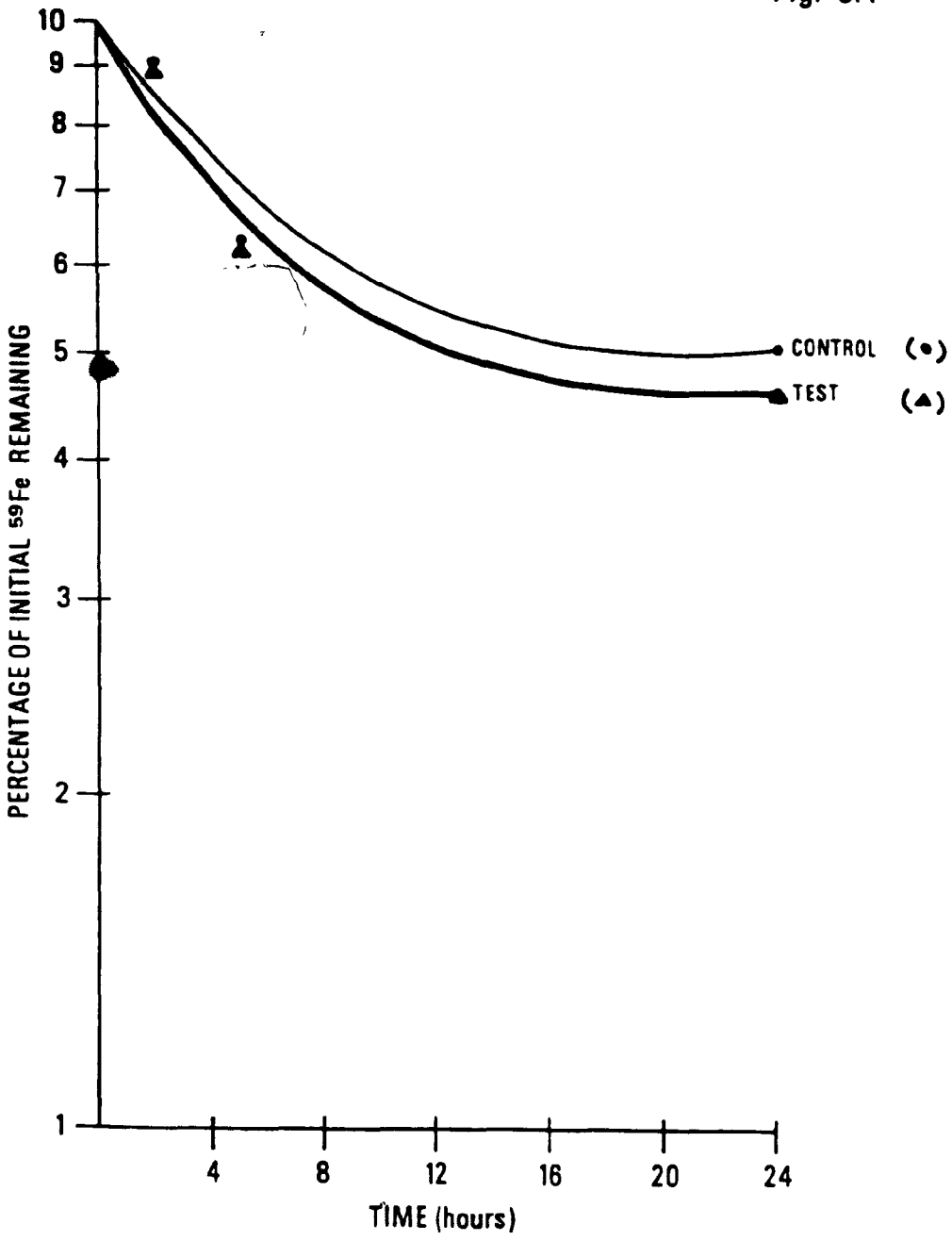
Figure 4

Binding of Lactoferrin to Sepharose



Sepharose-Lactoferrin complex

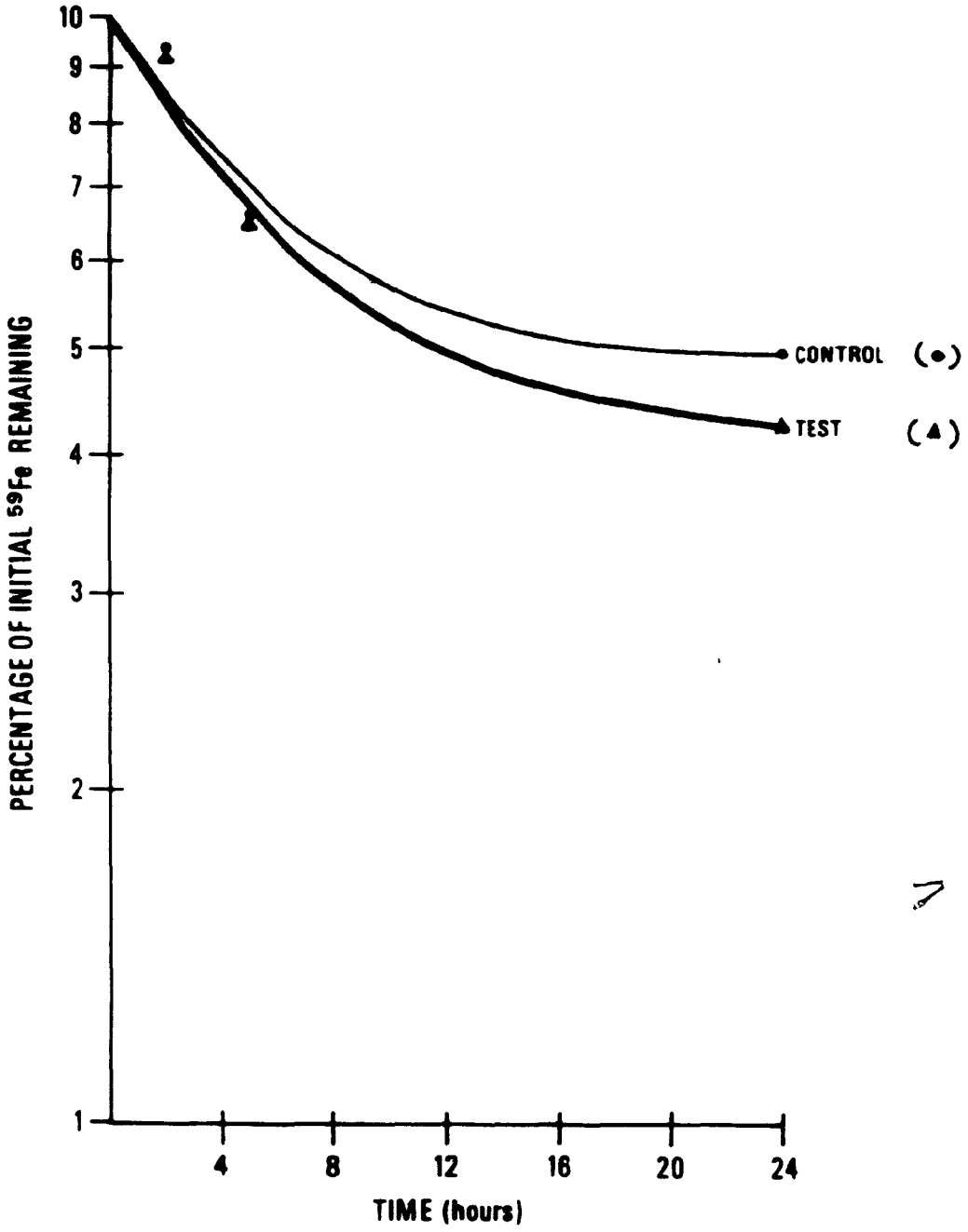
Fig. 5A



The maximum transfer of ^{59}Fe by dialysis.
Control samples contained no apolactoferrin in the dialysis chamber.
Test samples contained apolactoferrin in the dialysis chamber.

Ligand, 1.0 mM Citrate
Buffer, 10 mM Tris-HCl, pH 6.0

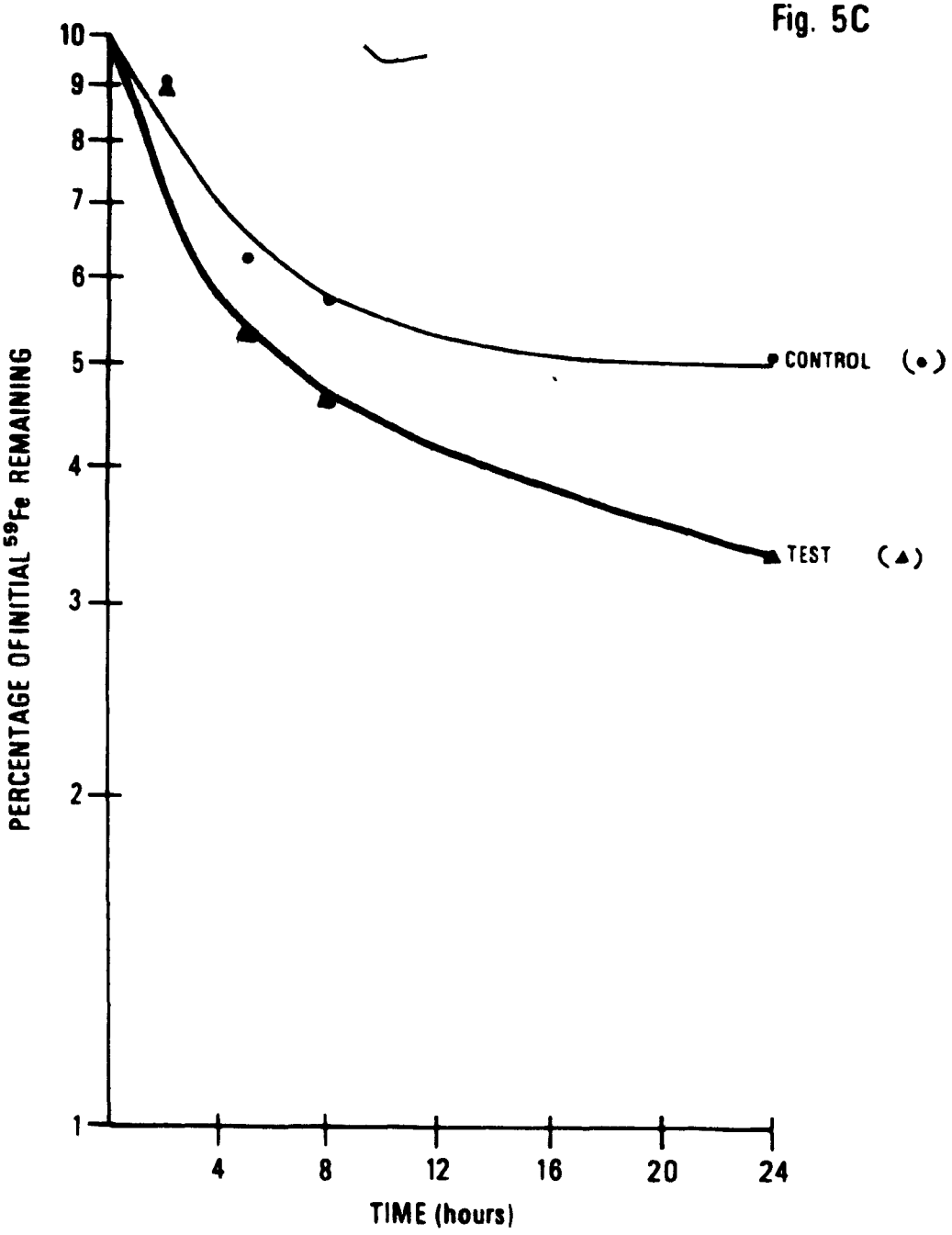
Fig. 5B



The maximum transfer of ^{59}Fe by dialysis.
Control samples contained no apolactoferrin in the dialysis chamber.
Test samples contained apolactoferrin in the dialysis chamber.

Ligand, 1 mM NTA
Buffer, 10 mM Tris-HCl, pH 6.0

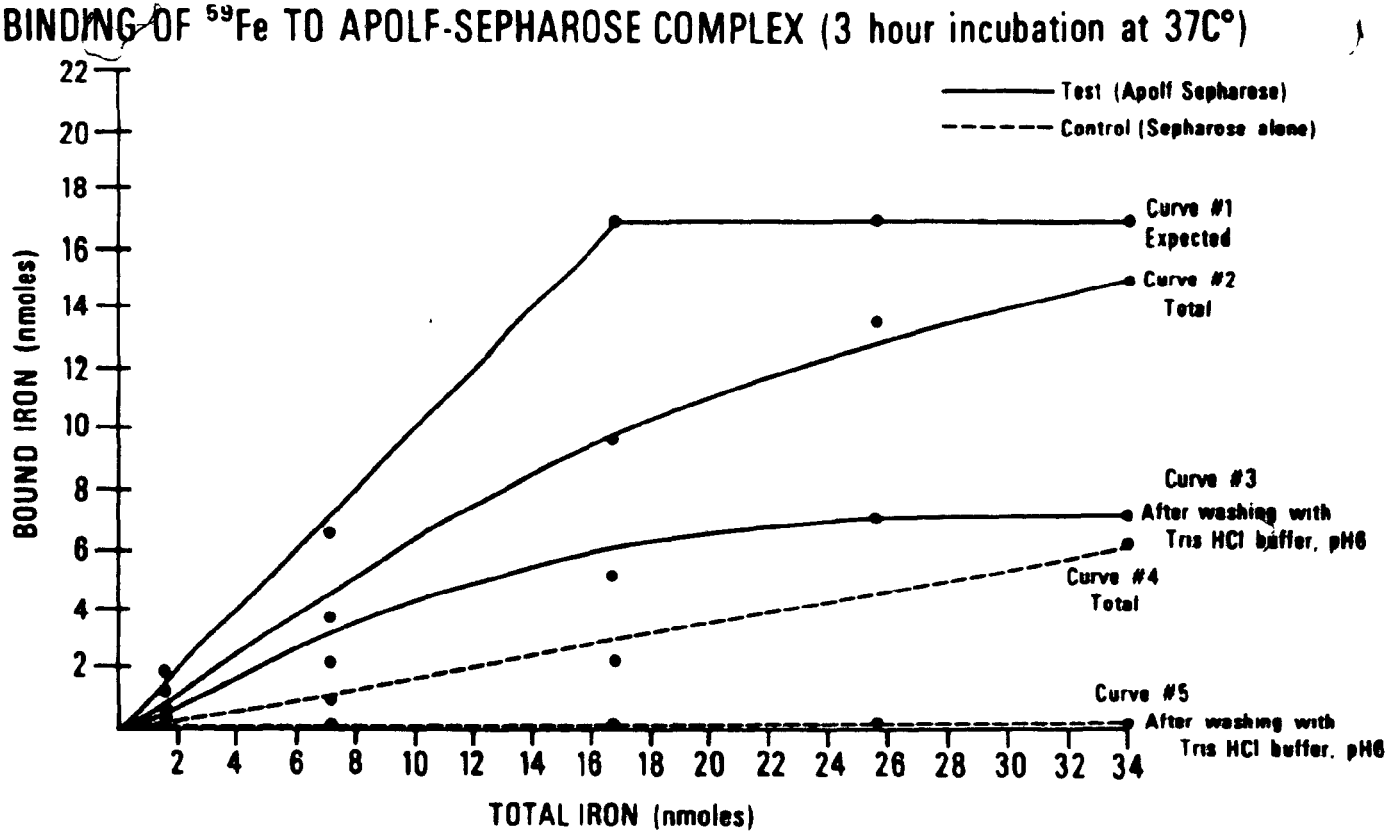
Fig. 5C



The maximum transfer of ^{59}Fe by dialysis.
Control samples contained no apolactoferrin in the dialysis chamber.
Test samples contained apolactoferrin in the dialysis chamber.

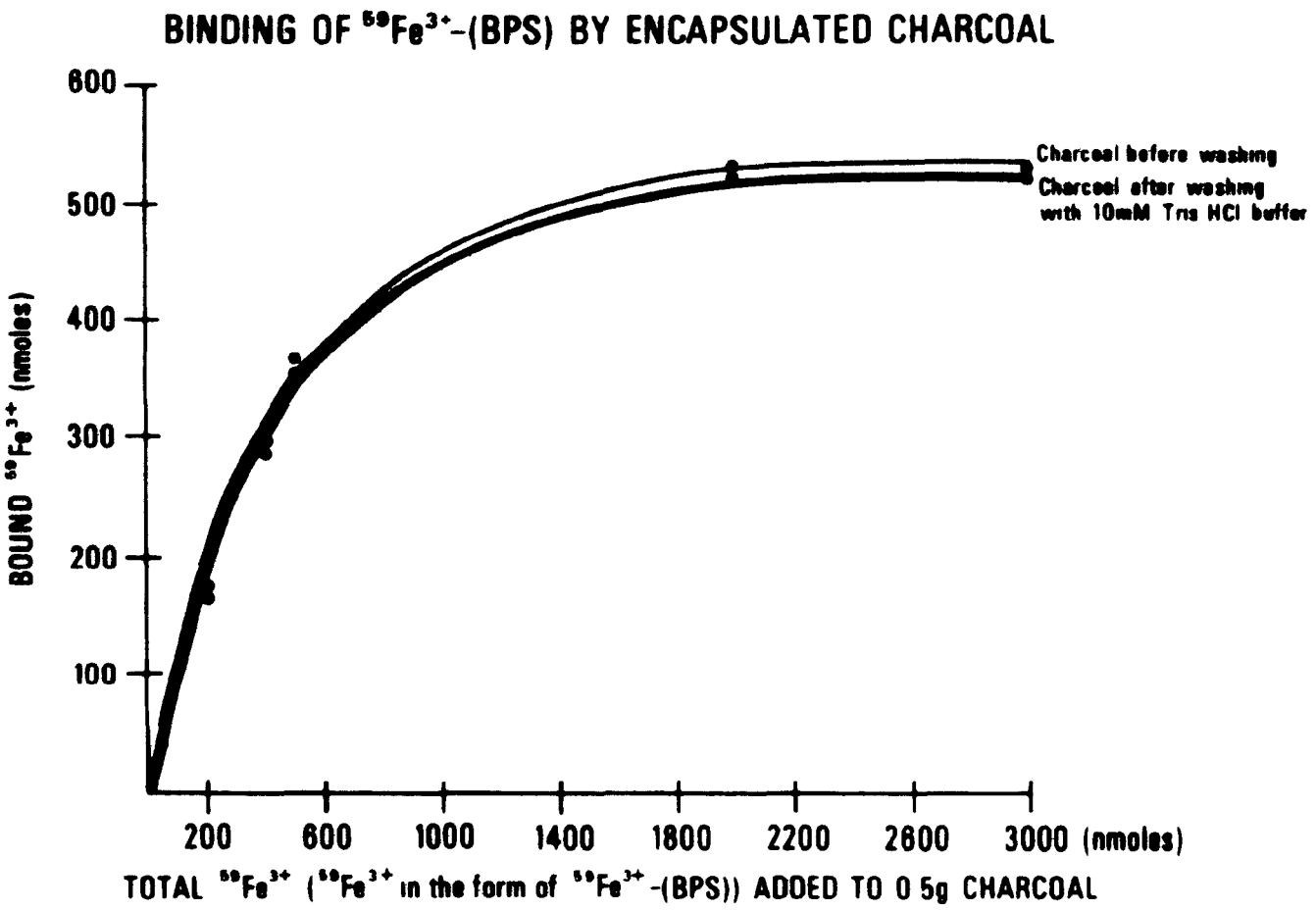
Ligand, 1 mM Pyrophosphate
Buffer, 10 mM Tris-HCl, pH 6.0

Fig. 6



Iron-binding capacity of apolactoferrin bound to activated sepharose.
The experiments were performed as described under Materials and Methods.

Fig. 7



Iron-binding capacity of encapsulated activated charcoal.

TABLE 1

Removal of ^{59}Fe from $^{59}Fe^{3+}$ Tf by dialysis.

Buffer pH	Ligand conc. (mM)	Ratio of apolactoferrin to transferrin	Percentage of initial ^{59}Fe transferred from $^{59}Fe^{3+}$ Tf to apolactoferrin							
			0 Hr		1 Hr		5 Hr		24 Hr	
			C	T	C	T	C	T	C	T
7.5	0	1:1	-	0.2	-	0.2	-	1.4	-	20.2
7.5	1.0	1:1	-	0	-	0.3	-	5.4	-	25.2
7.5	10.0	1:1	-	0.1	-	1.3	-	12.7	-	40.8
7.5	50.0	1:1	-	0	-	1.9	-	16.4	-	46.4
7.0	0	1:1	-	0	-	0.5	-	8.4	-	29.3
7.0	1.0	1:1	-	0.1	-	0.7	-	13.5	-	49.9
7.0	10.0	1:1	-	0.1	-	2.0	-	21.0	-	46.5
7.0	50.0	1:1	-	0	-	2.6	-	23.0	-	47.4
6.5	0	1:1	0	0	0.9	0.7	9.4	9.4	29.3	31.0
6.5	1.0	1:1	-	0	-	2.6	-	15.9	-	42.4
6.5	10.0	1:1	-	0	-	3.8	-	24.9	-	49.5
6.5	50.0	1:1	-	0	-	4.8	-	25.9	-	49.5
6.0	0	1:1	0.1	0	1.8	1.7	10.3	11.8	32.5	38.7
6.0	1.0	1:1	-	0	-	3.6	-	16.7	-	45.6
6.0	10.0	1:1	-	0	-	6.4	-	26.8	-	48.7
6.0	50.0	1:1	-	0	-	5.2	-	25.3	-	48.7
6.0	0	100.1	0.1	0.1	5.2	6.9	22.4	28.9	34.0	42.8
6.0	1.0	100.1	0.1	0	10.0	9.1	37.0	37.2	49.2	53.6
6.0	10.0	100.1	0.1	0.1	11.4	12.3	44.4	45.6	49.4	57.3
6.0	50.0	100.1	0.1	0.1	16.6	14.3	46.4	44.7	50.0	50.3

Ligand; Citrate, Buffer; 10 mM Tris-HCl.
Control samples contained no apolactoferrin in dialysis chamber as described under Materials and Methods.
C = Control, T = Test

TABLE 1 (continued)

Removal of ^{59}Fe from $^{59}Fe^{3+}$- Tf by dialysis.

Buffer pH	Ligand conc. (mM)	Ratio of apolactoferrin to transferrin	Percentage of initial ^{59}Fe transferred from $^{59}Fe^{3+}$ Tf to apolactoferrin							
			0 Hr		1 Hr		5 Hr		24 Hr	
			C	T	C	T	C	T	C	T
6.0	0	1:1	0	0	3.3	3.0	14.9	14.4	38.8	39.8
6.0	1.0	1:1	0	0	4.1	4.2	21.6	23.0	46.7	47.7
6.0	10.0	1:1	0	0	6.5	6.4	29.4	28.9	48.3	47.5
6.0	50.0	1:1	0	0	8.3	7.8	30.3	30.8	47.6	48.2
6.0	0	10:1	0	0	-	-	15.1	15.2	38.3	41.6
6.0	0.1	10:1	0	0	-	-	20.6	20.6	44.3	46.6
6.0	0.5	10:1	0	0	-	-	21.5	22.3	45.9	47.6
6.0	1.0	10:1	0	0	-	-	22.9	22.9	47.0	47.0
	10.0	10:1	0	0	-	-	28.6	27.3	48.4	47.8
6.0	0	100:1	0.1	0.1	4.2	4.4	25.9	27.1	43.1	59.6
6.0	1.0	100:1	0.1	0.1	7.4	7.1	33.9	34.2	50.5	58.1
6.0	10.0	100:1	0.1	0.2	12.6	11.8	42.9	42.3	49.6	51.3
6.0	50.0	100:1	0.1	0.1	13.2	14.8	43.8	44.8	48.9	50.8

Ligand, NTA; Buffer, 10 mM Tris-HCl.
Control samples contained no apolactoferrin in dialysis chamber as described under Materials and Methods.
C = Control, T = Test

TABLE 1 (continued)

Removal of ^{59}Fe from $^{59}Fe^{3+}$ Tf by dialysis.

Buffer pH	Ligand conc. (mM)	Ratio of apolactoferrin to transferrin	Percentage of initial ^{59}Fe transferred from $^{59}Fe^{3+}$ Tf to apolactoferrin							
			0 Hr		1 Hr		5 Hr		24 Hr	
			C	T	C	T	C	T	C	T
6.0	10.0	10:1	0	0	8.0	13.4	36.5	49.2	49.5	56.5
6.0	50.0	10:1	0	0	9.1	10.4	34.7	36.5	49.9	52.3
6.0	0	100:1	0.1	0.1	5.9	7.3	23.6	25.6	31.8	44.8
6.0	1.0	100:1	0.1	0.1	6.3	9.7	27.6	43.8	48.1	71.4
6.0	10.0	100:1	0.1	0.1	15.2	7.9	45.1	36.7	49.8	65.2
6.0	50.0	100:1	0.1	0.1	8.0	10.0	28.8	36.2	48.7	54.9
6.0	0	100:1	0.1	0.1	2.5	7.9	21.7	30.0	38.5	47.6
6.0	1.0	100:1	0.1	0.1	11.9	6.8	43.9	45.6	49.6	65.7
6.0	10.0	100:1	0.1	0.1	11.5	15.6	45.5	52.5	50.0	56.3
6.0	50.0	100:1	0.1	0.1	12.5	11.2	39.6	40.9	49.4	52.4
6.0	0	100:1	0	0.1	6.9	8.7	37.7	35.4	49.0	48.8
6.0	1.0	100:1	0.1	0.1	12.2	12.9	43.1	49.9	50.1	62.5
6.0	10.0	100:1	0.1	0.1	16.0	16.0	47.3	51.9	49.9	60.0
6.0	50.0	100:1	0.1	0.1	16.0	14.6	42.9	43.8	50.1	53.2

Ligand, pyrophosphate; Buffer, 10 mM Tris-HCl
Control samples contained no apolactoferrin in dialysis chamber as described under Materials and Methods.
C = Control, T = Test

TABLE 2

The transfer of ^{59}Fe from $^{59}Fe^{3+}$ Tf (in whole blood) to apolactoferrin (in saline) using Gambro plate dialyser.

Time (Hr)	pH of blood	Percentage of the initial ^{59}Fe transferred to apolactoferrin	Percentage of the initial ^{59}Fe remaining in whole blood
0	7.2	0.3	99.7
½	6.7	4.2	95.8
1	6.5	9.6	90.4
2	5.9	19.6	80.4
3	5.9	22.3	77.7

Pumps; Blood compartment, Sigmamotor pump (flow rate, 10 ml/min).
Dialysate compartment, Varistaltic pump (flow rate, 100 ml/min).
The experiments were performed as described under Materials and Methods.

TABLE 3

Transfer of ^{59}Fe From $^{59}Fe^{3+}$ Tf To
Apolactoferrin By Direct Contact In Solution.

(N=2)

Time (Hr)	Percentage of the initial ^{59}Fe transferred to apolactoferrin	Percentage of the initial ^{59}Fe remaining bound to transferrin
0	89.0	11.0
½	92.1	7.9
1	89.1	10.9
2	91.6	8.4

Conditions, 10 mM Tris-HCl buffer, pH 6.0 and 1 mM Pyrophosphate.
Ratio of apolactoferrin to transferrin = 10:1
After the ^{59}Fe exchange, apolactoferrin and transferrin were separated by cellulose acetate electrophoresis as described under Materials and Methods.

TABLE 4

Transfer of ^{59}Fe from $^{59}Fe^{3+}$ Tf to apolactoferrin-sepharose.

Sample	Incubation Time (Hr)	Apolactoferrin (nmoles)	Transferrin (nmoles)	Transferrin ^{59}Fe added (nmoles)	^{59}Fe transferred to lactoferrin-sepharose	
					Total ^{59}Fe (nmoles)	^{59}Fe after washing beads (nmoles)
					%	%
Control	1	-	0.24	0.48	0.22 (45.8)	0.01 (2.1)
Test	1	48	0.24	0.48	0.48 (100)	0.45 (93.)
Control	3	-	0.24	0.48	0.22 (45.8)	0.01 (2.1)
Test	3	48	0.24	0.48	0.46 (95.8)	0.45 (93.)

Ratio of apolactoferrin to transferrin = 200:1
Buffer, 10 mM Tris-HCl, pH 6.
Ligand, 1 mM Pyrophosphate.
Control = Sepharose alone.
Test = Apolactoferrin-sepharose complex.

TABLE 5A

Removal of Iron From Iron-saturated Transferrin By Different Combinations of Reducing Agents.

Reaction Mixture	#1	#2	#3	#4	#5
Serum (2.3 ug/ml)	+	+	+	+	+
1 mM Bathophenanthroline sulfonate	+	+	+	+	+
10 mM thioglycolate	+	+	+	+	-
26 mM Dithionite	-	-	+	+	+
10 mM Pyrophosphate	-	+	-	+	+
10 min	%	%	%	%	%
Fe removed (ug Fe/ml)	0.56 (23.7)	0.55 (23.3)	1.0 (42.4)	1.11 (47.0)	1.20 (50.8)
Final pH	7.5	7.5	6.8	6.8	6.9
30 min					
Fe removed (ug Fe/ml)	0.67 (28.4)	0.87 (36.9)	1.10 (46.6)	1.15 (48.7)	1.25 (53.0)
Final pH	7.5	7.5	6.8	6.9	6.9
60 min					
Fe removed (ug Fe/ml)	0.86 (36.4)	1.14 (48.3)	1.25 (53.0)	1.28 (54.2)	1.51 (64.0)
Final pH	7.6	7.5	6.8	6.9	6.9

All reagents were dissolved in saline, pH 7.4
+ indicates the presence of a reagent.
- indicates the absence of a reagent.
% = percentage of initial iron removed.

TABLE 5B

Reaction Mixture	#1		#2		#3		#4	
Serum (1.64 ug Fe/ml)	+		+		+		+	
1 mM Bathophenanthroline sulfonate	+		+		+		+	
26 mM Dithionite	+		-		+		-	
13 mM Dithionite	-		+		-		+	
10 mM Pyrophosphate	-		-		+		+	
10 min		%		%		%		%
Fe removed (ug Fe/ml)	0.38	(23.2)	0.34	(20.7)	0.89	(54.3)	0.92	(56.1)
Final pH	7.0		6.9		7.1		7.0	
25 min								
Fe removed (ug Fe/ml)	0.46	(28.0)	0.20	(12.2)	0.96	(58.5)	0.92	(56.1)
Final pH	6.9		6.9		7.0		7.0	
60 min								
Fe removed	0.66	(40.2)	0.47	(28.7)	1.25	(76.2)	1.13	(69.0)
Final pH	6.9		6.9		7.0		7.0	

TABLE 6

Removal of $^{59}Fe^{2+}$ bathophenanthroline sulfonate by dialysis after the reductive release of ^{59}Fe from $^{59}Fe^{3+}$ Tf - CO_3^{2-}.

Time (min)	Percentage of the initial $^{59}Fe^{2+}$ bathophenanthroline sulfonate removed by dialyzate
0	0
10	5.5
20	6.9
30	9.7
40	11.3
50	13.5
60	13.5
70	15.4
80	17.5
90	18.2
120	21.8
150	24.2
180	26.8

The experiments were performed as described under Materials and Methods.

TABLE 7

Removal of $^{59}Fe^{2+}$ bathophenanthroline sulfonate by encapsulated charcoal.

Time (min)	Percentage of the initial $^{59}Fe^{2+}$ bathophenanthroline sulfonate removed by encapsulated activated charcoal	
	A	B
0	0	0
10	98.9	99.1
30	99.5	99.5
70	99.5	99.5
120	99.8	99.7
180	100.0	100.0

Quantity of encapsulated charcoal used, A = 5 g, B = 10 g.
The experimental set up was as described under Materials and Methods.

TABLE 8

Iron transfer from $^{59}Fe^{3+}$ Tf to various chelating agents. Ethanol precipation study.

Reaction Mixture	Percentage distribution of ^{59}Fe									
	0 min		10 min		20 min		30 min		60 min	
	S	P	S	P	S	P	S	P	S	P
$^{59}Fe^{3+}$ Tf	0.8	99.2	1.3	98.7	3.9	96.1	1.8	98.2	2.5	97.5
$^{59}Fe^{3+}$ Tf 1 mM Pyrophosphate	0.9	99.1	0.5	99.5	0.9	99.1	2.9	97.1	1.6	98.4
$^{59}Fe^{3+}$ Tf 1 mM Pyrophosphate 4.5 mM Desferrioxamine	9.1	90.9	7.7	92.3	11.2	88.8	11.8	88.2	12.6	87.4
$^{59}Fe^{3+}$ Tf 1 mM Pyrophosphate 4.5 mM Tropolone	16.0	84.0	19.5	80.5	23.6	76.4	52.1	47.9	23.2	76.8
$^{59}Fe^{3+}$ Tf 1 mM Pyrophosphate 4.5 mM Catechol	1.8	98.2	0.4	99.6	3.2	96.8	3.1	96.9	2.0	98.0
$^{59}Fe^{3+}$ Tf 1 mM Pyrophosphate 4.5 mM Ethylenediamine	60.7	39.3	44.6	55.4	62.7	37.3	64.8	35.2	82.2	17.8

S = Supernatant (contained ^{59}Fe-chelating agent complex)
P = Precipitate (contained transferrin)

TABLE 9

Transfer of ^{59}Fe from $^{59}Fe^{3+}$ Tf in whole blood to ethylenediamine di-(O-hydroxyphenyl acetic acid) (EDDHPA).

Reaction Mixture	Percentage distribution of ^{59}Fe					
	10 min		30 min		60 min	
	S	P	S	P	S	P
$^{59}Fe^{3+}$ Tf in blood (19.4 uM ^{59}Fe)	89.0	11.0	89.5	10.5	89.1	10.9
$^{59}Fe^{3+}$ Tf in blood 500 uM EDDHPA	87.9	12.1	84.1	15.9	86.9	13.1
$^{59}Fe^{3+}$ Tf in blood 100 uM EDDHPA	88.3	11.7	89.2	10.8	88.2	11.8
$^{59}Fe^{3+}$ Tf in blood 10 uM EDDHPA	87.9	12.1	90.1	9.9	90.5	9.5
$^{59}Fe^{3+}$ Tf in blood 500 uM EDDHPA 10 mM Pyrosphast	45.6	54.4	39.8	60.2	30.3	69.7
$^{59}Fe^{3+}$ Tf in blood 100 uM EDDHPA 10 mM Pyrophosphate	47.4	52.6	38.3	61.7	27.2	72.8
$^{59}Fe^{3+}$ Tf in blood 10 uM EDDHPA 10 mM Pyrophosphate	73.5	26.5	69.0	31.0	67.2	32.8
$^{59}Fe^{3+}$ Tf in 0.1 M HEPES (12.9 uM ^{59}Fe)	35.4	64.6	36.5	63.5	31.9	68.1
$^{59}Fe^{3+}$ Tf in 0.1 M HEPES 500 uM EDDHPA	32.3	67.7	35.2	64.8	31.1	68.9
$^{59}Fe^{3+}$ Tf in 0.1 M HEPES 500 uM EDDHPA 10 mM Pyrophosphate	11.1	88.9	5.3	94.7	2.7	97.3

The formed ^{59}Fe-EDDHPA was removed by encapsulated activated charcoal as described under Materials and Methods.
S = Supernatant
P = Charcoal

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