Microsomal Ferritin Iron Release

in Relation to TCDD Toxicity

Microsomal Ferritin Iron Release in Relation to TCDD Toxicity

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Abstract

Previous experiments have demonstrated that the hepatoxicity of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) involves synergism with iron. It has been postulated that it is storage iron that plays a role in toxic effects observed with TCDD poisoning. It was hypothesized that TCDD induction of the Ah gene locus in some way leads to mobilization of storage iron from the iron storage protein ferritin. Microsomal ferritin iron release was investigated in intact microsomes and in a reconstituted system. Evidence is presented which suggests that NADPH-cytochrome c (P450) reductase is capable of donating electrons to exogenous flavin (FMN), and that at the same time, the reduced flavin thus generated is capable of effecting the release of iron from ferritin. While no direct evidence was obtained, the results do lend support to the hypothesis that TCDD toxicity could result from the mobilization of iron from ferritin by the TCDD induced microsomal electron transport system.

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List of Abbreviations Used

AHH	Aryl Hydrocarbon Hydroxylase
B-NF	Beta-Naphthoflavone
CO	Carbon Monoxide
DEAE	Diethylaminoethyl
DLPC	Dilauroylphophatidylcholine
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic Acid
FMN	Flavin Mononucleotide
$FMNH_2$	Reduced Flavin Mononucleotide
HCB	Hexachlorobenzene
ip	Intraparitoneal
LH	Lipophilic
MFO	Mixed Function Oxygenase
NADH	Reduced Nicotine Adenine Dinucleotide
NADPH	Reduced Nicotine Adenine Dinucleotide Phosphate
PB	Phenobarbital
PCBs	Polychlorinated Biphenyls
PCT	Porphyria Cutanea Tarda
SER	Smooth Endoplasmic Reticulum
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
Tris	Tris (hydroxymethyl) aminomethane

Introduction:

2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD), one of the most toxic small molecules known to man, belongs to the class of compounds known as chlorinated aromatic hydrocarbons. Also included in this group are hexachlorobenzene (HCB) and polychlorinated biphenyls (PCBs). Chlorinated aromatic hydrocarbons have become the subject of great concern over recent years, as awareness of the toxicity of these compounds has developed. Increasing levels of these environmental pollutants within the environment has prompted a great deal of research into the mechanism(s) through which these toxins exert their effects.

One of the primary target organs of chlorinated aromatic hydrocarbons is the liver. Histological examination of liver tissue obtained from rats treated with either HCB, PCBs, or TCDD show marked morphologic damage. Enlargement of centrilobular cells, vacuolization of the cytoplasm, fatty infiltration, increased endoplasmic reticulum, and focal cell necrosis are all typical morphologic changes associated with chlorinated aromatic hydrocarbon hepatotoxicity (1-6).

As well as causing the morphologic changes described above, HCB (7), PCBs (8), and TCDD (9) have also been found to cause porphyria in rats. The type of porphyria

observed in experimental animals treated with chlorinated aromatic hydrocarbons is biochemically similiar to the human disease Porphyria cutanea tarda (PCT). PCT is a hepatic porphyria with cutaneous manifestations. Unlike all other human porphyrias, which result from genetically inherited enzymic deficiencies, PCT can also be acquired through prolonged exposure to a variety of etiologic agents including ethanol and synthetic estrogens, as well as chlorinated aromatic hydrocarbons. Outbreaks of PCT, in human populations, have been attributed to exposure to HCB (10) and to TCDD (11).

Of the three toxic chlorinated aromatic hydrocarbons referred to, TCDD is by far the most potent. TCDD is 10,000 times more potent in elliciting the morphologic changes associated with chlorinated aromatic hydrocarbon hepatotoxicity than are HCB or PCBs (12).

TCDD, HCB, and PCBs are all inducers of the hepatic mixed function oxygenase system (MFO). However, while mg per kg quantities of HCB or PCBs are required for induction, only μg per kg quantities of TCDD are required. The potency of TCDD as a toxin correlates well with its potency as an inducer of the MFO system (13).

TCDD is a very potent inducer of aryl hydrocarbon hydroxylase (AHH) activity (14,15), as it specifically induces cytochrome P_1450, the liver microsomal monooxygenase

that mediates AHH activity; further, its liver toxicity has been found to be dependant upon AHH induction.

This dependence of TCDD toxicity upon AHH induction was demonstrated in experiments which showed that mice which are genetically unresponsive to AHH induction (DBA strain) are markedly less susceptible to TCDD toxicity, than those which are responsive to induction (C57BL/6 strain)(14). AHH induction was found to be a dominant trait (14), and has been attributed to an interaction between TCDD and a product of the Ah regulatory gene to form a complex which mediates AHH induction (15).

It has recently been established that iron plays an important role in the liver toxicity caused by TCDD. This was demonstrated by experiments which showed that iron deficiency protects against TCDD toxicity in mice (16), and is supported by the use of repeated venesection (withdrawing blood in order to deplete iron stores by stimulating synthesis of hemoglobin) as a means of treating PCT (17).

Although not proved, it was hypothesized that storage iron (ie. ferritin iron) is the source of the iron responsible for the toxic effects observed with TCDD poisoning. Investigations of tissue samples taken from patients with PCT (18) or from rats treated with HCB (19,20) demonstrated that hepatocytes which exhibit the red fluoresence characteristic of porphyrin accumulation (a

symptom of HCB or TCDD toxicity ie. porphyria) contain no stainable storage iron, while those which do not fluoresce, do contain stainable storage iron. These findings provide indirect support for the belief in the involvement of storage iron in the mechanisms leading to the toxic effects observed with TCDD toxicity.

Thus, roles for both iron and the induction of the Ah gene locus have been established, but the exact nature of these roles in the pathways which lead to the hepatotoxic effects observed with TCDD, remain unclear.

The accumulation of neutral lipid, a symptom of TCDD toxicity (21), is consistent with effects associated with lipid peroxidation processes. Because iron (Fe^{2+}) has been demonstrated to be important both as an initiator and as a promoter of microsomal lipid peroxidation (22,23,24), it has been postulated that a possible role which iron might play in TCDD toxicity, is that of inducing processes involving lipid peroxidation within affected cells. This theory is presently under investigation in this laboratory.

In order for storage iron to be involved in such reactions, presumably it must first be released, or mobilized, from its storage site within the cell. Iron is stored within cells in two different but related forms: (a) a soluble mobile fraction called ferritin, and (b) an insoluble deposit called hemosiderin. The difference in solubility

between the two forms is due to the protein envelope which surrounds the ferritin iron and prevents precipitation. Analysis of the physical charactertistics of the iron complexes present in each storage form indicates that the iron present in each form is very similiar in nature (25,26,27). In fact, there is a great deal each storage form is very similiar in nature. of evidence which suggests that hemosiderin may be formed by the partial denaturation of ferritin protein molecules (28,29,30). Whatever storage form the iron is in, it may be stored for long periods of time, or be mobilized for use by the cell almost immediately. Approximately one third of the body's iron reserves are found in the liver (31).

The liver is made up of several distinct cell types, of which the parenchymal and Kupffer cells comprise more than 85 percent of the total cell population and volume (32,33). The predominant form of storage iron present within the parenchymal cells is different from that found in the Kupffer cells. Ferritin predominates in parenchymal cells (95 % of the total liver ferritin iron stores), whereas hemosiderin predominates in Kupffer cells (34,35,36).

As well as differing in their predominant form of storage iron, parenchymal cells and Kupffer cells also appear to differ in their response to the toxin TCDD. The morphologic and biochemical changes caused by TCDD are

associated with corresponding changes that take place within parenchymal cells, rather than Kupffer cells (37,38). Because ferritin is the predominant form of storage iron in the parenchymal cells, it would seem likely then that ferritin, rather than hemosiderin, would be the form of storage iron to be involved in the hepatotoxicity caused by TCDD.

Iron is stored in ferritin as Fe^{3+}, as ferric oxyhydroxide crystals complexed with small amounts of phosphate. These crystals are found within the central cavity of the ferritin protein molecule. This cavity is formed by the hollow spherical arrangement of the protein's 24 subunits (39). The number of iron atoms present within a ferritin molecule ranges from zero atoms in apoferritin, to approximately 4500 atoms in fully loaded ferritin (39).

The mechanisms by which ferritin accumulates and releases its iron are at present unknown. However, it does seem clear from the evidence so far accumulated, that reduction of the iron atoms from the Fe^{3+} state to the Fe^{2+} state must precede release from the ferritin molecule.

It has been demonstrated that several biological reductants are capable of ellicting the release of iron from ferritin (40,41,42). Of the many low molecular weight reductants tested, reduced flavins, dithionite, cysteine, glutathione, and ascorbate were found to be the most

effective (in terms of iron release rate), in that order of efficiency (40). Because of the high rate at which reduced flavin mononucleotide ($FMNH_2$) mobilized iron from ferritin, it was suggested (42) that $FMNH_2$ would be the most likely reducing agent in tissues to be physiologically signifigant.

The release of iron from ferritin also appears to depend upon relatively free access to the center of the ferritin molecule for the reductant. The reductants presumably gain access to the ferritin iron core in the cavity of the protein by means of any of the six pores that exist within the protein shell (39). Once inside the protein shell, the reductants presumably transfer their reducing equivalents directly to the ferric iron, yielding Fe^{2+}, which permits the release of the iron from ferritin. That reduced flavins penetrate to the ferritin iron core in order to mobilize iron from ferritin was demonstrated in experiments which showed that reduced flavins linked to agarose beads are capable of mobilizing iron from synthetic iron cores, but are incapable of mobilizing iron from ferritin (41). This is further supported by the finding that apoferritin contains no specific binding site for reduced or oxidized flavins (41). Both of these findings support the suggestion that the reduced flavins must penetrate the ferritin molecule to the iron core, and once there, transfer their reducing equivalents directly to the ferric iron atoms present.

Measurements of the width of the intersubunit channels of ferritin (the six pores) indicate that these channels are indeed wide enough that reduced flavins would be capable of penetrating the ferritin protein molecule and gaining direct access to the ferric iron core (39).

Ferritin iron release by both mitochondrial and microsomal subcellular fractions, in the presence of exogenous flavin mononucleotide (FMN), has been demonstrated by several investigators (43,44). In all cases, the function of the mitochondrial or microsomal subcellular fraction was thought to be the generation of reduced flavin, which in turn reduces the ferritin iron, allowing for its release.

The ferritin iron release process invoked by these subcellular fractions was found to be extremely oxygen sensitive. It is strongly inhibited by oxygen concentrations greater than 2-3 μM O_2 (44). This inhibition is presumably due to autoxidation of the reduced flavins by O_2, creating a competition between O_2 and ferritin iron for the reducing equivalents via the flavins.

While the concentration of O_2 in the vicinity of the mitochondria has never been determined, the K_m of the cytochrome oxidase system for O_2 has been estimated (45) to lie between 0.02 μM O_2 and 0.50 μM O_2, depending upon the metabolic state of the mitochondria. Thus, the O_2 concentration in the vicinity of the mitochondria could very

well lie below the levels which would inhibit ferritin iron release, and yet still be high enough to support respiring mitochondria.

The concentration of O_2 in the vicinity of the microsomal subcellular fraction has not yet been determined. It is however conceivable that effectively anaerobic conditions may exist for the release of iron from ferritin by reduced flavins generated by microsomes. The parenchymal cells first affected by TCDD toxicity are the zone 3 parenchymal cells (37). Zone 3 parenchymal cells lie adjacent to efferent veins, while zone 1 parenchymal cells lie adjacent to afferent blood vessels (46). Between zones 1 and 3 lies an ill-defined zone 2. It is possible that significant differences in O_2 tension exist between these zones. Zone 3, being closest to the veins, would presumably have the lowest O_2 tension. Zone 3 parenchymal cells may very well provide a suitable environment for the mobilization of ferritin iron by a reduced flavin generating system.

Ulvik and Romslo (47) were successful in demonstrating that under anaerobic conditions, and in the presence of added flavin, mitochondria respiring on succinate are capable of releasing iron from ferritin, and at the same time, that ferrochelatase incorporates this iron into heme. In later experiments by the same group, evidence was advanced to suggest that ubiquinone serves as the point along the

mitochondrial electron transport chain at which the added FMN was draining its reducing equivalents (48). These experiments served to provide necessary information required to outline a possible molecular mechanism through which mitochondria are capable of mobilizing ferritin iron for heme synthesis. It is possible that a similar mechanism is operating in microsomes; that is to say, a mechanism whereby free cytosolic FMN drains reducing equivalents from some point along the microsomal electron transport chain, and in turn passes them on to the ferritin iron, reducing it, and allowing for its release.

Evidence exists to suggest that there is a cytosolic pool of free flavins within cells (49,50,51). Such a pool would serve as an excellent source for the flavin required for the proposed mechanisms of ferritin iron release by subcellular fractions.

The fact that the microsomal subcellular fraction has been demonstrated to be capable of mobilizing ferritin iron *in vitro*, could be of great importance in TCDD induced liver toxicity, especially if microsomal ferritin iron release can be attributed to a process regulated by, or a product of, the Ah gene locus.

TCDD induction causes large amounts of cytochrome P_1450 to be synthesized and incorporated into the smooth endoplasmic reticulum (SER). TCDD induction differs from phenobarbital (PB) induction not only in that different forms

of cytochrome P450 are induced (PB induces the cytochrome P450 form rather than the cytochrome P_1450 form which is induced by TCDD), but also in that PB leads to the induction of other SER components (such as NADPH-cytochrome c (P450) reductase) whereas TCDD induction does not. Another difference is that TCDD induction is a response to an inducer which is an extremely poorly metabolized substrate (if at all) for the pathway induced, whereas PB is metabolized, albeit slowly, by the induced MFO system. TCDD has a half life of 17 days in mice. There is no evidence for metabolism of TCDD by mice (52).

TCDD induction leads to significantly increased levels of cytochrome P_1450 being synthesized and incorporated into the SER membrane, but no metabolism of TCDD to a less toxic or excreted product takes place; ie. once synthesized, the cytochrome is part of the SER membrane, but lacks a substrate. It could be hypothesized that because the induced microsomal electron transport system is not occupied with the metabolism of the inducer (TCDD), as is the case with PB, it is available to participate in a process in which it may not normally be involved. Such a process could involve the release of iron from ferritin. This could be hypothesized because only TCDD induction leads to toxicity, whereas PB induction appears not to.

The major aim of this thesis was to investigate the

molecular mechanism by which ferritin iron is released by the microsomal subcellular fraction, in the hope of relating this mechanism to the mechanism through which TCDD exerts its hepatotoxic effects.

The mechanism of microsomal ferritin iron release was investigated using microsomal fractions obtained from PB induced, TCDD induced, and control rat livers, as well as in a reconstituted system in which the required microsomal membrane components were recombined in order to obtain a functional microsomal ferritin iron mobilizing system.

Materials and Methods

1. Chemicals

All reagents used were of commercial reagent grade or better and were used without further purification. Horse spleen ferritin obtained from Calbiochem (San Diego, California) was used as the ferritin iron source in all assays.

2. Animals

Experiments performed in the investigation of the mobilization of ferritin iron were conducted using microsomes or microsomal proteins purified from livers of treated or untreated male rats. In all cases, young adult male Sprague Dawley rats weighing 175-200 g were used.

3. Treatment Schedules

i) Phenobarbital Induction

Treatment with phenobarbital consisted of twice daily intraparitoneal (ip) injections of 40 mg/kg PB in saline for 3 days. The animals were starved overnight and sacrificed on the 4th day.

ii) Long Term TCDD Induction

Long term TCDD induction consisted of 3 injections (ip) of TCDD in corn oil, given at 1 week intervals. The first injection was 10 µg/kg TCDD, while the second and third were 2.5 µg/kg TCDD. One week after the third injection of TCDD, the animals were sacrificed. The animals were starved overnight prior to sacrifice.

iii) Short Term TCDD Induction

Short term TCDD induced animals recieved 1 injection (ip) of 10 µg/kg TCDD in corn oil and were sacrificed 1 week later. The animals were starved overnight prior to sacrifice.

4. Preparation of Microsomes

Microsomes were prepared according to the method of Mitoma et al (53), with slight modifications. The animals were sacrificed by decapitation and the livers were perfused in situ with a 150 mM KCl solution containing 10 mM EDTA, prior to removal. Twenty-five % (w/v) liver homogenates were then prepared in the above KCl-EDTA solution. The liver homogenates were centrifuged at 9,000 xg for 20 minutes. The supernatants were removed and then centrifuged at 100,000 xg for one hour. The resulting microsomal protein pellets were then washed with and resuspended in 0.1 M sodium phosphate buffer pH 7.4. The microsomal protein pellet obtained from 3 g of rat liver was resuspended in 2.0 ml buffer.

5. Measurement of Cytochrome P450

Cytochrome P450 was assayed by measuring the CO difference spectra of dithionite reduced suspensions of microsomes (or solubilized microsomal proteins) according to the method of Omura and Sato (54).

6. Measurement of NADPH-cytochrome c reductase Activity

NADPH-cytochrome c reductase activity was assayed using the method of Williams and Kamin (55). One unit of activity is defined as 1 nmole cytochrome c reduced per ml per minute.

7. Measurement of Benzphetamine-O-demethylase Activity

Benzphetamine-O-demethylase activity was determine according to the method of Cochin and Axelrod (56).

8. Measurement of Aryl Hydrocarbon Hydroxylase (AHH) Activity

AHH activity was measured according to the procedure of Poland et al (57). One unit of activity is defined as 1 picomole 3-OH Benz-a-pyrene formed per minute.

9. Measurement of Protein

Protein was measured spectrophotometrically according to the method of Sutherland et al (58), using bovine serum albumin as the standard.

10. Preparation of Oxygen Free Nitrogen

Oxygen free nitrogen was prepared by passing commercial grade N_2 gas through a scrubbing assembly consisting of three gas wash bottles. The first wash bottle contained 0.1 M Cr^{+2} in 2.4 N HCl over zinc amalgam, which was prepared according to the method of Meites (59). The second wash bottle contained deionized water, and the third contained the buffer used for the ferritin iron release assay system. The second and third wash bottles are included in the system, in order that any nebulized fluid, which is acidic after passing through the first wash bottle, can be raised to pH 7.4. Thus, when this gas is mixed into the ferritin iron release assay mixture, the pH of the assay system can be maintained.

11. Measurement of Microsomal Ferritin Iron Release

Microsomal ferritin iron release was measured using an assay similar to that employed by Ulvik and Romslo (44), in their investigation of mitochondrial ferritin iron release. The assay system was modified in that NADPH, rather than NADH, was used as the source of reducing equivalents for the microsomal electron transport chain. An NADPH generating system (glucose-6-phosphate and glucose-6-phosphate dehydrogenase) was also added, in order to maintain the concentration of NADPH in the assay system.

In a final volume of 2.0 ml, an assay mixture was prepared containing 4.0 mg microsomal protein, 150 μM NADPH, 1.0 mM 2,2'- bipyridyl, 350 μM Fe (present as ferritin iron), 1 mM glucose, 1 mM glucose-6-phosphate, 0.2 units glucose oxidase, 0.2 units glucose-6-phosphate dehydrogenase, and 25 mM Tris-HCl buffer pH 7.4. These components were added to the base of a Thunberg cuvette. The side arm of the Thunberg cuvette held 0.1 ml of a 5.0 mM solution of FMN in 25 mM Tris-HCl buffer pH 7.4. This yielded a final concentration of 250 μM FMN.

Anaerobic conditions were achieved by evacuating (degassing) and flushing the cuvette three times with oxygen free nitrogen. The cuvette was then brought to 37°C in a temperature controlled cuvette holder and a baseline (see

below) was recorded. Ferritin iron release was initiated by tipping the FMN into the assay mixture from the side arm of the cuvette.

The mobilization of ferritin iron was monitored spectrophotometrically as $A_{522}-A_{551}$, using the dual beam mode of the Perkin-Elmer 356 spectrophotometer (see page 25).

The rate of microsomal ferritin iron release was determined from the linear portion of the change in absorbance versus time plots, obtained within the first 8 minutes following addition of the flavin to the assay mixture (see Figure 2). The rate of iron release in nanomoles Fe^{+2} released per minute per milliliter was calculated from a standard curve of absorbance at 522 nm minus the absorbance at 551 nm versus $Fe^{+2}(bipyridyl)_3$ concentration.

The approach described above was confirmed using the split beam mode of the Perkin-Elmer 356 spectrophotometer. This method employs difference spectroscopy: two identical samples were prepared, except that in one sample, the reference sample, NADPH was omitted. Both samples were made anaerobic and warmed to 37°C in a temperature controlled cuvette holder. A baseline was recorded and the FMN was then tipped into the reaction mixtures. The change in the difference in absorbance at 530 nm, between the sample cuvette (containing NADPH) and the reference cuvette (NADPH omitted), was monitored versus time. A standard curve of

absorbance at 530 nm versus $Fe^{+2}(bipyridyl)_3$ concentration was used to calculate the rate of microsomal ferritin iron release.

12. Separation and Partial Purification of Cytochrome P450 and NADPH-cytochrome c (P450) reductase

Depending upon whether cytochrome P450 or cytochrome P_1450 was desired, young adult male rats were induced with either PB or TCDD. The animals were starved overnight prior to sacrifice.

Microsomal pellets were prepared as previously described, except that the microsomal pellets were washed with and resuspended in 0.2 M sodium phosphate buffer pH 7.7 containing 30 % (v/v)glycerol, 0.1 mM dithiothreitol (DTT), and 0.1 mM EDTA. The resuspended microsomes were frozen in liquid nitrogen until used for purification of the cytochrome and the reductase.

All purification procedures were carried out at 4° C. A flow rate of 0.5 ml per min was used for all column elutions.

When required, microsome samples were thawed and diluted to 10.0 mg/ml protein with buffer. A 10 % (v/v) solution of the nonionic detergent Renex 690 was then added dropwise, with stirring, to a final concentration of 1.5 %.

To the clear solubilized microsomal protein solution, protamine sulphate (1.5 % w/v) was then added dropwise, with stirring, to a final concentration of 0.03 %. The solution was then centrifuged at 100,000 xg for 1 hour.

The supernatant obtained was then applied to a 2.6 x 50 cm DEAE-Sephadex column which had previously been equilibrated with 0.1 M sodium phosphate buffer pH 7.7 containing 20 % glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.15 % Renex 690. The column was then washed with 250 ml of 0.1 M sodium phosphate buffer pH 7.7 containing 20 % glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.1 % (w/v) sodium deoxycholate.

Cytochrome P450 and cytochrome b_5 pass unbound through the column. Wash fractions containing these proteins were pooled and made 0.5 % (w/v) with respect to sodium cholate. The cytochrome P450 containing sample was then dialyzed overnight against 6 l of 10 mM sodium phosphate buffer pH 7.7 containing 20 % glycerol, 0.1 mM DTT, 0.1 mM EDTA, 0.15 % Renex, and 0.5 % sodium cholate.

NADPH-cytochrome c (P450) reductase was eluted from the column with a 1.0 litre linear 0-0.5 M gradient of KCl in the above wash buffer. The reductase elutes at approximately 0.25 M KCl. Fractions containing NADPH-cytochrome c reductase activity were pooled and concentrated by pressure dialysis against 10 mM sodium phosphate buffer pH 7.4. Aliquots of the concentrated reductase solution were then frozen in liquid

nitrogen until used in reconstitution experiments.

Following dialysis, the cytochrome P450 containing solution was applied to a 3.2 x 50 cm DEAE-cellulose column previously equilibrated with 10 mM sodium phosphate buffer pH 7.7 containing 20 % glycerol, 0.1 mM DTT, 0.1 mM EDTA, 0.15 % Renex, and 0.5 % sodium cholate. The column was washed with 250 ml of 10 mM sodium phosphate buffer pH 7.7 containing 20 % glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.15 % Renex. Cytochrome P450 was eluted with a 1.0 litre linear 0-0.5 M gradient of KCl in wash buffer. The cytochrome elutes at approximately 0.1 M KCl.

Fractions eluted from the column which contained cytochrome P450 were immediately pooled and applied to a 2.2 x 40 cm LH-sephadex column which had been previously equilibrated with 10 mM sodium phosphate buffer pH 7.7 containing 50 % glycerol, in order to remove detergent. Cytochrome P450 washes through the column while detergent is retained. Cytochrome P450 fractions were pooled and concentrated by pressure dialysis against 10 mM sodium phosphate buffer pH 7.4. Aliquots of the concentrated cytochrome P450 samples were frozen in liquid nitrogen until used for reconstitution experiments.

13. Method of Reconstitution

Reconstitution experiments were performed using Coon's method of reconstitution of MFO activity (57). Appropriate amounts of partially purified reductase and cytochrome were added to the base of a Thunberg cuvette. Lipid was then added and the solution was mixed. The mixture was allowed to stand at room temperature for 2-3 minutes. The remaining assay components were then added and the assay was performed. The amounts of cytochrome P 450, cytochrome $P_1$450, or NADPH-cytochrome-c (P450)-reductase used in each experiment are indicated in the appropriate table or figure legend. The lipid used in all reconstitution experiments was Dilauroylphosphatidylcholine (DLPC). Lipid was always added to a final concentration of 0.1 mg/ml.

14. Statistics

Statistical signifigance was determined using Student's t test.

Results

1. Development of the Microsomal Ferritin Iron Release Assay

As stated earlier, mobilization of ferritin iron by both the mitochondrial and microsomal subcellular fractions has been demonstrated by several investigators (43,44). While the release of ferritin iron by mitochondria has been investigated, and a mechanism for this phenomenon has been proposed (48), very little work has been devoted to the study of the same phenomenon as it occurs within the microsomal subcellular fraction.

In order to examine the microsomal ferritin iron release process, a system similiar to that employed by Ulvik and Romslo (44) in their investigation of mitochondrial ferritin iron release was used. The microsomal electron transport chain was supplied with reducing equivalents and the rate of formation of $Fe^{2+}(bipyridyl)_3$ complexes was then followed spectrometrically after FMN had been added to the assay system.

The results of repetitive scanning using difference spectroscopy are shown in Figure 1 (NADPH was ommitted from the reference cuvette). The increase in absorbance at 530 nm is due to the formation of the $Fe^{2+}(bipyridyl)_3$ complexes, as

the ferritin iron is mobilized by the microsomal ferritin iron release system.

The release of ferritin iron by microsomes was followed using the two difference spectroscopy modes of the Perkin-Elmer 356 spectrometer. In the split beam mode, a beam of light of the desired wavelength is split and passed through two cuvettes. Both cuvettes are prepared identically except that in the reference cuvette one assay component (NADPH) is omitted. Thus, the reaction can proceed only in the second cuvette, the sample cuvette. The difference in absorbance of light, at the desired wavelength, between the two cuvettes is then monitored by the spectrometer.

In the dual beam mode, two beams of light of different wavelength are passed through a single cuvette. The wavelengths of light used are chosen so that at lambda 1, the reference beam wavelength, there is less change in absorbance of light as the reaction is proceeding, than at lambda 2, the signal beam wavelength, where the net increase or decrease in absorbance is a function of the formation or disappearance of the reaction component being assayed. The absorbance of light at lambda 2 minus the absorbance at lambda 1 is monitored by the spectrophotometer.

Using the split beam mode, and 2.0 mg control microsomal protein per ml, an average rate of 42.64 (SD=0.86) nmole Fe^{2+} released/min/ml was obtained. When the dual beam

mode was used, under otherwise identical assay conditions, a rate of 44.38 (SD=0.66) nmole Fe^{2+} released/min/ml was obtained. Both methods for following the reaction yielded essentially the same rates of release of iron from ferritin ($p<0.01$). The dual beam mode was chosen for use in subsequent assays, as it is necessary to prepare only one sample for each assay.

Figure 2 shows a typical change in absorbance versus time plot obtained when following microsomal ferritin iron release using the dual beam mode. In order to determine the rate of iron release the slope of the linear portion of the absorbance versus time plots, obtained within 10 min of adding the flavin, was used. The rate of iron release was calculated using an extinction coefficient of 4.25 $mM^{-1}cm^{-1}$, which was determined from an absorbance versus $Fe^{2+}(bipyridyl)_3$ concentration standard curve.

After approximately 8-10 min, the slope of the absorbance versus time plots began to decrease. This decrease was attributed to decreases in the ferritin iron and bipyridyl concentrations. After 10 min, more than 1/3 of the added ferritin iron had been mobilized by the microsomal ferritin iron release system.

Figure 2 also shows that there is a lag phase following flavin addition, which precedes ferritin iron release. The duration of the lag phase was found to be

dependant upon both the amount of protein added (see Figure 5) and the concentration of flavin added to the system. The lag phase is thought to represent the time necessary to generate a reduced flavin concentration sufficiently high to initiate ferritin iron release. Ulvik and Romslo attributed the latency period observed with mitochondrial ferritin iron release to the same phenomenon. They found support for this hypothesis by demonstrating that when reduced flavins are generated by dithionite, instead of by mitochondria, the steady state rate of iron release was obtained within seconds; that is, no lag phase was observed.

As with the mitochondrial ferritin iron release system, the presence of O_2 in the assay system was found to severely inhibit the release of iron from ferritin by microsomes. This is presumably due to the autoxidation, by O_2, of the reduced flavins generated by the microsomes. For this reason all assays were performed under anaerobic conditions.

In the previous investigations which demonstrated ferritin iron release by the microsomal subcellular fraction, NADH was used as the source of reducing equivalents supplied to the microsomal electron transport chain. Figure 3 demonstrates that when NADH is replaced by NADPH, in the assay system, a doubling of the rate of ferritin iron release is observed. An average rate of 23.42 (SD=0.96) nmole

Fe^{2+}released/min/ml was obtained with NADH, whereas an average rate of 44.38 (SD=0.66) nmole Fe^{2+}released/min/ml was obtained with NADPH. It would seem then, that NADPH is the preferred source of reducing equivalents for the microsomal ferritin iron release pathway.

The rate of microsomal ferritin iron release was found to have a linear relationship with respect to the concentration of iron, added as ferritin iron at the start of the reaction, within the range of concentrations tested. This is illustrated in Figure 4.

The effect of increasing the microsomal protein concentration on the rate of ferritin iron release is shown in Figures 5 and 6. The three traces shown in Figure 5 demonstrate that both the duration of the lag phase observed, and the rate at which ferritin iron is mobilized are dependent upon the concentration of microsomal protein added. The results show that an increase in microsomal protein concentration leads to a decrease in the lag phase time as well as an increase in the rate of release of ferritin iron.

Figure 6 shows the relationship between the rate of ferritin iron release and the concentration of added microsomal protein. These results show a linear relationship between protein concentration and rate of ferritin iron release at protein concentrations greater than 0.5 mg/ml protein. A concentration of 2.0 mg/ml microsomal protein was

chosen for use in all subsequent assays.

The results presented in Figures 5 and 6 demonstrate that the mobilization of ferritin iron is dependent upon some component of the microsomes, as no ferritin iron release was observed in the absence of microsomal protein. This microsomal component likely serves as the point along the microsomal electron transport chain from which the added flavin drains its reducing equivalents for ferritin iron release. This is the same role as that which was suggested for the mitochodrial electron transport chain component ubiquinone, in the proposed mechanism of mitochondrial ferritin iron release.

The dependence of the rate of iron release upon the concentration of added flavin was also investigated, and the results are shown in Figure 7. The reaction was found to reach a maximum rate of release at approximately 125 μM FMN. No ferritin iron release was observed in the absence of added flavin. The results shown in Figure 7 were obtained using control (uninduced) microsomes. The shapes of the curves obtained for induced microsomes were found to be the same, but the concentration of added flavin required to obtain maximal ferritin iron release rate was found to be higher for the induced microsomes. A concentration of 250 μM FMN was found to be sufficient to yield the maximum rate of ferritin iron release with both induced and uninduced microsomes.

This concentration was used in all subsequent assays in order that all reactions would proceed at a maximal rate irrespective of the source of the microsomal protein used.

From the results obtained in this section the final assay conditions were established. All assays were performed using 2.0 mg microsomal protein per ml and an initial ferritin iron concentration of 350 μM Fe^{3+}. Both these values lie on the linear portions of the respective concentration versus iron release rate curves. A concentration of 250 μM FMN was chosen in order that the concentration of added flavin would not be rate limiting. Glucose and glucose oxidase were added as an O_2 scavenging system in order to ensure low O_2 concentrations in the assay system. Glucose-6-phosphate and glucose-6-phosphate dehydrogenase were added to serve as an NADPH generating system in order to maintain the NADPH concentration in the assay system. Together with bipyridyl and the 25 mM Tris-HCl pH 7.4 buffer, these components made up the ferritin iron release assay used for this investigation.

Figure 1

Figure 1 shows the results of repetitive scanning using difference spectroscopy. Two identical samples were prepared, except that NADPH was omitted in the reference cuvette. The initial scan was made immediately following addition of the flavin to the samples. Scanning was then repeated at t= 2, 4, 6, and 8 minutes. The increase in absorbance at 530 nm is due to the formation of $Fe^{2+}(bipyridyl)_3$ complexes as the iron is released from ferritin. The assay system used contained 350 μM ferritin iron, 2.0 mg uninduced microsomal protein per ml, 250 μM FMN, and 150 μM NADPH.

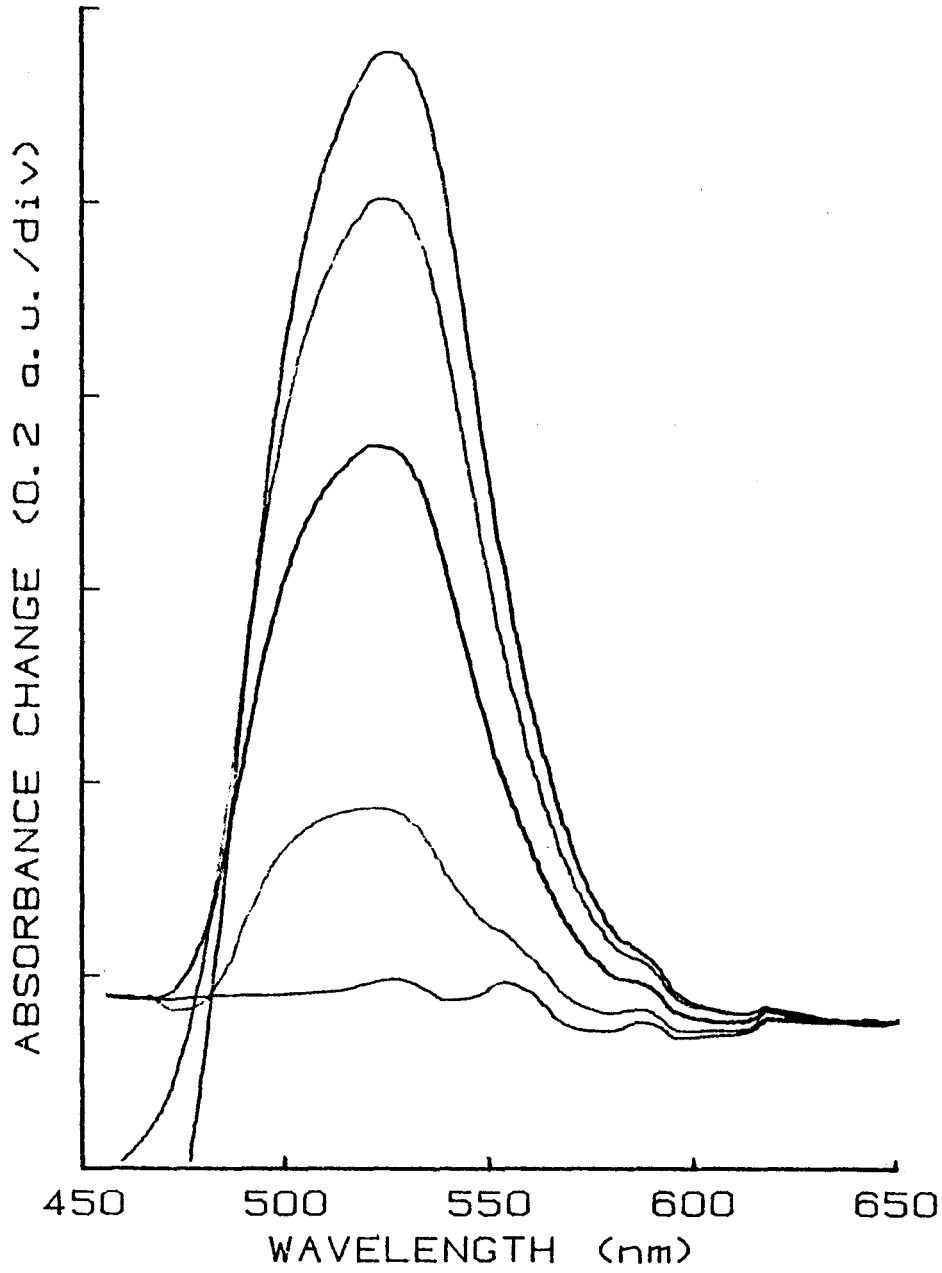

ABSORBANCE CHANGE (0.2 a.u./div)
450
500
550
600
650
WAVELENGTH (nm)

Figure 2

Figure 2 represents a typical change in absorbance versus time plot obtained when following the release of ferritin iron using the dual beam mode of the Perkin-Elmer 356 spectrometer. This figure demonstrates that there is a lag phase which preceeds the release of iron from ferritin. The linearly increasing portion of the change in absorobance versus time plot was used to calculate the rate of release of iron from ferritin. This plot was obtained using an assay system containing 350 μM ferritin iron, 2.0 mg uninduced microsomal protein per ml, 250 μM FMN, and 150 μM NADPH.

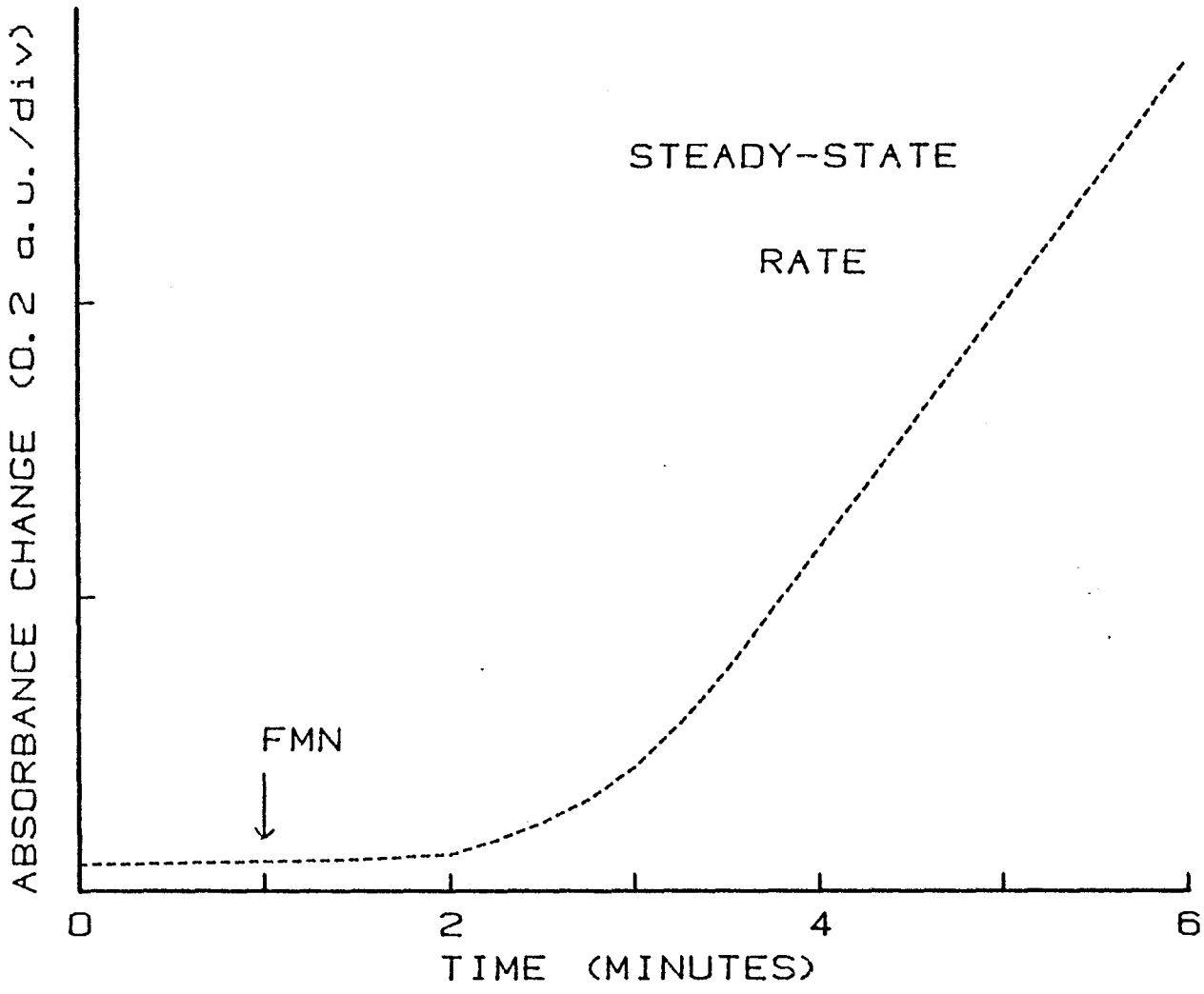

ABSORBANCE CHANGE (0.2 a.u./div)
STEADY-STATE
RATE
FMN
0
2
4
6
TIME (MINUTES)

Figure 3

Figure 3 demonstrates the effect of using NADPH, rather than NADH, as the source of reducing equivalents for the microsomal ferritin iron release pathway. A doubling of the rate of release of iron is observed. A rate of 23.42 (SD=0.96) nmole $Fe^{=2}$ released/min/ml was obtained with NADH, whereas a rate of 44.38 (SD=0.66) nmole Fe^{2+} released/min/ml was obtained with NADPH. Each assay contained 350 μM ferritin iron, 2.0 mg uninduced microsomal protein per ml, and 250 μM FMN. NADH or NADPH was added to a final concentration of 150 μM.

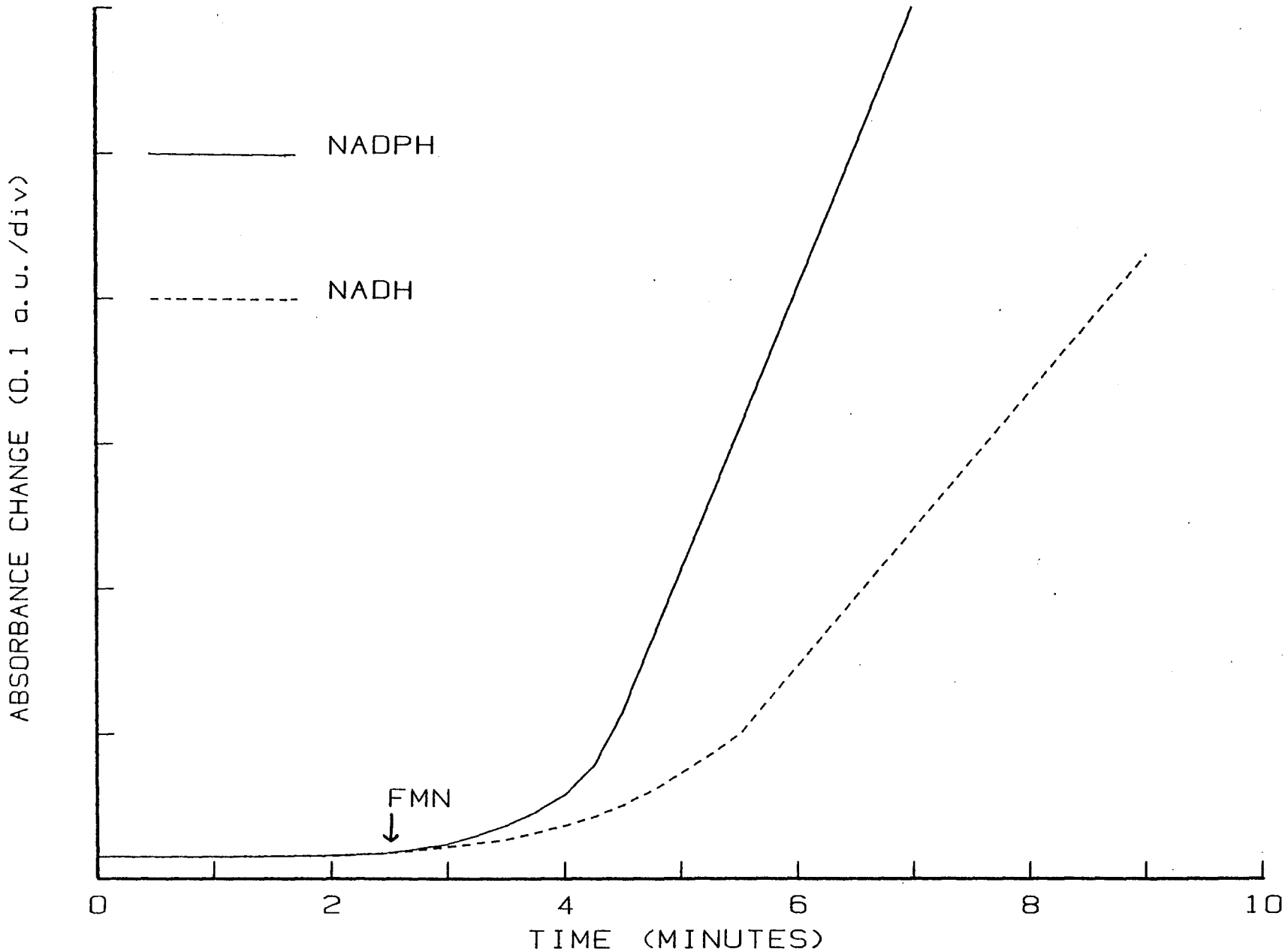

NADPH
NADH
FMN
ABSORBANCE CHANGE (0.1 a.u./div)
0
2
4
6
8
10
TIME (MINUTES)

Figure 4

Figure 4 illustrates the relationship between the initial ferritin iron concentration and the rate at which iron is mobilized from ferritin by microsomes supplied with NADPH and FMN. The straight line was fitted to the points using linear regression ($r=0.99$). Each assay contained 2.0 mg uninduced microsomal protein per ml, 250 μM FMN, and 150 μM NADPH.

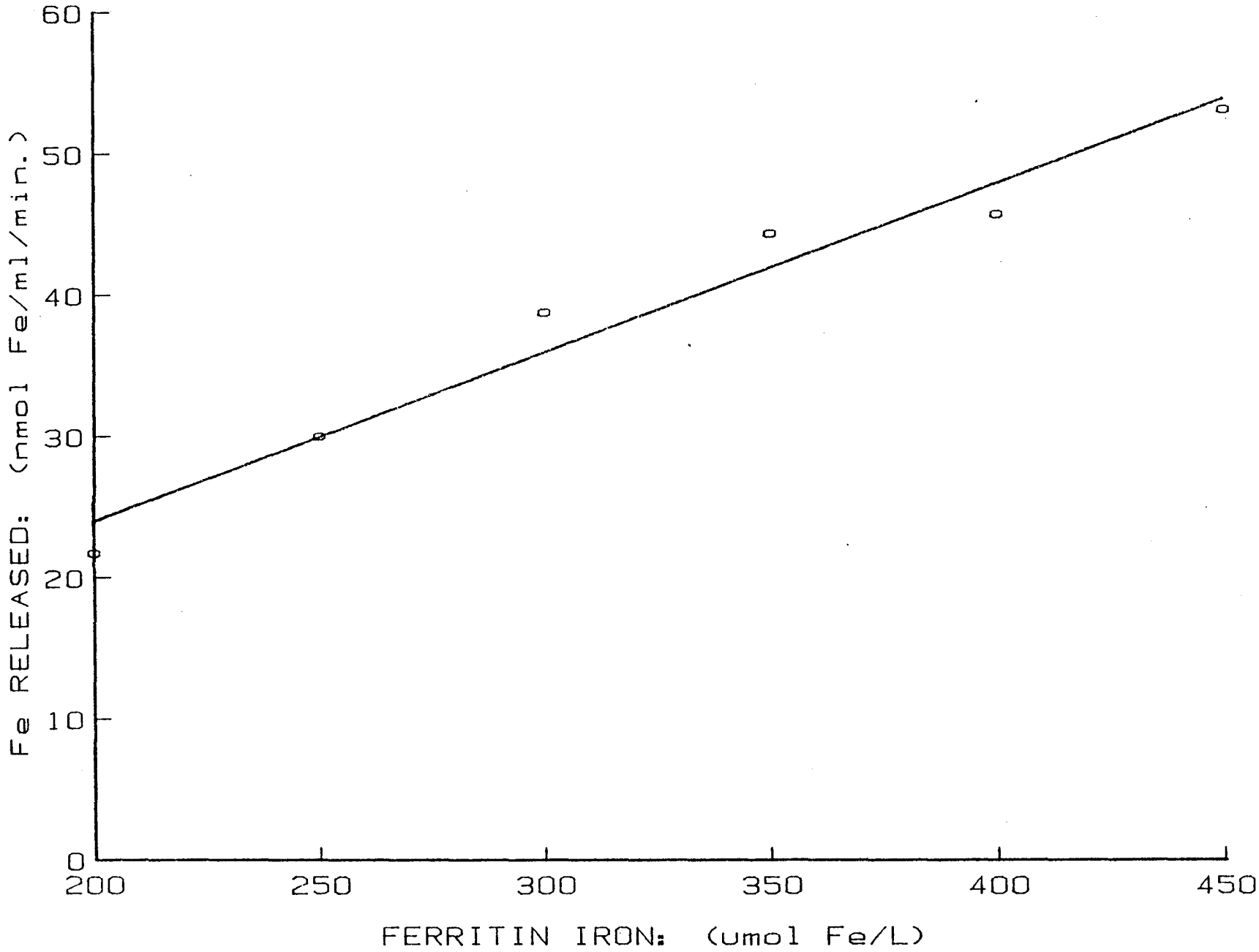

60
50
40
30
20
10
0
Fe RELEASED: (nmol Fe/ml/min.)
200
250
300
350
400
450
FERRITIN IRON: (umol Fe/L)

Figure 5

Figure 5 demonstrates the effect of varying the concentration of microsomal protein on the change in absorbance versus time plots obtained when monitoring ferritin iron release using the dual beam mode of the Perkin-Elmer 356 spectrometer. This figure shows that as the concentration of protein increases, so does the rate at which iron is mobilized from ferritin. This figure also demonstrates that the duration of the lag phase, which precedes ferritin iron release, also depends upon the concentration of microsomal protein. As the concentration of protein increases, the duration of the lag phase decreases. Each assay contained 350 μM ferritin iron, 250 μM FMN, and 150 μM NADPH.

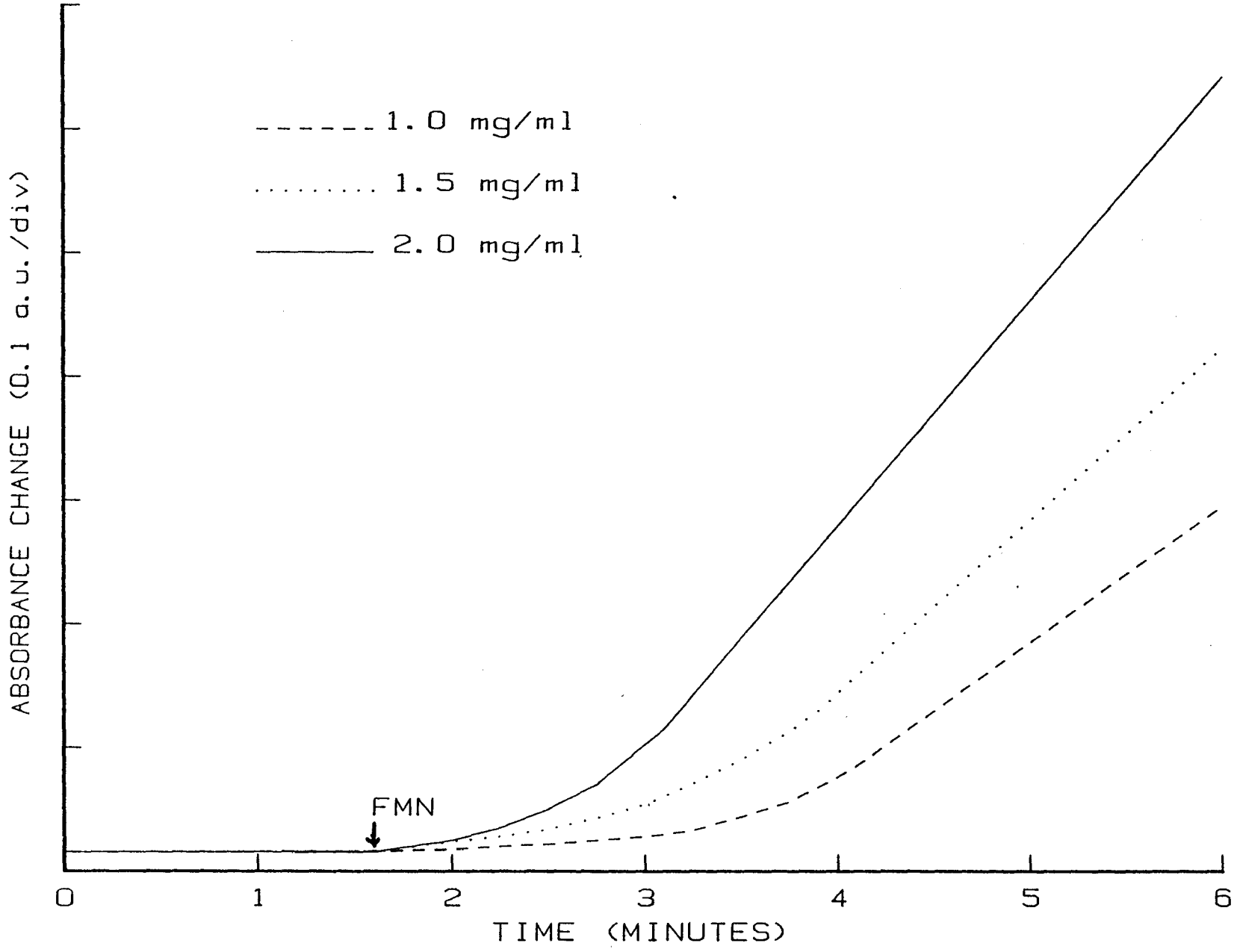

1.0 mg/ml
1.5 mg/ml
2.0 mg/ml
ABSORBANCE CHANGE (0.1 a.u./div)
FMN
0
1
2
3
4
5
6
TIME (MINUTES)

Figure 6

Figure 6 illustrates the relationship between the concentration of microsomal protein and the rate at which iron is released from ferritin. A linear relationship is observed for protein concentrations greater than 0.5 mg protein per ml. Each assay contained 350 μM ferritin iron, 250 μM FMN, and 150 μM NADPH.

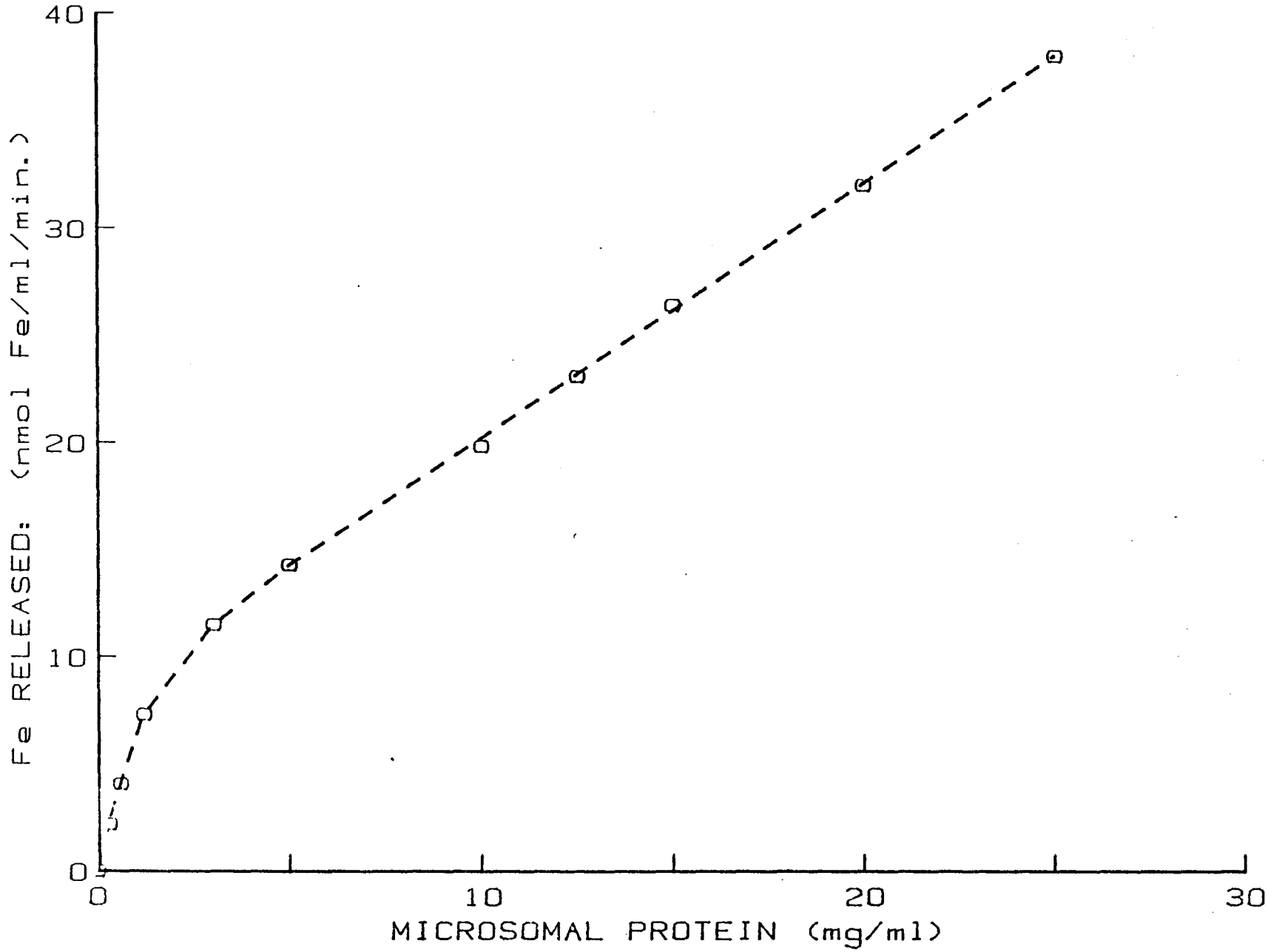
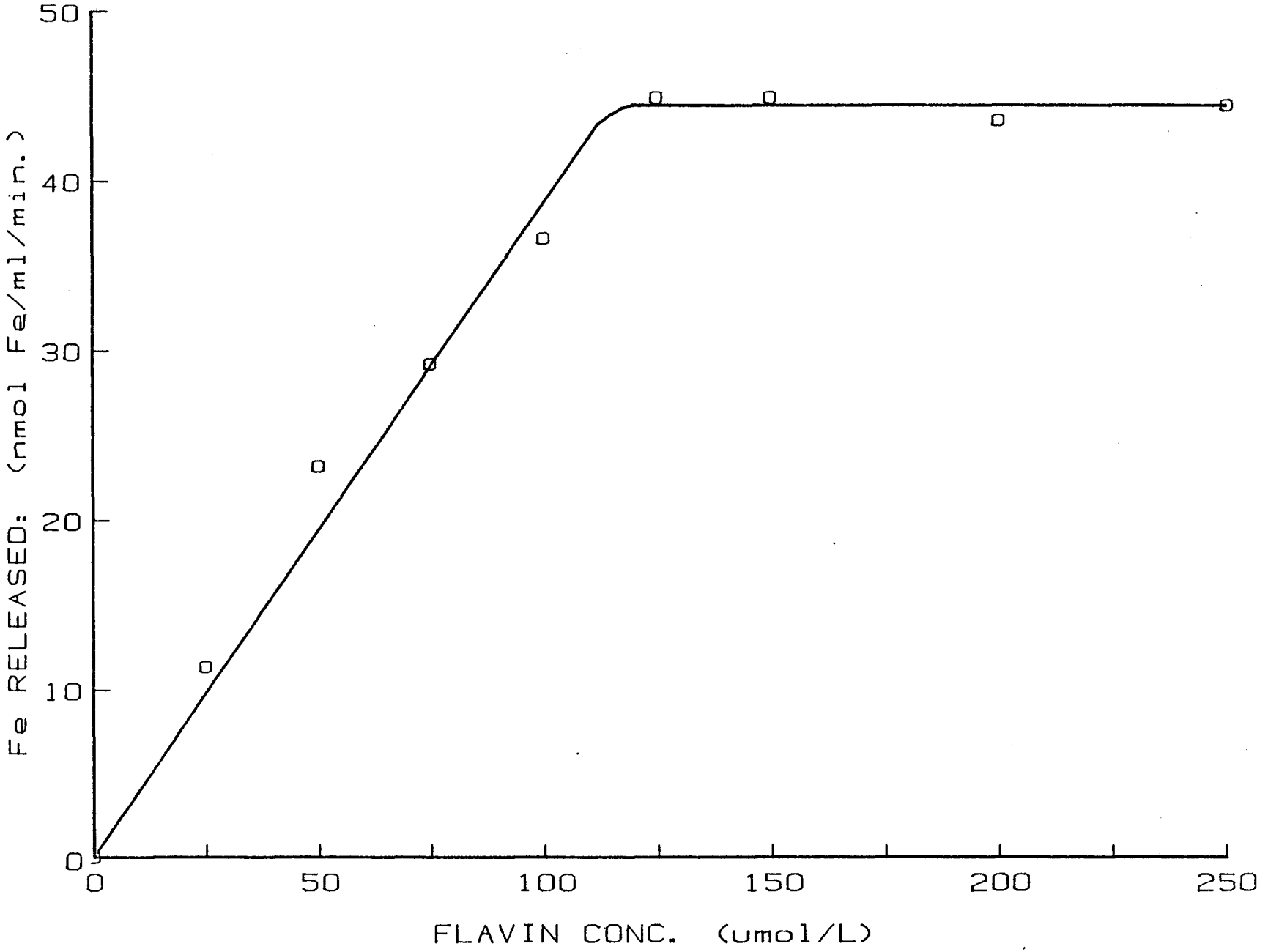

40
30
20
10
0
Fe RELEASED: (nmol Fe/ml/min.)
0
10
20
30
MICROSOMAL PROTEIN (mg/ml)

Figure 7

Figure 7 demonstrates the effect of added flavin concentration on the rate of microsomal ferritin iron release. A maximal rate of iron release is achieved at approximately 125 μM FMN. Each assay contained 350 μM ferritin iron, 250 μM FMN, 150 uM NADPH, and 2.0 mg uninduced microsomal protein per ml.


50
40
30
20
10
0
Fe RELEASED: (nmol Fe/ml/min.)
0
50
100
150
200
250
FLAVIN CONC. (umol/L)

2. Investigation of Ferritin Iron Release Using Intact Microsomes

To attempt to determine the microsomal electron transport protein responsible for ferritin iron release, the rate of release of ferritin iron by induced and uninduced microsomal protein fractions was investigated.

The levels of cytochrome P450 (or cytochrome P_1450), reductase activity, and total microsomal protein were determined for each microsome sample used in this investigation. The results of the characterization of the microsomal protein samples are shown in Table 1.

The rate of release of iron from ferritin by these microsomal protein samples was then determined using assay conditions under which the microsmal protein responsible for ferritin iron release would presumably become the rate determining factor. The rate of ferritin iron release was calculated for each microsomal protein sample investigated, and expressed in terms of the respective protein, reductase activity, and cytochrome P450 (or cytochrome P_1450) content of the microsomes. The results of these experiments are presented in Table 2.

The results of these experiments suggest that the rate determining microsomal protein in the microsomal

ferritin iron release pathway is cytochrome P450. Cytochrome P450 is indicated as being the rate limiting component in control, in short term TCDD induced, and in PB induced microsomes, as all three of these microsomal fractions yielded the same rate of release of ferritin iron when the rate was expressed as nmole Fe^{2+} released/min/nmole cytochrome P450 ($p<0.01$). The rates of iron release obtained with these fractions were not comparable when expressed in any other manner.

The fact that the long term TCDD induced microsomes did not follow the same pattern (ie. did not yield the same rate when expressed per nmole cytochrome P450 ($p<0.01$)), was attributed to the high ratio of molecules of cytochrome P_1450 per unit of NADPH-cytochrome c (P450) reductase activity determined for these microsomes (see Table 1). It appears that the lack of induction of the reductase by TCDD has an effect upon the rate of ferritin iron release by long term TCDD induced microsomes. Long term TCDD induction results in significantly increased levels of cytochrome P_1450 present in the microsomes, when compared to control values, but the level of reductase activity does not increase. Thus, there are more molecules of cytochrome P450 per unit reductase activity (more than double) in long term TCDD induced microsomes than in control microsomes. It is possible that, in the case of long term TCDD induced microsomes, NADPH-

cytochrome c (P450) reductase has become the rate limiting microsomal electron transport protein in the microsomal ferritin iron release pathway.

The results presented in Table 2 also show that the form of cytochrome P450 present in the microsomes appears to have no effect upon the rate of microsomal ferritin iron release. Both short term TCDD induced and control microsomes exhibit the same rate of iron release when expressed per nm cytochrome P450, even though the predominating form of cytochrome P450 present is different. Cytochrome P450 predominates in control microsomes, whereas cytochrome P_1450 predominates in short term TCDD induced microsomes.

Thus, the results of these experiments indicate that, while the form of cytochrome P450 present appears to have no effect upon the rate of microsomal ferritin iron release, the amount of cytochrome present does appear to have an effect. The results also suggest that there may be some critical value for the ratio of molecules of cytochrome P450 per unit reductase activity which when exceeded, allows NADPH-cytochrome c (P450) reductase, rather than cytochrome P450, to become the rate limiting component in the microsomal ferritin iron release pathway.

Table 1

Table 1 shows the results of the characterization of the microsomal protein samples used for the investigation of microsomal ferritin iron release. These values represent the mean of six determinations.

TABLE 1 Characterization of the Microsomal Protein Samples Used

Microsomes	Protein (mg/ml)	Cyt. c. Red.Act. (unit/ml)	Cyt.P450 Conc.(μM)	nmol P450 per unit Red.
Control	12.3±0.5	2.09±0.12	11.6±0.2	5.6
Short term TCDD induced	13.7±0.7	1.96±0.11	13.4±0.4	6.8
Long term TCDD induced	11.8±0.6	1.46±0.12	18.2±0.3	12.5
PB induced	25.0±0.7	4.52±0.14	37.4±0.4	8.3

Table 2

The mean rates of microsomal ferritin iron release (n=6) shown in Table 2 were determined using 2.0 mg/ml microsomal protein prepared from PB induced, TCDD induced, and control rat livers. The results of the characterization of the microsomal protein samples used to prepare the data presented in Table 2 are shown in Table 1. Each assay contained 350 μM ferritin iron, 250 μM FMN, and 150 μM NADPH.

TABLE 2 Rates of Iron Release at Saturating Flavin Concentration

nmol Fe^{2+} released per minute

Sample	Per ml	Per mg protein	Per nmol cytochrome	Per unit reductase
Control Microsomes	44.38±0.66	22.20±0.32	23.48±0.34	130.92±1.92
Short term TCDD induced microsomes	46.54±1.36	23.60±1.26	23.98±0.70	163.86±4.80
Long term TCDD induced microsomes	57.92±2.54	28.98±1.26	19.12±0.84	238.4±10.4
PB induced microsomes	69.92±1.20	34.50±0.60	23.18±0.40	191.86±3.30

3. The Effect of Inhibitors of Cytochromes P450 and Cytochrome P_1450

The results obtained in the preceding section suggested that cytochrome P450 is the microsomal electron transport protein from which the added FMN drained its reducing equivalents for ferritin iron release. In order to verify this finding, the effect of various inhibitors of cytochromes P450 and cytochrome P_1450 on the rate of microsomal ferritin iron release was investigated. The inhibitors SKF-525A and 7,8-benzoflavone were chosen for use because these compounds are specific inhibitors of cytochrome P450 and cytochrome P_1450, respectively. Hexobarbital, although a substrate rather than an inhibitor, was chosen because this compound has been shown to bind to the active site of cytochrome P450. Carbon monoxide was chosen because it inhibits both cytochrome P450 and cytochrome P_1450 mediated reactions by binding to the heme moiety of the cytochrome, rather than the substrate binding site. It was postulated that, if the cytochrome is the microsomal electron transport protein from which the added flavin drains its reducing equivalents for ferritin iron release, then the presence of these inhibitors in the assay system should result in a decrease in the rate of microsomal ferritin iron

release. PB and TCDD induced microsomes were used in this investigation, in order that microsomes used would contain one predominant form of the cytochrome, rather than a mixture of several different forms, allowing for better interaction between the inhibitors and the specific form of cytochrome which they inhibit. The results of these experiments are shown in Table 3.

It can be seen that none of the inhibitors tested exhibited an effect on the rate of release of iron from ferritin by microsomes. This lack of inhibition suggests that cytochrome P450 is not the microsomal electron transport protein from which the added FMN drains its reducing equivalents for the release of ferritin iron. If cytochrome P450 does not serve as the donor of electrons to FMN in the microsomal ferritin iron release pathway, the most likely alternative would be NADPH- cytochrome c (P450) reductase. It was decided that the best method to be used in order to determine the roles of both cytochrome P450 and the reductase in the microsomal ferritin iron release process, would be to separate and partially purify these proteins from microsomes and then attempt to reconstitute a functional microsomal ferritin iron mobilizing system.

Table 3

Table 3 shows the results of attempts to inhibit microsomal ferritin iron release using inhibitors of cytochrome P450 and P_1450. No inhibition of ferritin iron release was observed with any of the inhibitors tested. Each assay contained 2.0 mg microsomal protein per ml, 350 μM ferritin iron, 250 μM FMN, and 150 μM NADPH. SKF-525A, 7,8-benzoflavone, and hexobarbital were added to a final concentration of 100 μM.

TABLE 3 The Effect of Inhibitors of Cytochrome P450 and P_1450

Inducer	Inhibitor added	Rate of iron release ($nmol\ Fe^{2+}/min/ml$).
TCDD	None	47.4
TCDD	SKF-525A	46.20
TCDD	7,8-benzoflavone	46.54
TCDD	Carbon monoxide	46.54
TCDD	Hexobarbital	46.04
PB	None	69.38
PB	SKF-525A	69.38
PB	7,8-benzoflavone	68.10
PB	Carbon monoxide	69.66
PB	Hexobarbital	68.20

4. Separation and Partial Purification of Cytochrome P450, Cytochrome P_1450, and NADPH-cytochrome c (P450) reductase from Microsomes

Although it is possible to separate cytochrome P450 and NADPH-cytochrome c (P450) reductase by simple chromatography of detergent solubilized microsomes on DEAE-cellulose (61), the reductase fractions obtained using this method were found to contain cytochrome b_5 as an impurity. Because a hemoprotein free reductase fraction was desired, an alternative method for the separation of these proteins was required. The purification method used was a combination of the method of Dignam and Strobel (62) for the purification of the reductase, and the method of Warner et al (63) for the purification of the cytochromes. The results of the separation and partial purification of the proteins using this method are shown in Tables 4 and 5. Cytochrome P450 was purified from PB induced microsomes, cytochrome P_1450 was purified from TCDD induced microsomes, and NADPH-cytochrome c (P450) reductase was purified from both PB and TCDD induced microsomes.

The specific content of the cytochrome P450 and cytochrome P_1450 fractions obtained were lower than was expected. Because the heme moiety of the cytochrome is not

covalently attached, it can be lost in the course of purification. This loss of heme from the cytochrome results in an apparent low recovery of cytochrome P450 or cytochrome P_1450, as the detection of the cytochrome is dependent upon the presence of the heme. The co-elution of apocytochrome P450 or apocytochrome P_1450 with the intact cytochrome P450 or cytochrome P_1450 results in a low specific content value (ie. nmoles cytochrome/mg protein) for the fractions isolated, because the protein measurements will reflect the presence of apocytochrome in the isolated fractions, but the CO binding spectra used to measure cytochrome P450, detects only the cytochrome itself. In order to obtain higher specific content values for the cytochrome fractions isolated, it was found necessary to prewash the columns with a heme containing solution prior to chromatography, in order to saturate any heme binding sites present on the columns. Warner et al saturated these sites by preparing their microsomes from nonperfused livers. The microsome fractions obtained in this way contain hemoglobin as an impurity. Hemoglobin does not bind to the DEAE columns, but will lose heme to the heme binding sites on the column. Because the heme binding sites are occupied by heme from hemoglobin, cytochrome P450 retains its heme moiety over the course of chromatography. This technique could not be used with our method as the chromatography of hemoglobin containing samples

resulted in a heme impurity co-eluting with the reductase fraction.

Very little heme appeared to be lost until the DEAE-cellulose step of the procedure. It is at this step that saturation of heme binding sites on the column becomes important. Saturation of these sites was attempted by prewashing the DEAE-cellulose column with a solution containing 1 μM hemin in equilibration buffer; however this was unsuccessful as the hemin tended to precipitate upon entering the column packing. It appears as if the hemin were not able to enter into the packing and saturate the heme binding sites on the column, as no increase in specific content of cytochrome P450 was obtained when samples were chromatographed on hemin pretreated columns. Because of the failure of this technique, and because a cytochrome of high specific content was not required for the reconstitution experiments which followed, attempts to increase the specific content of the cytochrome P450 and cytochrome P_1450 fractions isolated were not pursued.

The NADPH-cytochrome c (P450) reductase fractions isolated by this method were again somewhat lower in specific activity than was expected. Dignam and Strobel reported a specific activity of 4.30 units/mg protein for reductase fractions following chromatography on DEAE-Sephadex. Both procedures are the same up to and including the DEAE-Sephadex

chromatography step, except that Dignam and Strobel start with 5 to 10 times as much microsomal protein. It is possible that in scaling down their procedure, we were unable to maintain the same degree of purification.

Having obtained separation of the cytochromes and the reductase, with neither fraction contaminated by the other, the next step was to attempt the reconstitution of MFO activities using the protein fractions isolated. By demonstrating the reconstitution of MFO activities using the proteins isolated by our method, one also demonstates that the method of reconstitution, and proteins to be used for reconstitution, are suitable for use in the investigation of microsomal ferritin iron release using a reconstituted system.

Table 6 shows the results of reconstitution of aryl hydrocarbon hydroxylase and benzphetamine-O-demethylase activities using proteins isolated by our method of separation. The lipid used for the reconstitution of these activities was dilauroylphosphatidylcholine (DLPC). DLPC was chosen for use because it has been demonstrated to yield the highest activities in reconstituted MFO systems (64). The results reported in Table 6 are very similiar to the results obtained by Lu et al (65), when they reconstituted these MFO activities using proteins purified by a different method, but of similiar specific content.

The results shown in Table 6 show that the microsomal source of the reductase fraction used to reconstitute activity was unimportant. Reductase purified from either source appears to be capable of recombining effectively with both cytochrome P450 and cytochrome P_1450. Because of this finding, it was decided that only the reductase fraction separated from PB induced microsomes would be used for the reconstitution experiments designed to investigate the microsomal ferritin iron release process.

Having demonstrated that the proteins obtained by our method of separation could be used to reconstitute MFO activities, the next step in the investigation was to attempt to reconstitute microsomal ferritin iron release using the same system.

Table 4

Table 4 shows the results of the separation and partial purification of cytochrome P450 and NADPH-cytochrome c (P450) reductase from PB induced microsomes.

TABLE 4 Results of Separation and Partial Purification of Cytochrome P450 and NADPH-cytochrome-c-(P450)-reductase from PB Microsomes

	Cytochrome P450			NADPH-cyt-c (P450)-red.		
Fraction	Total nanomoles cyt.P450	Specific Content (nmol /mg)	Percent Recovery	Total Units cyt-c-red.	Specific Activity (unit/mg)	Percent Recovery
Microsomes	592	0.97	100	86	0.14	100
After detergent solubilization	583	1.00	98	87	0.15	101
After protamine sulphate precipitation	522	1.15	88	87	0.17	101
After DEAE-sephadex chromatography	523	1.40	88	24	3.60	28
After DEAE-cellulose chromatography	173	2.90	29	--	--	--

Table 5

Table 5 shows the results of the separation and partial purification of cytochrome P_1450 and NADPH-cytochrome c (P450) reductase from short term TCDD induced microsomes.

TABLE 5 Results of Separation and Partial Purification of Cytochrome P_1450 and NADPH-cytochrome-c (P450)-reductase from TCDD Microsomes

Fraction	Cytochrome P_1450			NADPH-cyt-c (P450)-red.		
	Total nanomoles cyt.P_1450	Specific Content (nmol/mg)	Percent Recovery	Total Units cyt-c-red.	Specific Activity (unit/mg)	Percent Recovery
Microsomes	475	0.72	100	73.7	0.11	100
After detergent solubilization	470	0.68	99	70.8	0.10	96
After protamine sulphate precipitation	459	0.94	97	65.7	0.14	89
After DEAE-Sephadex chromatography	472	2.20	99	26.3	2.10	36
After DEAE-cellulose chromatography	157	3.60	33	--	--	--

Table 6

Table 6 shows the results of attempts to reconstitute aryl hydrocarbon hydroxylase and benzphetamine-O-demethylase activities using microsomal proteins obtained using our method of purification. The levels of activity achieved are similiar to results obtained by Lu et al (65) using proteins of similiar specific activity. PB Red is reductase purified from PB induced microsomes. TCDD Red is reductase purified from TCDD induced microsomes. The results shown in Table 6 represent the average result obtained. Each assay was performed twice, in duplicate.

TABLE 6 Reconstitution of MFO Activities

(1) Reconstitution of Aryl Hydrocarbon Hydroxylase Activity

Cyt c Red. Activity (10^{-3} unit/ml)	Cyt $P_{(1)}450$ Content (nmol/ml)	Units AHH Activity
15.4 TCDD Red.	0.04 P_1450	59
15.4 TCDD Red.	0.05 P450	5
30.5 PB Red.	0.12 P_1450	126
30.5 PB Red.	0.10 P450	7

(2) Reconstitution of Benzphetamine-O-demethylase Activity

Cyt c Red. Activity (10^{-3} unit/ml)	Cyt $P_{(1)}450$ Content (nmol/ml)	nmol CH_2O formed in 10 min.
130 TCDD Red.	0.22 P_1450	--
130 TCDD Red.	0.21 P450	28.3
130 PB Red.	0.22 P_1450	--
130 PB Red.	0.21 P450	30.3

5. Reconstitution of Microsomal Ferritin Iron Release

To determine the microsomal electron transport protein responsible for ferritin iron release, cytochrome P450 or cytochrome P_1450 and NADPH-cytochrome-c (P450)-reductase were recombined with lipid, together and separately, and the release of ferritin iron by these reconstituted systems was assayed. The results of these experiments demonstrated that only the reductase need be recombined with lipid in order to observe release of iron from ferritin.

Figure 8 shows the dependence of the rate of release of ferritin iron on the amount of reductase activity present, both in intact microsomes and in the reconstituted system. These results indicate that the reductase is the microsomal electron transport protein from which the FMN drains its reducing equivalents for ferritin iron release. This is indicated by the fact that the curves obtained for both the intact microsomes and the reconstituted system are essentially identical.

Figure 9 shows the dependence of the duration of the lag phase upon the amount of reductase activity present, in both intact microsomes and the reconstituted system. Unlike the curves obtained for the rate of iron release versus

reductase activity (Figure 8), the two curves shown in Figure 9 are not identical. The duration of the lag phase observed with the reconstituted system (containing only the reductase) was always greater than that observed with intact control microsomes containing the same amount of reductase activity. This difference in the duration of the lag phase, between the two systems, suggests that the reconstituted system is in some way deficient. Because of the earlier results which suggested that cytochrome P450 was responsible for microsomal ferritin iron release (the data in Table 2), the effect of adding varying amounts of cytochrome P450 or cytochrome P_1450 to a reconstituted system containing a constant amount of reductase activity was investigated. The results of these experiments are shown in Table 7.

The results shown in Table 7 demonstrate that as cytochrome P450 or cytochrome P_1450 is added back into the reconstituted system, the duration of the lag phase observed decreases. The results also show an increase in the rate at which iron is released from ferritin at the highest level of cytochrome added back. These findings suggest that the cytochromes also play a role in the mobilization of iron from ferritin by microsomes, but the exact nature of this role is unclear. It is possible that, due to the presence of cytochrome P450 or cytochrome P_1450, the reconstituted membrane system is better able to bind one or more of the

reactants in the ferritin iron release system.

Through the use of reconstituted membrane systems, this investigation of microsomal ferritin iron release has provided evidence that NADPH-cytochrome c (P450) reductase serves as the microsomal electron transport protein responsible for the generation of the reduced flavin required for ferritin iron release. The results of this investigation also indicate that the cytochromes play an as yet undefined role in the microsomal ferritin iron release process.

Figure 8

Figure 8 shows the dependence of the rate of ferritin iron release upon the amount of NADPH-cytochrome c reductase activity present, in both intact microsomes and in reconstituted membrane systems containing reductase alone. Both systems exhibit the same relationship, indicating that the reductase is the microsomal electron transport protein responsible for microsomal ferritin iron release. Each assay contained 350 μM ferritin iron, 250 μM FMN, and 150 μM NADPH.

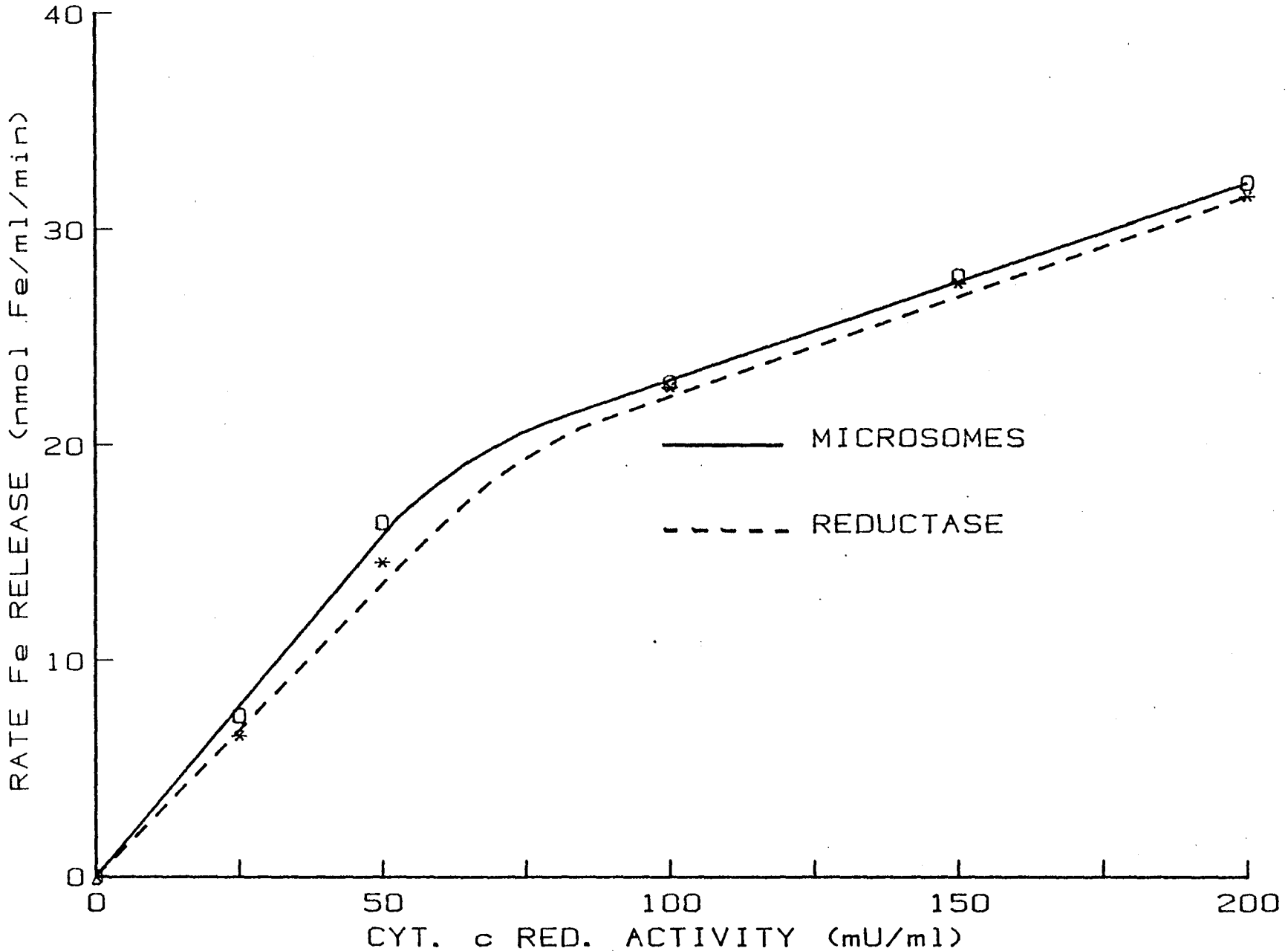

40
30
20
10
0
RATE Fe RELEASE (nmol Fe/ml/min)
0
50
100
150
200
CYT. c RED. ACTIVITY (mU/ml)
MICROSOMES
REDUCTASE

Figure 9

Figure 9 demonstrates the relationship between the duration of the lag phase preceeding ferritin iron release and the amount of NADPH-cytochrome c reductase activity present. The results obtained with both intact microsomes and reconstituted membrane systems containing reductase alone are shown. While the shape of the two curves are simliar, the duration of the lag phase was always greater with the reconstituted system. Each assay contained 350 μM ferritin iron, 250 μM FMN, and 150 μM NADPH.

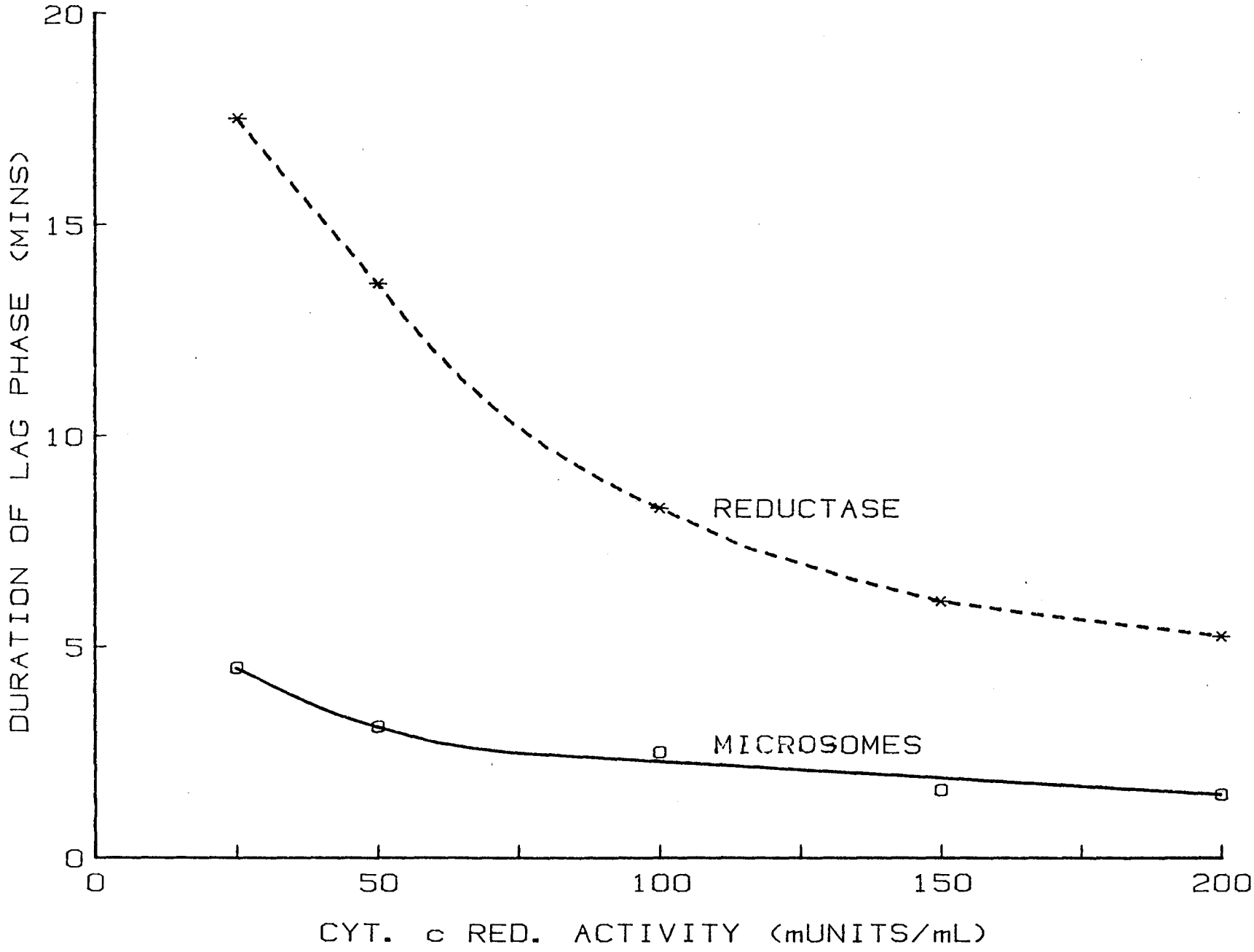

20
15
10
5
0
0
50
100
150
200
DURATION OF LAG PHASE (MINS)
CYT. c RED. ACTIVITY (mUNITS/mL)
REDUCTASE
MICROSOMES

Table 7

Table 7 shows the effect that the amount of cytochrome P450, or cytochrome P_1450, present in a reconstituted system has upon the duration of the lag phase preceding ferritin iron release. The effect of the cytochrome P450, or cytochrome P_1450, concentration on the rate of ferritin iron release is also shown. The cytochrome was reconstituted with a constant amount of reductase activity (0.1 unit/ml) and DLPC (200 μg). Each assay contained 350 μM ferritin iron, 250 μM FMN, and 150 μM NADPH.

TABLE 7 The Effect of Cytochrome P450 or P_1450 on Reconstituted Ferritin Iron Release

Cytochrome Added (nmol/ml)	Rate of Iron Release (nmole Fe/ml/min)	Lag Time (min.)
0.25 P450	21.68	6.0
0.50 P450	21.68	5.3
0.75 P450	23.54	4.0
1.00 P450	26.30	3.5
0.25 P_1450	21.68	6.4
0.50 P_1450	22.60	5.3
0.75 P_1450	22.60	4.2
1.00 P_1450	27.24	3.8
None	22.60	8.3

Discussion

The histologic damage observed with TCDD toxicity is similar to damage associated with lipid peroxidation processes. Ferrous iron has been demonstrated to be important as a catalyst of lipid peroxidation reactions (22,23,24). Thus, it has been postulated that the role of storage iron in the liver toxicity caused by TCDD is the initiation and promotion of lipid peroxidation processes. In order for storage iron to be involved in such processes, the iron would first have to be mobilized from the iron storage protein ferritin. With these ideas in mind, an investigation of a possible mechanism of microsomal ferritin iron release was undertaken.

Mobilization of ferritin iron by the microsomal subcellular fraction has been demonstrated by several investigators (43,44). In all cases, the release of iron from ferritin was attributed to the generation of reduced flavins by the microsomal fractions. The results obtained in this investigation support this hypothesis. Ferritin iron release could not be demonstrated in the absence of either microsomal protein or flavin. The requirement for flavin indicates that whatever microsomal protein is involved in the process, it does not interact directly with the ferritin molecule in

order to effect the release of iron from ferritin. Because only reduced FMN is capable of releasing iron from ferritin, and because the flavin is added to the assay mixture in the oxidized form, it seems reasonable to propose that the role of the microsomal protein in this process is the generation of the reduced flavins required for ferritin iron release.

In order for the toxic effects of TCDD to become manifest in treated animals, these animals must be responsive to induction of the Ah genetic locus (14,15); ie induction of AHH activity. Because NADPH is generally used as the source of reducing equivalents when assaying microsomes for MFO activities (54), it was postulated that NADPH may serve as a more suitable source of reducing equivalents for the microsomal ferritin iron release process. In the previous investigations of microsomal ferritin iron release, NADH had been used as the source of reducing equivalents supplied to the microsomal electron transport chain. The results of an experiment in which NADH was replaced by NADPH in the microsomal ferritin iron release assay system, showed that the presence of NADPH leads to a doubling of the rate at which iron is released from ferritin. This finding suggests that NADPH is the preferred source of electrons for the microsomal ferritin iron release pathway. That NADPH is the preferred electron source for the pathway was important in establishing a link between the microsomal ferritin iron

release process, and induction of the Ah genetic locus.

The first step taken in order to determine the microsomal protein responsible for ferritin iron release was to establish assay conditions under which, presumably, this protein would become the rate determing component in the ferritin iron release pathway. Having established these conditions (see the Results Section), the rate of release of iron from ferritin by induced and uninduced microsomal fractions was determined. The results indicated that cytochrome P450 is the rate limiting microsomal component in the ferritin iron release pathway, however some doubt was cast upon the validity of this interpretation of the results. Firstly, the rate of release of iron determined for long term TCDD induced microsomes was not in agreement with the values determined for the other microsomal fractions investigated, when expressed as nmole Fe^{2+} released/min/nmole cytochrome P450. This discrepency could be rationalized by attributing the difference to the fact that NADPH-cytochrome c (P450) reductase is not induced by TCDD. Due to the induction of the cytochrome, and not the reductase, the ratio of molecules of cytochrome P450 per unit of reductase activity determined for the long term TCDD microsomes was more than double the value determined for noninduced control microsomes. Thus, it could be suggested that, in the case of long term TCDD induced microsomes, the reductase becomes the rate limiting

microsomal component in the ferritin iron release pathway.

Another factor which cast some doubt upon the suggestion that cytochrome P450 was the rate determining microsomal component of the ferritin iron release pathway was the finding that the type, or form, of cytochrome P450 present appears to have no effect upon the rate of release of iron from ferritin. If electrons were being shuttled through cytochrome P450 to the flavin, one would have expected to observe some kind of effect. Assuming that cytochrome P450 acts as the electron donor in the ferritin iron release pathway, one would expect that the electrons donated to the flavin would be passed on through the active site of the cytochrome. Because of differences in substrate specificities between the two families of cytochrome P450, the affinity of the cytochromes for flavin would be expected to be somewhat different. The results obtained here however do not support any differences in affinity for flavin, as both forms of cytochrome P450 appear to mobilize ferritin iron at the same rate.

The lack of inhibition of ferritin iron release by cytochrome P450 and cytochrome P_1450 inhibitors confirmed the suspicions that interpretation of the data presented in Table 2 was in error. The results of the inhibitor study provided strong evidence suggesting that cytochrome P450 does not act as the donor of electrons to FMN in the microsomal ferritin

iron release pathway, as ferritin iron release was not inhibited by any of the inhibitors tested. In view of this finding it was postulated that NADPH-cytochrome c (P450) reductase would be the most likely alternative microsomal electron transport protein to be responsible for the production of the reduced flavin required for ferritin iron release. It was decided that the most effective means of investigating microsomal ferritin iron release would be to separate cytochrome P450 and NADPH-cytochrome c (P450) reductase from microsomes, and then attempt to reconstitute a functional microsomal ferritin iron mobilizing system.

Before reconstitution of ferritin iron release could be attempted, reconstitution of known MFO activities was investigated. The finding that the levels of reconstituted MFO activity achieved were similar to the levels of reconstiuted activity achieved by Lu et al (65), using proteins of similiar specific content, demonstrated that the partially purified proteins obtained through our method of purification were suitable for use in the investigation of microsomal ferritin iron release using a reconstituted system.

The results of the reconstitution experiments performed in order to investigate microsomal ferritin iron release demonstrated that NADPH-cytochrome c (P450) reductase is the microsomal electron transport protein responsible for

ferritin iron release. No iron release was observed in the absence of this protein, and the curve obtained for the rate of iron release versus the reductase activity present closely paralleled the curve obtained for intact control microsomes. NADPH-cytochrome c (P450) reductase has been demonstrated to be capable of donating electrons to several artificial electron acceptors besides its native electron acceptor, cytochrome P450 (66). Thus, it is not unreasonable to propose that this protein serves as the electron donor to FMN in the microsomal ferritin iron release pathway.

The difference in lag phase time observed for the reductase alone, compared with the lag phase observed for intact microsomes, suggested that there was some deficiency in the reconstituted microsomal ferritin iron mobilizing system containing reductase alone. Because of the earlier results obtained suggesting that cytochrome P450 served as the electron donor to FMN in the microsomal ferritin iron release pathway, it was suggested that perhaps cytochrome P450 performs a role in the ferritin iron release process. The results of an experiment in which various amounts of cytochrome P450 or cytochrome P_1450 were added to a reconstituted system containing a constant amount of reductase activity showed that the presence of cytochrome P450 in the reconstituted system caused a decrease in the lag phase time observed. The duration of the lag phase was found

to depend upon the amount of cytochrome P450 added back to the system. The more cytochrome P450 added back, the shorter the duration of the lag phase observed. At the highest level of cytochrome P450 added back, an increase in the rate of iron mobilization was also observed.

The duration of the lag phase is thought to represent the time necessary for the generation of a reduced flavin concentration sufficiently high to initiate ferritin iron release. It is possible that the role of cytochrome P450 is to serve as a membrane binding site for the added flavin. By doing so, cytochrome P450 might make the flavin more accessible to the reductase for electron transfer. Because the flavin would be more accessible to the reductase, the time required for the generation of a reduced flavin concentration sufficiently high to initiate ferritin iron release would likely decrease. In their proposed mechanism of mitochondrial ferritin iron release, Ulvik and Romslo (48) suggested that carbohydrate residues on the mitochondrial membrane surface may serve as sites at which the FMN binds in order to withdraw reducing equivalents from the mitochondrial electron transport chain component ubiquinone. Both cytochrome P450 and cytochrome P_1450 are glycoproteins, containing 1 glucosamine and 2 mannose residues per polypeptide chain (67). It is possible that the carbohydrate residues on the cytochromes may serve as the microsomal

membrane binding sites for FMN in the microsomal ferritin iron release pathway.

From the results of this investigation, a possible mechanism for the microsomal ferritin iron release process could be proposed. The results suggest that FMN may bind to the membrane at cytochrome P450 and is then reduced by NADPH-cytochrome c (P450) reductase. Once reduced, the flavin then migrates to the ferritin iron core, passes on its reducing equivalents to the ferric iron present, thus reducing it to the ferrous form, and allowing for its release from ferritin.

Induction of the MFO system by both PB and TCDD leads to an increase in the rate at which the microsomes will release iron from ferritin *in vitro* (see Table 2), but only TCDD induction leads toxicity *in vivo*. If the toxicity observed with TCDD results from the liberation of iron from ferritin *in vivo*, then there must be some difference in induction pattern which leads to release of iron from ferritin with TCDD induction, but not with PB induction. One difference in induction pattern which could lead to the toxicity observed with TCDD, but not with PB, is that TCDD is not metabolized by the cytochrome which it induces, whereas PB is. Thus, with TCDD induction, the induced microsomal electron transport system is not metabolically active and would therefore be free to particpate in a reaction in which

it may not normally be involved. With PB induction on the otherhand, the induced microsomal electron transport chain would be metabolically active (ie. metabolism of PB), and thus would not be free to participate in such a reaction.

Recently, experiments were performed in this laboratory in order to investigate the possibility that TCDD toxicity results from the lack of a metabolizable substrate. A cytochrome P_1450 substrate, namely Beta-naphthoflavone (B-NF), was given to mice in conjunction with treatment with TCDD. It was found that the inclusion of the cytochrome P_1450 substrate in the diet of TCDD treated mice resulted in partial protection against the morphologic changes associated with TCDD toxicity (68). In order to assess the significance of the protection against TCDD toxicity provided by B-NF, it must be borne in mind that TCDD is a much more potent inducer than is B-NF. While signifigant levels of cytochrome P_1450 and its associated MFO activities can be induced through oral administration of B-NF (69), prolonged exposure to B-NF is required. The level of induction achieved with the 5 % B-NF diet used in this experiment was equivalent to a dosage of approximately 1 µg/kg/week TCDD, whereas the dose of TCDD used to produce toxicity was 25 µg/kg TCDD, administered as a single injection (ip). It is possible that, because of the disparity in inducing abilities between the two compounds, the level of B-NF included in the diet was not sufficiently

high to allow for complete protection against the toxicity caused by TCDD.

While the results of this experiment in no way prove that TCDD toxicty results from the liberation of iron from ferritin, they do not rule out such a possiblity. The results provide support for the suggestion that TCDD toxicity may result from the participation of the induced MFO proteins in a process in which they may not normally be involved. Such a process could very well be the release of iron from ferritin, but this remains to be demonstrated.

Thus, in summary, the results documented in this thesis provide evidence that NADPH-cytochrome c (P450) reductase could serve as the microsomal electron transport protein from which FMN drains its reducing equivalents for ferritin iron release. This finding is important in that it provides a possible mechanism through which TCDD might exert its hepatotoxic effects. Although these results do not provide any direct evidence that TCDD toxicity results from the mobilization of storage iron from ferritin by the TCDD induced microsomal electron transport system, they do provide support for such a suggestion. It could be postulated that, owing to the lack of a metabolizable substrate, reducing equivalents are drained from the induced microsomal electron transport system by free cytosolic flavin, or possibly an as yet unidentified flavoprotein, and that these electrons,

rather than being utilized in the metabolism of the inducer TCDD, are instead being diverted to the mobilization of storage iron from ferritin. The lack of stainable storage iron in porphyric liver cells obtained from HCB treated rats (19,20) supports such a suggestion, as HCB and TCDD appear to cause toxicity via a similiar mechanism. Mobilization of storage iron would result in the presence of free unbound ferrous iron within the cell, which in turn could result in lipid peroxidation reactions causing membrane damage, and ultimately cell death. The presence of iron in the cytosol could also result in the development of the PCT like porphyria associated with TCDD toxicity. The porphyria caused by TCDD results from a decrease in the activity of the enzyme uroporphyrinogen decarboxylase, which allows this enzyme to become the rate limiting enzyme of the heme biosynthetic pathway (70). The evidence collected thus far suggests that porphyria develops because of synergism between TCDD and iron. Experiments have shown AHH responsiveness is necessary for the development of porphyria (14,15), and this has been interpreted to indicate that induction of cytochrome P_1450 (which leads to induction of heme synthesis) is required for the induction of porphyria. A synergistic role for iron is indicated by the finding that lower levels of liver iron clearly delay or prevent the porphyria from developing (16,37). Thus, a phenomenon such as the mobilization of

storage iron from ferritin could very well result in the hepatotoxic effects associated with TCDD poisoning.

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