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The incorporation of hemoglobin-heme into hepatic hemoproteins

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THE INCORPORATION OF HEMOGLOBIN-HEME INTO HEPATIC HEMOPROTEINS

by

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DISSERTATION

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ABSTRACT

Parenteral administration of heme often is highly beneficial in the treatment of acute hepatic porphyrias. In animals, exogenously administered heme can functionally reconstitute cytochrome P-450 destroyed by drugs, such as allylisopropylacetamide (AIA), during their cytochrome P-450-dependent oxidative metabolism. This suggested that heme might be useful in treating patients intoxicated with drugs that destroy cytochrome P-450. However, the high doses of heme required in these therapies are found to be toxic. In contrast, stroma-free hemoglobin has not only been therapeutically employed in hypo-volemic states without the toxic effects of heme, but is also primarily removed from the circulation by hepatocytes. If following hepatic uptake, hemoglobin would donate its heme moiety to the hepatic free heme pool, then it might serve as a therapeutic substitute for heme. Release of hemoglobin-heme into the hepatic free heme pool was therefore investigated by monitoring its incorporation into the hepatic hemoproteins, tryptophan pyrrolase and cytochrome P-450.

Following administration of hemoglobin to rats, heme saturation of tryptophan pyrrolase was significantly increased compared to that in non-hemoglobin treated controls, suggesting incorporation of hemoglobin-heme into this hemoprotein.

In order to determine whether hemoglobin would similarly donate its heme to hepatic cytochrome P-450, its incorporation into the hemoprotein was examined in isolated perfused rat livers and in intact rats, following AIA-mediated destruction of the prosthetic heme of cytochrome P-450 by AIA. Administration of hemoglobin was found to restore the content, as well as the functional activity of hepatic cytochrome P-450, as demonstrated by

the reversal of the AIA-mediated impairment of cytochrome P-450-dependent N-demethylation of ethylmorphine. In the absence of AIA treatment, hemoglobin did not significantly alter the level or activity of cytochrome P-450.

Confirmation that hemoglobin-heme had been incorporated into cytochrome P-450 was obtained by demonstrating the generation of radiolabeled adducts following injection of isotopic hemoglobin. Such adducts have been shown to be derived solely from the prosthetic heme moiety of cytochrome P-450 and AIA during cytochrome P-450-dependent oxidative metabolism of AIA, in a process termed "suicidal inactivation." On the bases of 1, the initial amount of methemoglobin present prior to injection of isotopic hemoglobin; 2, the intravascular rate of methemoglobin formation during the experimental period; and 3, the relative rate of hepatic uptake of isotopic heme from methemoglobin and that of heme bound prosthetically to hemoglobin, it was found that the larger fraction of radiolabeled AIA-adducts was derived from prosthetic heme of hemoglobin. Furthermore, such adducts were ascertained to be derived by AIA-mediated alkylation of hemoglobin-heme incorporated into cytochrome P-450 and not from hemoglobin catalyzed oxidation of AIA.

The above findings clearly demonstrate that hemoglobin-heme gains access to the hepatic free heme pool and is subsequently incorporated into hepatic hemoproteins. Therefore, hemoglobin administration may be a therapeutically safe and useful alternative to heme administration in the treatment of heme deficient states such as the acute hepatic porphyrias.

TABLE OF CONTENTS

PAGE

PAGE

PAGE

LIST OF FIGURES

LIST OF TABLES

INTRODUCTION

The catabolic disposition of hemoglobin-heme has been extensively investigated for more than fifty years. Although the specific steps involved in the degradation of hemoglobin are not completely understood, much information has been obtained regarding the anatomical disposition of hemoglobin and the enzymatic conversion of its prosthetic heme group to bile pigments.

Under normal physiologic conditions, hemoglobin is predominantly catabolized in the spleen, following phagocytosis of senescent erythrocytes by the splenic reticuloendothelial system (RES) (100,178). In addition to the spleen, the RES of liver and bone marrow are sites for removal of intact red cells (21,100). In contrast to hemoglobin from intact erythrocytes, extracorpuscular hemoglobin is removed primarily by liver parenchymal cells (21,80,104,106), with spleen and bone marrow accounting for only 5% and 10-15%, respectively (104). Thus, following hemolysis or parenteral administration of cell-free hemoglobin, the liver is the most important tissue in the catabolism of hemoglobin (99).

Independent of the site of degradation, hemoglobin-heme is catabolized to bilirubin, which if extrahepatic, is transported to the liver, conjugated with glucuronic acid, and excreted in the bile (146,178,179). Until recently, it was generally believed that heme from hemoglobin was quantitatively broken down to bilirubin and therefore not reutilized in the synthesis of new hemoproteins (33,109,146). Evidence has accumulated, however, which suggests that extracorpuscular hemoglobin, taken up by hepatocytes, may be reutilized in the synthesis of hepatic hemoproteins (125,179).

In the liver, heme required for hemoprotein formation is derived from the hepatic "free" heme pool (57,87,110,160). The heme content of this pool is finely controlled, in part by heme itself, which modulates its own

synthesis and degradation by regulating the rate limiting enzymes in heme synthesis and catabolism. It was previously shown that heme, exogenously administered to rats, is taken up by hepatocytes and gains access to the "free" heme pool (46,49,70,71,73). This is evidenced by the fact that administered heme: (1) inhibits delta-aminolevulinic acid (ALA)-synthetase, the rate limiting enzyme in heme synthesis (86,125,159); (2) induces heme oxygenase, the rate limiting enzyme in heme degradation (179); (3) is converted to bilirubin (110); and (4) is incorporated prosthetically into hepatic hemoproteins such as cytochrome P-450 (46,70,71) and tryptophan pyrrolase (11,74). Similarly, hepatic ALA-synthetase is repressed (179) and microsomal heme oxygenase is stimulated (125,179) following parenteral administration of hemoglobin, thus indicating that hemoglobin-heme may also gain access to the hepatic "free" heme pool.

Because parenterally administered heme is incorporated into the hepatic "free" heme pool, its use has been clinically exploited in the treatment of acute attacks of the hepatic porphyrias (52,108,127,129,185), a group of diseases generally resulting from in-born errors in the biosynthesis of heme. In addition, heme has been proposed as therapy in the treatment of patients intoxicated with chemicals that deplete hepatic heme by destroying the prosthetic heme moiety of cytochrome P-450 (73). Following destruction, the apoprotein moiety of cytochrome P-450 is apparently left largely intact and capable of incorporating heme, since exogenous administration of heme is found to result in functional reconstitution of the cytochrome (46,73).

Therapeutic use of heme is often limited, however, by the potential toxicity of the large doses of heme required. The most frequently reported toxicity following heme administration is a generalized vascular reaction, ranging from irritation to hemorrhage and thrombosis (5,52,115,119,129,132).

Renal failure has been observed following very large doses of heme (51). On the other hand, intravenous injection of stroma-free hemoglobin solutions have been used therapeutically for resuscitation of patients with severe hypovolemia, without the untoward effects observed with heme (61, 62,65,77,147,150,173). Thus, if heme from parenterally administered hemoglobin was incorported into the hepatic "free" heme pool to an extent comparable to that of heme, then it might serve as a feasible substitute for heme in the clinical treatment of acute hepatic porphyrias and of intoxications with cytochrome P-450 inactivating drugs. The incorporation of hemoglobin-heme into hepatic hemoproteins largely reflects the extent to which heme from hemoglobin enters the hepatic "free" heme pool. The incorporation of hemoglobin-heme into hepatic cytochrome P-450 and tryptophan pyrrolase was therefore examined in an animal rat model and constitutes the subject of this dissertation.

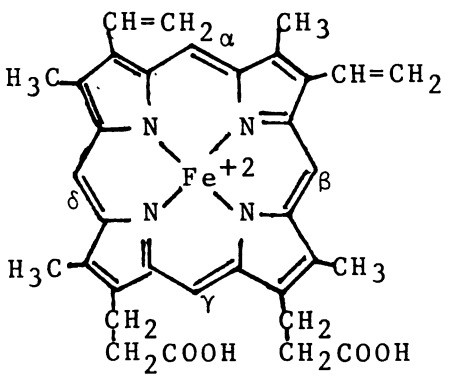
BACKGROUND

Heme

Heme Structure:

Heme, ferroprotoporphyrin IX, is the most abundant and widely distributed metalloporphyrin complex in the animal kingdom. Heme is composed of four pyrrole rings, joined together by four methylene bridges in an alternating double bond ring structure; methyl, vinyl, and propionic acid side chains are distributed on the pyrrole rings with iron being bound equally to the tertiary nitrogens of the rings (153) (Figure 1). There are sixteen possible isomers of this structure, but, ferroprotoporphyrin X is the only isomer found naturally.

Figure 1. Structure of Heme.



In the free form, heme is unstable and rapidly oxidizes to ferriheme (hemin if complexed with Cl^- or hematin if complexed with OH^-). Bound prosthetically to proteins, however, heme participates in a variety of reactions which are essential for maintenance of life. The diversity of heme interactions is attributable to its dual capacity to undergo reversible oxidation-reduction reactions, as well as its ability to bind and transport molecular oxygen.

Heme Synthesis and Regulation:

The primary site of heme synthesis is the bone marrow, where hemoglobin formation requires approximately 80% of the daily heme production (124). The liver is the second major site of heme synthesis, where heme is required for mitochondrial, microsomal and cytosolic hemoproteins. Because of the extended half-life of myoglobin ($t_{\frac{1}{2}}$ = 80 to 90 days), (3), the heme requirement for this hemoprotein is relatively small.

The formation of heme begins within the mitochondria, with δ-aminolevulinic acid (ALA)-synthetase catalyzing the condensation of glycine and succinyl CoA to δ-aminolevulinic acid (ALA) (Figure 2). ALA-synthetase is located in the mitochondrial matrix and is the rate limiting step in the biosynthesis of heme (86,125,159,180). Following synthesis, ALA leaves the mitochondria and is converted in the cytosol to porphobilinogen (PBG) by ALA-dehydratase. The combined actions of cytosolic uroporphyrinogen-1-synthetase and uroporphyrinogen cosynthetase convert PBG to uroporphyrinogen, which in turn, is decarboxylated by uroporphyrinogen decarboxylase to coproporphyrinogen (119). Coproporphyrinogen re-enters the mitochondria and is converted by coproporphyrinogen oxidase to protoporphyrinogen, which is oxidized to protoporphyrin IX by protoporphyrinogen oxidase. Finally, heme is formed by ferrochelatase-mediated insertion of iron into the porphyrin ring (28,119,180). Thus, heme is one of the very few prosthetic groups which is synthesized almost entirely within the animal (180).

Following its synthesis, heme is transferred from the mitochondria to the cytosol by mechanisms not yet clarified. It is belived that heme is then bound to cytosolic proteins and transported to the endoplasmic reticulum for the assembly of specific microsomal hemoproteins (192). Based on the binding affinity of heme for ligandin, this cytosolic protein is thought to be the primary heme carrier in liver cytosol (126).

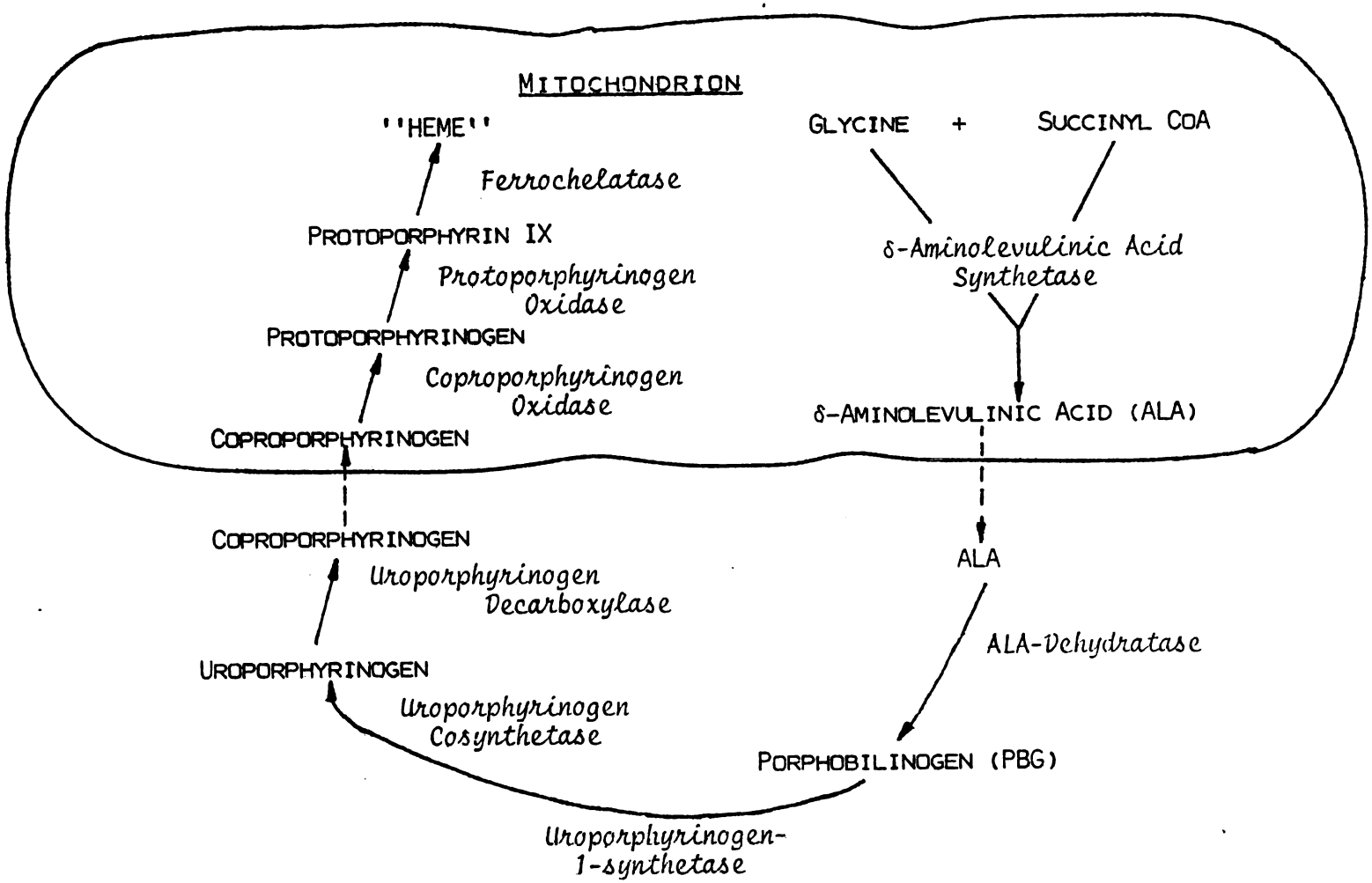


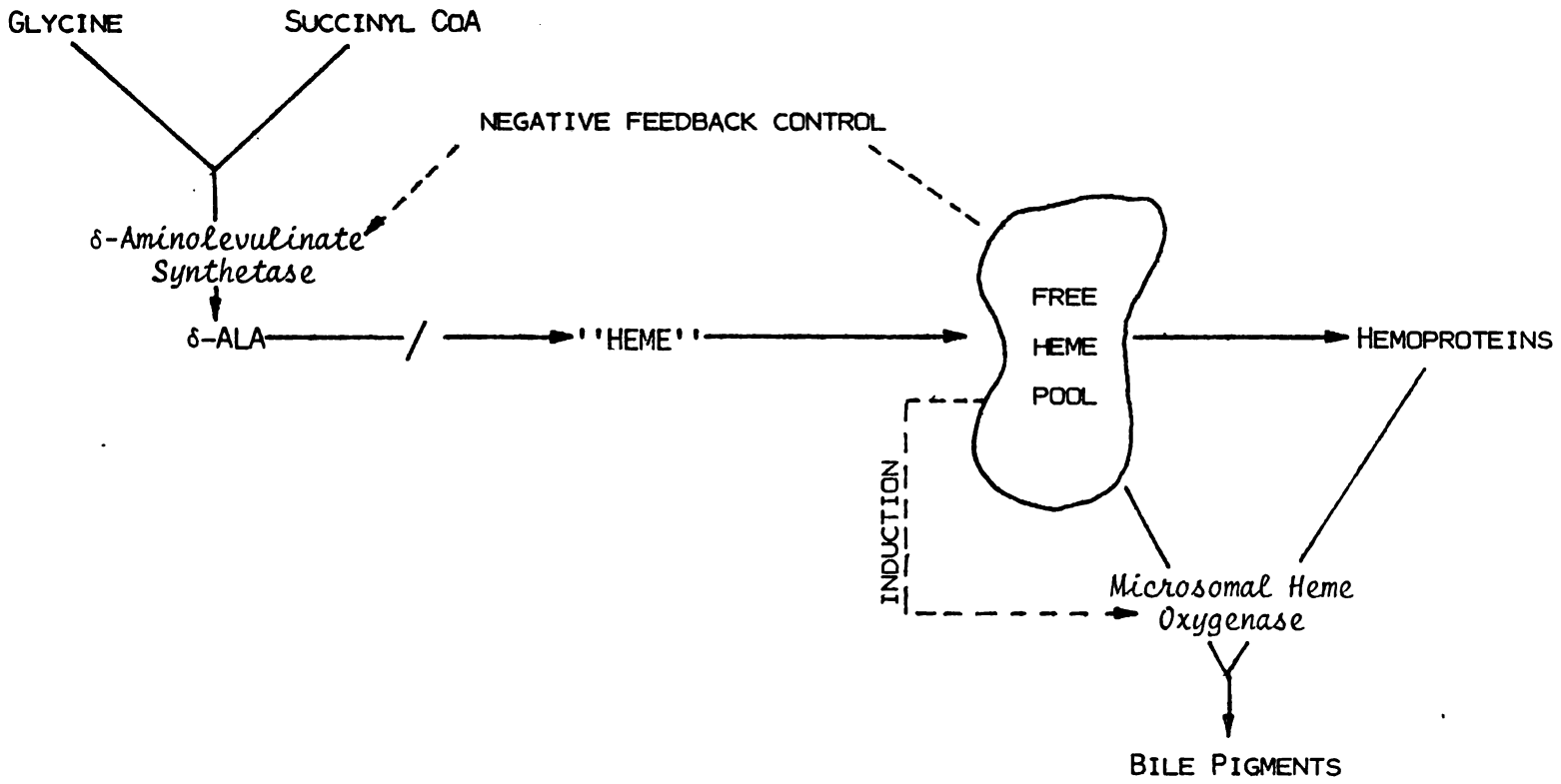
Figure 2: Biosynthesis of Heme

The rate at which heme is synthesized is controlled by the first enzyme in the heme synthetic pathway, ALA-synthetase (86,125,159). The activity and level of this enzyme is repressed by heme through a negative feedback mechanism. Heme also stimulates the activity of microsomal heme oxygenase, the rate limiting enzyme in heme catabolism. Since heme, in part, controls its own synthesis and degradation, a "regulatory" or "free" pool of heme has been postulated to exist within hepatocytes (57,87,110, 159) (Figure 3). This pool consists of newly synthesized or unassigned heme, as well as heme derived from the turnover of hemoproteins (160,161, 190). Heme required for hepatic hemoproteins synthesis is obtained from the free-heme pool; heme formed in excess of hemoprotein requirements is catabolized to bile pigments. The size and location of the free-heme pool, as yet, have not been established. Based on studies with hepatic tryptophan pyrrolase (discussed below) it is possible that unassigned heme is primarily located in the cytosolic fraction of the cell (8). Based on the concentration of heme required for repression of ALA-synthetase (87), and the loss of heme from tryptophan pyrrolase following administration of porphyrogenic drugs (8) (discussed below), the concentration of cytosolic heme was approximated to be 0.1 μM.

Porphyrias and Heme Therapy:

Abnormalities in heme biosynthesis result in the systemic illness known as porphyria. A comprehensive review of the porphyrias has been provided by Meyer and Schmid, 1978 (127). Porphyrias may be hepatic or erythropoietic in origin and result from a genetically determined deficiency of one of the intermediate enzymes involved in heme synthesis (32). Symptoms seen in acute hepatic porphyria include abdominal pain, vomiting, constipation, both central and peripheral neuropathies, and solar photosensitivity resulting in skin lesions (119). Clinically, porphyrias are

Figure 3. Regulation of Synthesis and Degradation of Heme by the Hepatic Free-Heme Pool.



classified as acute and non-acute; in the latter, the only presenting symptom is photosensitivity to sunlight.

Biochemically, porphyrias are classified on the basis of the particular intermediate enzyme deficiency (see Figure 2). The enzyme deficiency in acute intermittent porphyria is localized at uroporphyrinogen-1-synthetase; in hereditary corpoporphyria, coproporphyrinogen oxidase; in porhyria variegata, protoporphyrinogen oxidase; in cutaneous hepatic porphyria, uroporphyrinogen decarboxylase; in erythropoeitic protoporphyria, ferrochelatase; and in congenital porphyria, uroporphyrinogen cosynthetase (129). All of these enzyme deficiencies result in a reduction of heme synthesis, and therefore, by removing end-product repressions, ALA-synthetase activity is increased. Increased ALA-synthetase activity, in combination with an enzymatic block in the heme synthetic pathway, brings about an over-production of porphyrins and porphyrin precursors prior to the enzymatic block. Thus, the different porphyrias are characterized by a pattern of build-up of specific porphyrin molecules, and the particular type of porphyria is diagnosed, in part, by the isolation of these characteristic porphyrins in urine, feces, and blood (129).

In acute hepatic porphyrias, namely acute intermittent porphyria, porphyria variegata, and hereditary coproporphyria, a porphyric "crisis" may be caused by administration of drugs, such as barbiturates or estrogens (185), which not only increase the demand for heme, but also induce ALA-synthetase activity and therefore increase the production of porphyrins and porphyrin precursors.

In addition to the inherited disorder, porphyria may result, in the absence of a genetic predisposition, from exposure to porphyrinogenic chemical (acquired or chemical porphyrias) which induce or repress specific enzymes in the heme biosynthetic pathway (86,181). Among these compounds

are hexachlorobenzene (38), 2-allyl-2-isopropylacetamide (AIA) (181), 3, 5-diethoxycarbonyl-1,4-dihydrocollidine (DDC) (40, and references therein), griseofulvin (58), polychlorinated biphenyls (84,123), and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) (152). An excellent review of porphyrinogenic agents and their mechanisms of action has recently been provided (166).

The realization that ALA-synthetase activity is repressed by heme, via a negative feedback mechanism, led to the first therapeutic administration of heme in the treatment of acute hepatic porphyrias (29). Heme therapy produced a simultaneous chemical and clinical remission of acute intermittent porphyria and has since been employed routinely as the treatment of choice for hepatic porphyria (52,108,119,129,151).

Heme Catabolism:

Heme is normally catabolized by microsomal heme oxygenase with formation of equimolar amounts of bilirubin and carbon monoxide (110). Eighty percent of the daily bilirubin production is derived from hemoglobin-heme, with the remaining 15 to 20% derived from heme of hepatic hemoproteins (72,156). The disproportionately large amount of bilirubin formed from hepatic hemoproteins, relative to their heme content (less than 1% of total body heme, 153), is accounted for by the rapid rate of turnover of these hemoproteins (particularly cytochrome P-450) (33,162). The primary site of heme catabolism is the spleen, followed by bone marrow, liver, and kidney (176). As would be expected, the level of heme oxygenase activity in these tissue, is proportional to their relative participation in heme catabolism.

Heme is stereoselectively cleaved by heme oxygenase at the α-methene bridge of the protoporphyrin-IX ring (110,118). Heme oxygenase activity is dependent upon the presence of NADPH and molecular oxygen (161,177). The

first step in heme catabolism is the binding of heme to heme oxygenase to form a ferric heme-heme oxygenase complex (194, and references therein). Following reduction by NADPH-dependent cytochrome P-450 reductase, the complex binds molecular oxygen. Addition of a second electron from NADPH (or NADH) presumably activates oxygen to a reactive electrophile, which hydroxylates the α-methene carbon of the protoporphyrin ring, resulting in a hydroxyheme intermediate. Hydroxyheme is converted to an intermediate called 688 nm substance (193), releasing the α-methene carbon as carbon monoxide (194). Loss of heme iron and insertion of two atoms of oxygen into the porphyrin ring converts the 688 nm substance to biliverdin. The last step is the reduction of biliverdin by NADPH-dependent biliverdin reductase to form bilirubin. Following conjugation by UDPG-glucuronyl transferase, bilirubin is excreted in the bile (175).

The conversion of heme to bilirubin is quite rapid. Studies employing a molecular oxygen isotope ($^{18}O_2$) demonstrated the appearance of radiolabeled bilirubin ten minutes after exposure of animals to isotopic oxygen (33). Minor amounts of heme appear to be catabolized by a secondary pathway in which bilirubin is not an intermediate (19,89a,110). What metabolites are formed and their possible physiological significance is not known.

HEMOPROTEINS

In animals, heme is the prosthetic moiety for a diverse group of hemoproteins, including hemoglobin and myoglobin (oxygen transporting hemoproteins), catalase and peroxidase (hemoproteins which break down hydrogen peroxide), cytochromes b, c_1, c, and cytochrome oxidase (mitochondrial respiratory hemoproteins), tryptophan pyrrolase (a dioxygenase in tryptophan catabolism), cytochrome P-450 (a microsomal monooxygenase), and cytochrome b_5 (a microsomal hemoprotein involved in desaturation of fatty

acids). In vertebrates, hemoglobin-heme represents approximately 90% of the total body heme; about 10% is myoglobin and less than 1% is contained in all other hemoproteins (153).

Structually different forms of heme are found in both cytochrome oxidase and cytochromes "c". Cytochrome oxidase, composed of cytochromes a and a_3, contains heme "a" which has an extended farnesyl ethyl side chain in place of the vinyl function (153). Heme "c" is found in cytochromes c and c_1 and has cysteinyl groups substituted in place of vinyl groups (170). With the exception of cytochromes a and c, the heme moieties of all hemoproteins are structurally identical. Functional exchange or reutilization of prosthetic groups among hemoproteins, is believed to occur, but has not been demonstrated.

Because the subject of this investigation is the reutilization of the prosthetic heme of hemoglobin by hepatic tryptophan pyrrolase and cytochrome P-450, these three hemoproteins are discussed in detail in the following sections.

Hemoglobin:

Hemoglobin, an oxygen transporting hemoprotein, has been more extensively characterized than any other protein, and the relationships between its structure and function are now understood in great detail (148,149). Hemoglobin is a spheroidal molecule containing four heme moieties, the site of oxygen binding. Each heme is bound separately to a polypeptide chain, globin, and the four globin chains are noncovalenty bound together and occur as non-identical pairs (63). In hemoglobin A (adult human hemoglobin) there are two alpha and two beta chains. In addition to hemoglobin A, hemoglobins A_2 and F are present in humans (153). In rats, six distinct hemoglobins have been isolated (81,172). The heterogeneity of hemoglobins,

within and between species, is attributable to differences in the sequence of amino acids composing the specific globin chains. Qualitatively, differences in globin chains effect alterations in crystal form, solubility, affinity for oxygen, and absorption spectra of various hemoglobins (153). The structure of heme is, however, identical in all hemoglobin studied to date.

Hemoglobin synthesis:

Hemoglobin synthesis takes place within erythrocytes precursor cells, developing in the bone marrow. Synthesis of heme occurs in early erythroblasts, prior to the accumulation of globin-messenger RNA (82). Heme was shown to stimulate globin synthesis both in intact reticulocytes and in cell-free extracts, whereas, in heme-deficient states, globin synthesis is repressed (116,136). The translation of globin-mRNA is indirectly regulated by heme through heme inactivation of an inhibitor of a translation-initiation factor. Thus, heme and globin synthesis are coordinated throughout erythropoiesis, and following release of newly synthesized globin from polyribosomes, the hemoglobin tetramer is spontaneously assembled (116,136). The erythrocyte contains its full complement of hemoglobin prior to being released from the bone marrow into the circulation.

Intracellular formation of methemoglobin:

The normal life-span of the erythrocyte is approximately 120 days (34). During this time hemoglobin iron is slowly, but continuously, oxidized to the ferric state, forming methemoglobin, which is incapable of binding and transporting oxygen. Within the erythrocyte, methemoglobin is enzymatically converted back to hemoglobin by NADH-dependent methemoglobin reductase (164). Reduced NAD is supplied intracellularly through anaerobic glycolysis (153). In addition to methemoglobin reductase, small amounts of methemoglobin are reduced non-enzymatically by reducing agents such as

cysteine, reduced glutathione, and ascorbic acid (25,171). Thus, in combination, enzymatic and non-enzymatic mechanisms prevent the accumulation of methemoglobin within the erythrocyte to any appreciable extent; the normal methemoglobin content of human erythrocytes is less than 1.5% (68).

Fate of intravascular hemoglobin:

As discussed in the Introduction, erythrocytic hemoglobin is predominantly catabolized by phagocytic cells of the reticuloendothelial system, while extracorpuscular hemoglobin is degraded by hepatic parenchymal cells. Under normal conditions, the amount of hemoglobin released into serum is quite small (91) and therefore, catabolism of hemoglobin by hepatocytes is a relatively inconsequential pathway (79,94). In hemolytic states, however, the liver assumes a primary role in the degradation of hemoglobin. Following its release, hemoglobin dissociates into dimers (35). Depending upon the extent of hemolysis, hemoglobin may be cleared from the circulation by several different mechanisms:

Hemoglobin-haptoglobin complex: Free hemoglobin is rapidly bound by haptoglobin, a glycoprotein found in the serum of man and other mammals (34). The hemoglobin-haptoglobin complex is sufficiently large to prevent renal filtration of hemoglobin. Synthesized in the liver, haptoglobin is present in serum at concentrations one-hundred fold greater than the normal level of circulating hemoglobin (4,34). Like hemoglobin, haptoglobin is a tetrameric molecule, consisting of two heavy and two light chains (88). Hemoglobin binds non-covalently to haptoglobin in a molar ratio of 1:1, forming a soluble, stable complex (101). However, binding occurs with dimers of hemoglobin and not the tetramer (133). The specific molecular interaction of hemoglobin with haptoglobin is not yet fully understood and is currently an active area of research (88,102,103,183, 184). Recent

evidence indicates that a specific receptor for the hemoglobin-haptoglobin complex may exist within liver parenchymal cell membranes (105). Binding of hemoglobin to haptoglobin may alter the molecular configuration of the carrier protein and thus, allow recognition of the complex by the receptor. Once formed, the hemoglobin-haptoglobin complex is rapidly removed ($t_{\frac{1}{2}}$ = 10 to 30 min.) in toto by hepatocytes (21,76,79). Since haptoglobin is not returned to the circulation, but is degraded along with hemoglobin, serum haptoglobin rapidly becomes depleted in pathological states involving severe hemolysis (95). In the absence of haptoglobin, secondary transport mechanisms assume the primary role of hemoglobin clearance.

Methemalbumin and hemopexin: Hemoglobin released from the intracellular environment of the erythrocyte undergoes autooxidation to methemoglobin (78). If not bound to haptoglobin, methemoglobin is unstable and dissociates into its hemin and globin residues (36,97). Free hemin is rapidly complexed with serum proteins, albumin and hemopexin. As with haptoglobin, these proteins function to prevent renal filtration of the relatively small heme molecule, allowing the body to salvage heme iron (94).

Heme is initially bound to albumin, which serves as a depot for heme storage within the vasculature (97,131). Because of its relative abundance and multiple binding sites, the heme carrying capacity of albumin is far greater than that of hemopexin. In addition to a primary high affinity site (k_D-10^{-8}), albumin possesses as many as ten non-specific binding sites for heme (2,15).

Heme is subsequently transferred to hemopexin, which has a much higher heme-binding affinity than albumin (k_D=10^{-13}M) (131). Hemopexin, in turn, transports heme to the liver parenchymal cells where it is taken up and catabolized (97,130). The uptake of heme by hepatocytes is a receptor-

mediated process (167,168). As with haptoglobin, binding of heme causes a change in the tertiary structure of the protein which is thought to allow recognition of the heme-hemopexin complex by the heptocyte receptor (17, 130). Unlike haptoglobin, hemopexin is not degraded in the hepatocyte along with heme, but is recycled into the circulation (53,168,169).

Two separate mechanisms for hepatic uptake of heme have been described in isolated hepatocytes: A "specific" transport system which is saturable and has a high affinity for heme, and a "selective" transport system which is not saturable and has a relatively lower heme affinity (169). Although both processes require energy for transport of heme, the former is thought to be the hemopexin receptor-mediated system. Whether or not the "selective" transport system exists in vivo has not been established.

Renal filtration of hemoglobin: If hemolysis is severe, hemoglobin is released into the circulation in amounts exceeding the serum-haptoglobin-binding capacity, and unbound hemoglobin is removed from the serum, in part, by glomerular filtration (34). Because the glomerular permeability of hemoglobin is far greater than albumin, a molecule of comparable size, it is thought that hemoglobin is filtered as dimers (35). Upon entering the kidney, hemoglobin is reabsorbed by the proximal tubular epithelium and rapidly catabolized (66,111). One hour following injection of ^{14}C-heme-labeled hemoglobin, only 15% of the radioactivity contained in renal tubular cells was recovered as hematin (37).

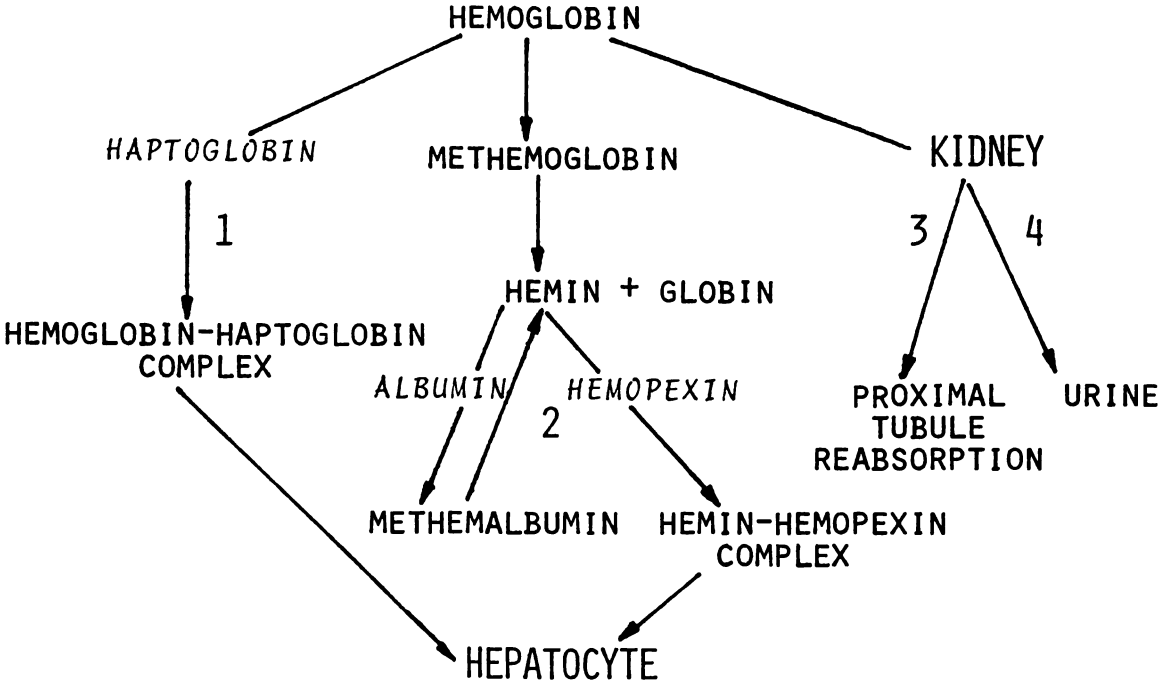
The breakdown of hemoglobin within renal tubular cells results in the accumulation of iron in the form of ferritin and hemosiderin (37,154). The ability of animals to mobilize this iron appears to vary among species. Mobilization of renal iron in humans is very limited and thus the iron is relatively unavailable for reutilization by the bone marrow (157). In comparison, rats have been shown, albeit slowly ($t_{1/2}$ =30 days), to recycle

up to 70% of the hemoglobin-iron deposited within the kidneys (154).

Only when the amount of filtered hemoglobin exceeds the reabsorption capacity of the proximal tubules will hemoglobin appear in the urine (37). Thus, mammalian organisms have developed an elaborate scheme for conservation of physiological iron stores (94) (Figure 4).

In summary, following hemolysis, hemoglobin is initially bound by haptoglobin and transported to the liver (Fig. 4, 1). If the capacity of haptoglobin is exceeded, hemoglobin iron may be salvaged by binding of heme residues by albumin and hemopexin (Fig. 4, 2). If hemoglobin is cleared by glomerular filtration, it is reabsorbed by cells of the proximal tubules (Fig. 4, 3). Only when this last line of defense is surpassed or compromised, is hemoglobin lost in the urine (Fig. 4,4).

Figure 4. The Fate of Intravascular Hemoglobin



Tryptophan Pyrrolase:

Tryptophan pyrrolase (L-tryptophan 2,3-dioxygenase) is the rate-limiting enzyme in the degradation of tryptophan by the kynurenine-nicotinic acid pathway (9). This enzyme is a cytosolic hemoprotein, widely distributed in nature, and is composed of four subunits of equal molecular weight containing two g-atoms of copper and two moles of heme per mole of tetramer (31). Catabolic activity of tryptophan pyrrolase is dependent upon copper being in a reduced state (Cu^{+}); the heme moieties may be in either the ferric or ferrous form. Molecular oxygen preferentially binds to ferroheme, however, if heme is oxidized, oxygen will bind to Cu^{+} (31).

The pyrrole ring of L-tryptophan is oxidatively cleaved by tryptophan pyrrolase to form formylkynurenine (9). Formylkynurenine is hydrolyzed to kynurenine which, in turn, is converted to a series of intermediates and by-products. All metabolites of L-tryptophan, except glutaryl CoA, are excreted in urine (153).

Hepatic tryptophan pyrrolase is present as holo- and apoenzyme in humans, rats, mice, pigs, turkeys, and chickens (9,74), whereas, in guinea pigs, gerbils, hamsters, cats, rabbits, ox, sheep, and frogs, tryptophan pyrrolase exists only as holoenzyme (9,12). In animals which contain both the apo- and holo- form of the enzyme, the activity of tryptophan pyrrolase is enhanced by administration of glucocorticoids, L-tryptophan, and the cofactor heme (9,12,163). Glucocorticoids stimulate the synthesis of new apoenzyme, possibly by increasing the amount of enzyme specific mRNA, while L-tryptophan stabilizes the enzyme, decreasing the rate of enzyme degradation without affecting the rate of enzyme synthesis (24,163). Administration of heme results in constitution of the catalytically inactive apo-tryptophan pyrrolase to the functional holoenzyme (8,11,74). In animals which lack the apo-tryptophan pyrrolase, glucocorticoids do not induce

enzyme activity and L-tryptophan is relatively more toxic (9,10,24).

The fact that tryptophan pyrrolase exists, in certain species, as apoenzyme has provided a means for examining the size and regulation of the hepatic free heme pool. Altering the intracellular heme concentration directly, by administration of heme (11,74), or indirectly, by perturbation of the synthesis and/or degradation of heme (8,20,189), results in measurable changes in the heme-saturation of tryptophan pyrrolase. Small changes in the general homeostasis of heme alter heme-saturation of tryptophan pyrrolase. Thus, this enzyme appears to be intimately associated with the hepatic free heme pool and may play an important role in the regulation of intracellular heme.

Cytochrome P-450 and Cytochrome P-450-Dependent Mixed Function Oxidases:

Cytochrome P-450 is the terminal oxidase of a microsomal enzyme system generally referred to as microsomal mixed function oxidases or cytochrome P-450 monooxygenases. This hemoprotein, rather than being a single enzyme, is a group of isozymes with different, but overlapping, substrate specificities. In addition to cytochrome P-450, mixed function oxidases consist of cytochrome P-450 reductase, an NADPH-dependent flavoprotein which is unusual because it contains two flavin molecules, FMN and FAD. Complexed together, the reductase and cytochrome P-450 form an electron transport chain which participates in the catabolism of a great number of structurally diverse compounds, including a variety of lipid-soluble drugs and chemicals (xenobiotics), as well as endogenously synthesized steroids, fatty acids, thyroxine, prostaglandins, and bile acids. Over the past two decades, cytochrome P-450 and cytochrome P-450-dependent mixed function oxidases have been extensively investigated and many comprehensive reviews are currently available (24,42,50,96,122,135,188). The following is presented as a general overview of this complex system.

Cytochrome P-450 is widely distributed in nature, occurring in virtually all tissues of animals, as well as in plants and microorganisms. In mammals, cytochrome P-450 is found intracellularly bound to membranes of the endoplasmic reticulum (ER), primarily the smooth ER, with the exception of the adrenal cortex, where it is contained within mitochondria. The greatest concentration of cytochrome P-450 is found in liver, with lesser quantities having been demonstrated in lung, kidney, adrenals, testes, ovaries, intestine, epidermis, aorta, and blood platelets.

It is currently believed that within the membrane of the ER, clusters of cytochrome P-450 molecules complex with one molecule of cytochrome P-450 reductase, either in a rosette fashion around the reductase, or by the random movement through the membrane. Fully three-fourths of the reductase flavoprotein is thought to extend outside of the membrane, while cytochrome P-450, which is difficult to solubilize in pure form, is believed to be located deep within the membrane. The heme prosthetic group of cytochrome P-450 is bound within a relatively open, hydrophobic cleft on the surface of the apoprotein. A loose heme-protein attachment occurs through hydrophobic forces, Coulombic attractions and a coordinate-covalent linkage of the pentacoordinate position of iron to a thiolate anion of a cysteine residue of the apoprotein.

Reconstitution studies have demonstrated that fully activated microsomal mixed function oxidases require the presence of cytochrome P-450, cytochrome P-450 reductase, the cofactor NADPH, a heat-stable-lipid factor (phosphatidylcholine) and molecular oxygen. The initial step in the oxidative catabolism of substrates by cytochrome P-450-dependent mixed function oxidases is the binding of the lipophilic substrate to the active site of the enzyme. As binding occurs, the ligand at the hexacoordinate position (presumably water) of heme-iron is displaced. An electron, provided by NADPH through cytochrome P-450 reductase, reduces heme-iron to the

ferrous state, enabling the prosthetic group to bind molecular oxygen. Addition of a second electron, provided by NADPH, or NADH through cytochrome b_5, initiates reactions, which as yet are not completely understood, resulting in the transfer of one atom of oxygen to substrate with the other oxygen being released as water. Employing this general mechanism, cytochrome P-450-dependent mixed function oxidases effect a variety of biochemical transformations including aliphatic and aromatic hydroxylations, N-oxidations, sulfoxidations, epoxidations, N-, S-, and O-dealkylations, desulfurations, and deaminations. In the absence of oxygen, cytochrome P-450, through direct electron transfer, carries out reductions of azo-, nitro-, N-oxide, and epoxide groups. Generally, these transformations result in, or lead to, detoxication of substrates, but in certain cases, compounds may be activated to more toxic substance, or transformed to mutagens and/or carcinogens.

Many of the substrates of cytochrome P-450-dependent mixed function oxidases stimulate de novo synthesis of this enzyme system, thus enhancing their own metabolism. Specifically, levels of cytochrome P-450, cytochrome P-450 reductase, membrane phospholipid, and consequently, the amount of smooth ER, are increased subsequent to administration of various xenobiotics. The mechanisms controlling these events are quite complex and, as yet, not completely understood.

Different substrates induce different forms of cytochrome P-450. Several isozymes have been purified and characterized and may be distinquished on the basis of not only their substrate specificities, but also by their spectral and physical properties (50, and references therein). The most familiar inducers of mixed function oxidases are phenobarbital and 3-methylcholanthrene, and substrates have been commonly grouped as being catabolized by phenobarbital-inducible (cytochrome P-450) or 3-methyl-

cholanthrene-inducible (cytochrome P-448) forms of the cytochrome.

Two schools of thought have emerged regarding the question of how many forms of cytochrome P-450 exist. One school advocates that the different forms of cytochrome P-450 are relatively limited, and that the diversity of substrate specificity seen with cytochrome P-450 dependent mixed function oxidases is attributable to overlapping substrate specificities of these few cytochromes. A second group of investigators postulate that induction of cytochrome P-450 by different substrates may be analogous to the formation of immunoglobulins following exposure to various antigens; i.e., specific antigens stimulate production of specific antibodies. It is now realized that hundreds of xenobiotics are capable of inducing their own metabolism. Although similar, these inductions are not precisely phenobarbital-like or 3-methylcholanthrene-like. This observation, through implication, suggests that there may be potentially hundreds, or even thousands, of structually distinct forms of cytochrome P-450. The isolation and identification of various forms of cytochrome P-450 is currently a very active area of research. Which of these hypotheses is correct will undoubtedly be resolved in the near future.

Certain chemicals, rather than increasing the concentration of cytochrome P-450, cause a loss of the enzyme, either by inhibiting its formation, by increasing its degradation, or through a combination of both processes. The majority of chemicals which prevent formation of cytochrome P-450 do so by disrupting heme biosynthesis (discussed above), while chemically mediated degradation of cytochrome P-450 may occur through a variety of mechanisms. More than any other hepatic hemoprotein, cytochrome P-450 is particularly susceptible to chemical destruction. A review of the various factors contributing to the specific chemical lability of this enzyme has been published (58). A brief description of these factors is

given below.

The prosthetic heme of cytochrome P-450 is attached to the apoprotein through a thiol ligand, making the cytochrome susceptible to attack by heavy metals and other sulfydryl reagents. Blockade or oxidation of the thiol group disrupts the stability of the enzyme, converting cytochrome P-450 to cytochrome P-420, an inactive form of the enzyme.

The structural and functional integrity of cytochrome P-450 is dependent upon the physical organization of the cytochrome within the hydrophobic environment of the membrane of the endoplasmic reticulum. Chemical agents disrupting this membrane, such as detergents, alcohols, and lipid peroxidizing substances, cause conversion of cytochrome P-450 to cytochrome P-420.

Chemicals which stimulate microsomal heme oxygenase activity, such as metals (including Co, Cd, Cr, Cu, Fe, Hg, Ni, Pb, Sn, and Zn, (121,158), endotoxin (20), and heme itself (23,125,174), elicit a loss of cytochrome P-450, presumably by depleting the prosthetic heme of the cytochrome. The mechanism(s) by which an increase in heme oxygenase activity produces accelerated loss of cytochrome P-450 has not, as yet, been clearly defined.

Finally, as a result of its role in the catabolism of xenobiotics, cytochrome P-450 is a primary target for destruction by toxic chemicals. Upon binding at, or near, the active site of the enzyme, many chemicals are oxidized to reactive intermediates, and these derivatives, rather than the parent substrates, cause the destruction of cytochrome P-450. Based on the specific targets of the reactive intermediates, three classes of agents have been differentiated: (1) Metabolically activated allyl containing compounds such as the porphyrinogenic agent, allylisopropylacetamide (AIA, 2-isopropyl-4-pentenamide), specifically destroy the prosthetic heme moity of the cytochrome. (2) The apoprotein of cytochrome P-450 is the primary target of reactive sulfur free radicals, produced by oxidative desulfura-

tion of certain sulfur containing compounds, for example, carbon disulfide and parathion. In addition, reactive sulfur binds to microsomal proteins producing centrilobular degenerative changes in hepatocytes. (3) Catabolism of carbon tetrachloride produces a reactive intermediate which diffuses throughout the cell, nonspecifically destroying endoplasmic reticulum, other membranes and cell constitutents leading to cell death and liver necrosis.

Of these compounds, allyl containing chemicals, specifically AIA, are particularly important to the current investigation. The selective destruction of cytochrome P-450-heme by AIA with consequent generation of an intact apocytochrome was exploited experimentally in this investigation to demonstrate the incorporation of hemoglobin-heme into hepatic cytochrome P-450.

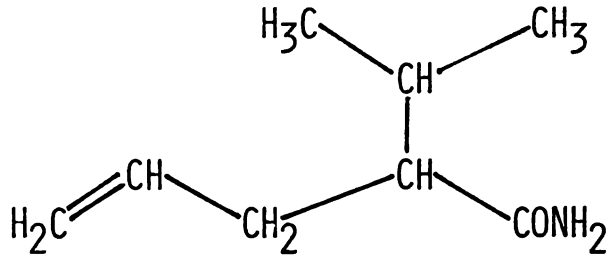
2-Allyl-2-isopropylacetamide (AIA): Suicidal inactivation of cytochrome P-450:

Autocatalytic destruction of cytochrome P-450-heme has been demonstrated not only with AIA, but with a variety of allyl containing compounds, including the therapeutic agents norethisterone (139), secobarbital and allobarbital (113), the anesthetic fluroxene (30), the porphyrinogenic agent 3,5-diethoxycarbonyl-1, 4-dihydrocollidine (DDC) and its 4-ethyl analog (DDEP) (40, and references therein), and industrial agents such as vinyl chloride (89), ethylene and acetylene (138,140,186). Structurally, the functional group common to these substrates and that required for destruction of cytochrome P-450 heme, is a terminal, unsaturated, carbon-carbon bond. The bond may be an olefinic, an allenic, or an acetylenic function (142). A new class of agents, the heterocyclic compounds 5,6-dichloro-1,2,3-benzothiadiazole (DCBT) and 1-amino-benzotriazole (ABT), have recently been shown to cause autocatalytic destruction of cytochrome

P-450 (142, and references therein). The destructive capacity of these compounds lies in the heterocyclic ring, which is fragmented during catalysis, producing reactive intermediates.

Among those compounds containing allyl functions, AIA (Figure 5) has been most extensively studied and is considered the prototype for this

Figure 5. Structure of 2-Allyl-2-Isopropylacetamide (AIA)



group of chemicals. AIA selectively destroys the heme of the phenobarbital-inducible isozyme of cytochrome P-450 (13,30,141). Administration of AIA to rats in vivo caused a 60% loss of the phenobarbital-inducible isozyme, while only destroying 17% of the 3-methylcholanthrene isozyme (142). With the exception of catalase (1,14,155,174), other hemoproteins are not measurably affected by AIA (60,112). The depressant effect of AIA on catalase is thought to be indirect, being attributed to disruption of heme homeostasis within the hepatocyte. In addition, the apoprotein of cytochrome P-450 is apparently left largely intact (18,98), since exogenous administration of heme results in functional reconstitution of the cytochrome (discussed below).

That loss of cytochrome P-450 occurs through catalytic inactivation of

the enzyme is evidenced by: (1) AIA-mediated loss of cytochrome P-450-heme is enhanced by pretreatment with phenobarbital (55); (2) inactivation of cytochrome P-450 depends upon the presence of NADPH (56) and oxygen (141); and (3) SKF-525-A, an inhibitor of cytochrome P-450 enzyme activity, decreases the extent of cytochrome P-450 destruction (56). AIA-mediated destruction of cytochrome P-450 differs, however, from that of carbon tetrachloride (discussed above), in that the destructive event is "suicidal". By definition, this means that the catalytically produced species must destroy the enzyme within the confines of the active site of the enzyme (141, and reference therein), not diffusing to other molecular environments, as is the case with carbon disulfide and carbon tetrachloride (58). AIA metabolites were shown to react specifically with the heme of cytochrome P-450 and not with heme present in the cytosol or bound to other hemoproteins (45). Suicidal destruction is further defined as being a saturable, pseudo-first-order kinetic process which is competitively inhibited by other suitable substrates. The catalytically-derived, destructive intermediate should bind covalently to the enzyme and not be trapped by nucleophiles (e.g., glutathione). That AIA-mediated destruction of cytochrome P-450 meets these qualifications, has been amply demonstrated (141).

Destruction occurs through 1:1 covalent binding of an AIA metabolite to the prosthetic heme moiety of cytochrome P-450 (143,144), resulting in the formation of abnormal porphyrins, commonly called "green pigments" (56). Formation of pigments <u>in vivo</u> or in isolated perfused livers is responsible for the characteristic brownish-green discoloration of liver which is seen after treatment with AIA. The metabolism-inactivation partition ratio for AIA, an expression of the efficiency of destruction of cytochrome P-450 by AIA, is relatively low, with 230 to 320 molecules of

AIA metabolized for every molecule of enzyme inactivated (141,142). Non-destructive metabolites of AIA, isolated subsequent to secondary metabolism, include 2-isopropyl-4, 5-dihydroxypentanamide (AIA glycol), 2-isopropyl-4, 5-dihydroxypentanoic acid- -lactone (AIA lactone), and two additional, as yet, unidentified products (165).

AIA-porphyrin adducts (green pigments) have been isolated, purified and extensively characterized (60,120,143,182). These abnormal porphyrins, isolated by high-pressure liquid chromatography, spectrophotometrically exhibit absorption maxima at 417, 512, 545, 594, and 652 nm; stabilization of adducts with zinc shifts absorption maxima to 431, 547, 591, 634, and 669 nm (143). Structural investigations have indicated that the adduct is an N-alkylated protoporphyrin IX (140,143,144). The postulated mechanism for formation of this species is that cytochrome P-450 mediated oxygen transfer to the unsaturated carbon-carbon bond of AIA is interrupted by the carbonyl oxygen of the amide function, thereby blocking normal epoxidation of the double bond (143,144). Intramolecular cyclization of AIA produces an alkylating species, which alkylates the porphyrin ring.

Reconstitution of autocatalytically destroyed cytochrome P-450:

AIA-mediated inactivation of cytochrome P-450 mixed function oxidase results in impaired metabolism of not only the suicide inhibitor, but of other substrates administered concomittantly (70,71,73). Structural and functional reconstitution of the inactivated cytochrome has been accomplished by exogenous administration of heme (27,46,70,71,73. Reconstitution of cytochrome P-450 was most pronounced for the phenobarbital-inducible isozyme (70), that isozyme selectively destroyed by AIA. Heme increased the content of cytochrome P-450 by 46% in phenobarbital pretreated rats, while producing only a 6% elevation of enzyme levels in 3-methyl-cholanthrene pretreated animals. Moreover, functional restoration

of monooxygenase activity was greatest for oxidases stimulated by phenobarbital; N-demethylation of ethylmorphine was enhanced 60% when heme was administered concomittantly with AIA. Similarly, the delayed plasma disappearance of AIA, resulting from autocatalytic destruction of cytochrome P-450 by this agent, was reversed by administration of heme (73).

That heme is prosthetically incorporated into cytochrome P-450 was evidenced by; (1) heme, in the absence of AIA, had no effect on levels or activity of cytochrome P450 (70); (2) reconstitution was independent of de novo synthesis of apocytochrome P-450, as cyclohexamide did not depress the effect of heme (70); and (3) radiolabeled AIA-porphyrin adducts were isolated from rats treated with AIA and ^{3}H-heme (formation of this adduct requires the prosthetic heme of a functional cytochrome P-450 enzyme) (45, 46). Thus, it was suggested that heme may be therapeutically useful for enhancing drug catabolism in patients intoxicated with chemicals that destroy cytochrome P-450 (73).

EXPERIMENTAL RATIONALE AND SPECIFIC AIMS

The feasibility of substituting hemoglobin for heme in clinical therapy of heme-deficient states will be evaluated, following parenteral administration, by examining its ability to donate heme for incorporation into the hepatic hemoproteins tryptophan pyrrolase and cytochrome P-450. These particular hemoproteins were selected because they are convenient models for scrutiny of functional heme-protein assembly. They normally exist partly as apo-proteins or can be experimentally stripped of heme to yield apoproteins. In rats, tryptophan pyrrolase is present as apo-and holoenzyme in approximately equal proportions. Exogenous administration of heme *in vivo*, or addition of heme *in vitro*, results in functional constitution of apotryptophan pyrrolase to the holoenzyme. Accordingly, the level of enzyme activity reflects the extent to which tryptophan pyrrolase is saturated with heme. Therefore, measuring the level of tryptophan pyrrolase activity, following parenteral administration of hemoglobin to rats, is a convenient determinant of whether hemoglobin-heme is incorporated into this enzyme.

Cytochrome P-450 is not normally present, to any appreciable extent, as apoenzyme, hepatic synthesis of heme being adequate to meet the requirements of this hemoprotein. However, this enzyme can be pharmacologically stripped of its heme by treatment with AIA. Autocatalytic destruction of the cytochrome P-450-heme moiety, in the course of metabolic activation of AIA, generates a relative excess of heme-stripped apocytochrome P-450 and results in the formation of N-alkylated heme adducts. As discussed in the Background, exogenously administered heme can reconstitute cytochrome P-450 which has been destroyed by AIA. Therefore, the use of AIA to strip cytochrome P-450 heme permits examination of whether hemoglobin-heme is incorporated into the stripped apocytochrome P-450. In

this, two separate approaches may be used: First, the structural, as well as functional, reconstitution of cytochrome P-450 may be determined by measuring its content and activity, following treatment with hemoglobin. Secondly, because metabolic activation of AIA results in the formation of an AIA-porphyrin adduct, formation of radiolabeled adducts may be monitored as an index of prosthetic incorporation of isotopic hemoglobin-heme into cytochrome P-450. If this apocytochrome P-450 would initially reassemble with radiolabeled heme derived from hemoglobin, subsequent re-exposure of the reconstituted cytochrome to AIA would result in the production of radiolabeled AIA-porphyrin adducts which can be isolated, purified and quantitated. Since formation of such adducts occurs only from prosthetically incorporated heme, and not from non-specifically bound heme, formation of radiolabeled adducts would conclusively demonstrate that hemoglobin-heme is incorporated into hepatic cytochrome P-450.

Accordingly, the specific aims of this investigation are:

I. To determine if hemoglobin-heme is incorporated into hepatic tryptophan pyrrolase.

II. To examine whether hemoglobin-heme is incorporated into hepatic apocytochrome P-450 by:

A. Establishing whether cytochrome P-450 is structurally and functionally reconstituted by hemoglobin-heme and,

B. Determining if radiolabeled hemoglobin-heme is incorporated into AIA-porphyrin adducts formed following destruction of the reconstituted cytochrome P-450-heme by AIA.

MATERIALS AND METHODS

Materials: Allylisopropylacetamide (AIA) and ^{14}C-AIA (specific activity 7.4 mCi/mmol) were generously donated by Hoffman-La Roche Inc., Nutley, N.J. through the courtesy of Drs. W.E. Scott and J.J. Burns. Delta-(2, 3-H)-aminoleuvulinic acid hydrochloride (specific activity 30 Ci/mmol) was obtained from Research Products International Corp., Mount Prospect, Ill. Heme (equine hemin, type III), bovine serum albumin (fraction V), glutathione, nicotinamide, L-tryptophan, and glucose-6-phosphate dehydrogenase were purchased from Sigma Chemical Co., St. Louis, Mo. Ethylmorphine was supplied by Merck Chemical Division, Rahway, N.J. and phenobarbital (sodium salt) by Mallinckrodt Inc., Paris, Ky. Sodium taurocholate and p-chloro-N-methylaniline were obtained from Calbiochem, San Diego, Ca. Phenylhydrazine hydrochloride and 2, 4-pentanedione were purchased from Eastman Kodak, Rochester, N.Y. All other chemicals and solvents were reagent grade quality.

Animals: Male, Sprague-Dawley rats were obtained from Simonsen Labs, Gilroy, Ca. and housed two per cage in a room maintained at 20-25°C with a 12 h dark-light cycle. Water and laboratory rat chow (Ralston Purina Co., St. Louis, Mo.) were provided ad libitum, unless otherwise stated.

General Methods

Preparation of Rat Hemoglobin

Whole rat blood was collected from the abdominal aorta in 1 ml heparinized saline. Erythrocytes were separated from plasma by centrifugation (6,000 x g for 10 min) and washed three times with Na_2HPO_4 + KH_2PO_4 buffer (0.1 M pH 7.4). Following lysis of erythrocytes in a cell disruption chamber (Parr Instruments, Moline, Ill.; 1,000 p.s.i. nitrogen for 30 min),

hemoglobin was separated from erythrocyte stroma by centrifugation at 27,000 x g for 20 min. Following quantitation (see below), the desired concentration of hemoglobin was prepared by diluting with phosphate buffer (0.1 M).

Preparation of Hemin:

Crystalline hemin, either commercially purchased equine hemin or radiolabeled rat hemin, was dissolved in a small volume of NaOH (0.1 N) and then neutralized by diluting to the desired concentration with phosphate buffer. Solutions were prepared fresh for all experiments, were protected from light by wrapping the container with aluminum foil, and were stored in ice until used.

Preparation of Radiolabeled Hemoglobin:

To maximize the amount of radioactivity contained in the heme moiety of hemoglobin, a highly radiolabeled, heme-specific precursor, 2,3-^{3}H-ALA, was employed in the synthesis of radiolabeled hemoglobin. Previous investigators have employed isotopic glycine in the synthesis of hemoglobin (146,160); glycine, however, serves as a precursor for both heme and globin moieties. Thus, following catabolism of globin, isotopic glycine could theoretically be reutilized in the synthesis of other proteins, including apocytochrome P-450. Specifically labeling the heme of hemoglobin with 2,3-^{3}H-ALA, precluded possible isotopic glycine incorporation into apocytochrome P-450.

Isotopic hemoglobin was synthesized, as previously described (83), by incubating rat reticulocytes with ^{3}H-ALA. To stimulate production of reticulocytes, male Sprague-Dawley rats (300-350 g) were injected subcutaneously with phenylhydrazine (0.3 mmol/kg) every other day for ten days. Fresh solutions of phenylhydrazine (0.25 mM) were prepared by dissolving phenylhydrazine in 0.1 ml NaOH (1 N), diluting with distilled water and

neutralizing to pH 7.4 with concentrated HCl. The reticulocyte-enriched blood was collected, and erythrocytes washed and lysed as described for non-radiolabeled (native) hemoglobin. A 20 ml suspension of lysed erythrocytes was added to an incubation medium containing 20 ml rat plasma (freshly prepared), phosphate buffer (0.15 M, pH 7.55, 6 ml), penicillin 50,000 units, streptomycin (10 mg), $FeSO_4 \cdot 7H_2O$ (10 mg), glucose (300 mg), and ^{3}H-ALA (5 mCi) in 2.5 ml isotonic saline. This mixture was aerobically incubated for 3 h at 37°C in a shaking waterbath, after which, the pH was adjusted to 9.0 by addition of NaOH (0.1N), and a clear supernatant of ^{3}H-hemoglobin was collected by centrifugation at 27,000 x g for 20 min. The pH of the supernatant was adjusted to 7.0 with acetic acid (0.1 N) and the solution allowed to stand at 4°C for at least 24 h to permit rat hemoglobin to crystallize. Crystals were collected by centrifugation, washed five times with distilled water and stored at 4°C until used. Just before ^{3}H-hemoglobin administration, crystals were suspended in phosphate buffer containing EDTA (1 mM) and dissolved by titrating to pH 9 with NaOH (0.1N). Undissolved hemoglobin was removed by centrifugation at 27,000 x g for 10 min.

Methemoglobin, formed during synthesis and storage, was converted to oxyhemoglobin by gel filtration through a sodium dithionite reducing column (64). A 41 x 2.5 cm column (total volume 225 ml) of Sephadex G-25, fine (Pharmacia Fine Chemicals, Uppsala, Sweden) was equilibrated overnight with phosphate buffer containing EDTA. Sodium dithionite, 0.2 g dissolved in approximately 2 ml buffer, was added to the column and washed in with an additional 2 ml buffer. ^{3}H-Hemoglobin (2-4 ml) was then added to the column and eluted in the void volume as oxyhemoglobin; sodium dithionite was retained by the Sephadex column. The percentage of methemoglobin remaining in the hemoglobin solution was quantitated spectrophotometrically (discussed below).

Preparation of ^{3}H-Hemin:

^{3}H-hemin was extracted from ^{3}H-hemoglobin by the method of Labbe and Nishida, 1957 (107). An aliquot of crystalline ^{3}H-hemoglobin, containing heme (3.5 mg), was dissolved (pH 9) and added, drop wise, to 12 parts $SrCl_2$/ acetone solution composed of $SrCl_2 \cdot 6H_2O$ (2% in glacial acetic acid (1 part) and acetone (3 parts). The mixture was briefly boiled to precipitate protein. Hemin and protein were separated by filtering through Watman (No. 4) filter paper (Wand R. Balston LTD., England). The filtrate, containing hemin was concentrated by heating to 102°C and then allowed to cool for 1 h. Crystallized hemin was collected by brief centrifugation and subsequently washed two times with 50% acetic acid and one time each with distilled water, ethanol and diethyl ether; crystals were dried overnight under vacuum. The purity of hemin isolated by this method was verified by thin-layer chromatographic analysis (TLC) on Silica Gel 60 F_{254} plates (E. Merck, Darmstadt, Germany), using commercially obtained equine hemin as the reference standard. Employing a mobile phase of hexane:n-propanol:glacial acetic acid (10:5:1.5 v/v/v), ^{3}H-hemin migrated in a single band with the same R_f value (0.33) as the hemin standard.

Quantitation and Radioassay of Hemin and Hemoglobin:

Concentrations of hemin and hemoglobin were determined by difference spectrophotometry after converting hemin or hemoglobin-heme to their corresponding pyridine hemochromogens (69). A freshly prepared solution (1:5, v/v) of pyridine in NaOH (0.1N, 2 ml) was added to hemin or hemoglobin (1 ml), the mixture vortexed, and the solution divided equally into reference and sample cuvettes. The reference solution was oxidized by adding $K_3Fe(CN)_6$ (3 mM, 20 ul) and the sample solution reduced by sodium dithionite. Solutions were mixed by inverting the cuvettes several times, and the hemochromogen difference spectrum was determined between 500-600 nm, using an

Aminco DW-Dual beam scanning spectrophotometer (American Instruments Co., Silver Spring, Md.) Concentrations of hemin were calculated from the absorbance difference (557-541 nm) using delta E = 20.7 mM^{-1} (69). The concentration of hemin samples could be obtained directly, whereas hemoglobin concentration required dividing the value obtained for the pyridine-hemochromogen by 4; i.e., 4 moles heme per mole hemoglobin. Although hemoglobin concentrations are conventionally quantitated by the cyanmethemoglobin method (68), the pyridine hemochromogen assay was used for convenience. Values obtained for hemoglobin using the pyridine hemochromogen assay compared favorably with those obtained using the cyanmethemoglobin method, with less than 10% variability.

Radioactivity of hemin or hemoglobin was determined from 50-100 ul aliquots of known heme concentrations. Samples were placed in scintillation vials and dissolved in Hydroxide of Hyamine 10-X 1 ml, to which 10 ml of Dimilume-30 scintillation cocktail was added (Packard Instrument Co., Downers Grove, Ill.) Samples were counted in a Beckman LS 7000 liquid scintillation spectrometer (Beckman Instrument, Inc., Irvine, Ca.) The efficiency of counting was determined from a previously prepared quench curve. Radioactivity was expressed as disintegrations per min (DPM)/nmol heme.

Characterization and Authentication of Radiolabeled Hemoglobin:

Synthesized, radiolabeled hemoglobin was determined to be free of isotopic contamination from radiolabeled ALA or heme precursors, by comparison of specific activities of ^{3}H-hemoglobin (expressed in terms of spectrally determined heme content) and of ^{3}H-hemin which was isolated from this hemoglobin. The respective specific activities, 45,630 $\pm$ 4,440 and 49,800 $\pm$ 5,530 dpm/nmol (mean values $\pm$ S.D.) were not significantly different ($p<0.2$), verifying that all radioactivity was contained in heme.

Furthermore, the possible isotopic contamination of ^{3}H-hemoglobin with radiolabeled heme, bound non-specifically to hemoglobin, was investigated using gel filtration chromatography (described above). Specific activities before (48,320 dpm/nmol) and after (44,610 dpm/nmol) chromatography were not significantly different ($p < 0.5$), demonstrating that radiolabeled heme of hemoglobin was prosthetically bound.

Determination of methemoglobin contamination: The percent methemoglobin present in synthesized, radiolabeled and native hemoglobin solutions was determined spectrophotometrically by a standard method (68) as follows: Aliquots of hemoglobin solution were pipetted into two tubes. Hemoglobin in one tube was converted completely to methemoglobin by addition of $K_3Fe(CN)_6$ (0.6M, 0.1 ml) while the other tube received 0.1 ml of phosphate buffer. The absorbance difference (at 629 nm) of each solution was determined before and after the addition of 20 µl of KCN (0.8M). The percent methemoglobin content was calculated by dividing the value obtained for the methemoglobin solution by that obtained for the hemoglobin solution to which the buffer was added. The methemoglobin content of isotopically labeled hemoglobin, following reduction with a sodium dithionite reducing column, ranged from 2-6%, while freshly prepared native hemoglobin generally contained less than 1% methemoglobin.

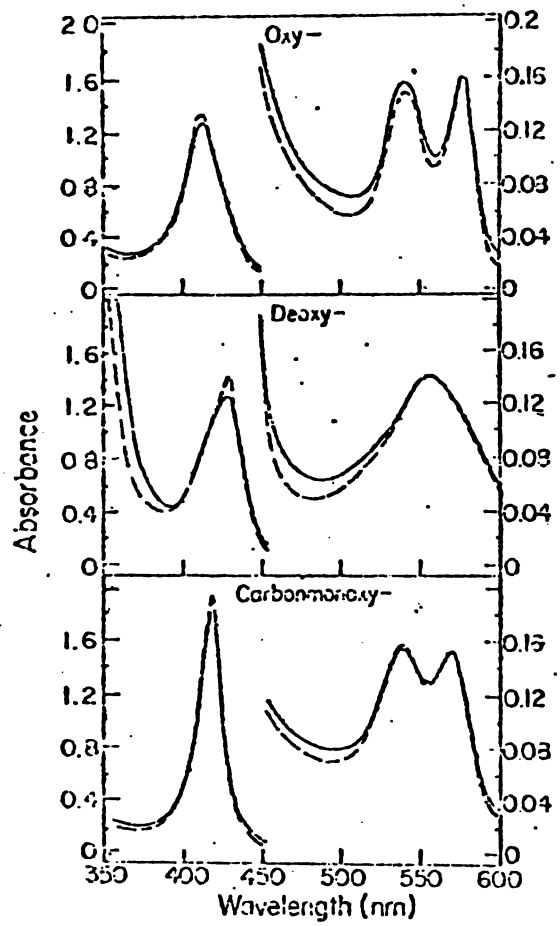
Comparative absorption spectra of native and isotopic hemoglobins: The authenticity of radiolabeled hemoglobin was established by spectrophotometric comparison (Hewlet Packard, Model 8450A, UV-VIS spectrophotometer with a 75-25B plotter) with freshly prepared native hemoglobin. Absorption spectra for ^{3}H-hemoglobin (oxy-, deoxy-, and carbonmonoxy- forms) were identical to those obtained for native hemoglobin (Figure 6).

Oxygen binding studies: Radiolabeled hemoglobin was chracterized physiologically by comparing its oxygen dissociation curve (ODC) with that of native hemoglobin. Oxygen binding properties of isotopic and native hemoglobins were determined using an Aminco Hem-O-Scan Oxygen Dissociation Analyzer (American Instrument Co.) (92). Carbon dioxide-free gases were employed and the pH (7.4), ionic strength (0.1 M phosphate buffer + 1 mM EDTA), temperature (37°C), and hemoglobin concentration (218-253 μ M) were controlled in these assays. The analyses (previously described) 16) were performed by placing 2 μl of hemoglobin solution on a cover slip and covering with a gas-permeable membrane. After inserting slips into the instrument, solutions were automatically deoxygenated by exposure to humidified nitrogen. Deoxyhemoglobin was then equilibrated with an atmosphere of continuously increasing oxygen measured with a Clark electrode. Simultaneously, the concentration of oxy- and deoxyhemoglobin was determined with a dual wavelength spectrophotometer and the percentage oxygenation recorded.

Oxygen binding affinities, expressed as P_{50} and Hill coefficients were calculated from the ODC. By definition, P_{50} is the partial pressure of oxygen at which hemoglobin is 50% saturated with oxygen; the relationship of P_{50} value to oxygen binding affinity is inverse, i.e., a decrease in values of P_{50} represents an increase in oxygen affinity, and vice versa. The Hill coefficient is an expression of the cooperative binding of oxygen among hemoglobin-heme moieties (6,41,43).

The oxygen affinity (P_{50}) of isotopic hemoglobin was significantly greater ($p<0.05$) than that of native hemoglobin (Table I), whereas the cooperativity (Hill coefficient) of isotopic hemoglobin was significantly decreased ($p < 0.005$) relative to that of native hemoglobin. Reduced cooperative binding of oxygen may be attributable to prior oxidation of

Figure 6. Comparison of Absorption Spectra of Oxy-, Deoxy-, and Carbonmonoxy- Forms of Isotopic and Native Hemoglobin Preparations.



Solid and dashed lines represent isotopic and native hemoglobin, respectively. For details, see Methods.

^{3}H-hemoglobin during its synthesis and storage. Previous investigators have observed similar reduced cooperativity of synthesized human hemoglobin, as compared to hemoglobin that had never been oxidized to methemoglobin (191). As a consequence of reduced cooperativity, the ODC was shifted to the left, resulting in a decreased value of P_{50} (higher oxygen affinity).

Although the cooperativity of synthesized, isotopic hemoglobin was different from that of native hemoglobin, in all likelihood, this did not influence results of experiments investigating the incorporation of hemoglobin-heme into hepatic cytochrome P-450. As is discussed below, oxygenation of hemoglobin, i.e., binding of oxygen to the heme moiety, prevents the dissociation of heme from hemoglobin (36). Since the affinity of isotopic hemoglobin for oxygen was increased relative to that of native hemoglobin (Table I), heme undoubtedly remained prosthetically bound to globin chains of hemoglobin.

Specific Methods

Incorporation of Hemoglobin-Heme into Rat Liver Tryptophan Pyrrolase:

Animal treatment and preparation of homogenates: Rats (155-210 g), fasted overnight, were injected with 1.0 ml of freshly prepared hemoglobin (350-400 nmol) via the penile vein; controls received buffer only. Animals were killed by decapitation at specific times from 0-180 min after injection. Livers were excised, weighed, perfused with ice-cold isotonic KCl, and reweighed. A fifty percent (w/v) homogenate was prepared by mincing and homogenizing livers in 5-10 ml of phosphate buffer. The homogenate was filtered through gauze, and additional phosphate buffer was added to bring the homogenate to two volumes of that of the perfused liver. Homogenates were stored in ice until assay.

Table 1. Oxygen Binding Properties of Isotopic and Native Rat Hemoglobins.

Preparation	n	P_{50}	Hill Coefficient
Isotopic Hemoglobin	3	16.3 ± 1.1	1.76 ± 0.03
Native Hemoglobin	4	19.7 ± 1.2	2.02 ± 0.06

Mean values ± S.D.
N = number of observations.
See Methods for details.

Assay of tryptophan pyrrolase activity: The activity of tryptophan pyrrolase was determined by the method of Feigelson and Greengard, 1961 (74), as modified (11,44), in which the formation of kynurenine from L-tryptophan is monitored. Homogenate (3 ml) was added to each of the two reaction flasks containing 12.0 ml of phosphate buffer (0.2 M, pH 7.0), distilled water (12.5 ml), and L-tryptophan (1.5 mM 2.5 ml). Hemin, (0.2 M, prepared as described above) was added to one reaction flask to allow determination of total enzyme activity (holo + apo), while the other flask, in which holoenzyme activity was measured, received no hemin. Reactions were carried out aerobically for 60 min at 37°C in a shaking waterbath. At times 0 and 60 min, duplicate aliquots were removed from reaction mixtures and added to tubes containing 2.0 ml of ice-cold trichloroacetic acid (0.9 M) to stop the reaction. The tubes were vortexed and the precipitated protein was sedimented by brief centrifugation (5,000 x g) and the supernatant (2.5 ml) was pipetted into tubes containing 1.5 ml of NaOH (0.6 N). Absorbance values were determined (within 40 min after addition of NaOH) at 365 nm using a Carl Zeiss spectrophotometer (model PMQ II, Oberkochen/Wuertt, West Germany). The amount of kynurenine formed was calculated from absorbance (60-0 min.) values using an extinction coefficient of 4.54 nM^{-1}. The activities were expressed as µmol kynurenine/g liver/h. The percent heme saturation of tryptophan pyrrolase at various times following injection of hemoglobin was calculated by dividing the holoenzyme activity by the total activity.

Isolated Perfused Liver Experiments:

A recirculating, isolated, perfused liver system, described by Gollan et al; (1981), was employed to examine the incorporation of hemoglobin-heme into cytochrome P-450. This system, coupled with the use of an artificial blood substitute (described below), was ideally suited for these studies

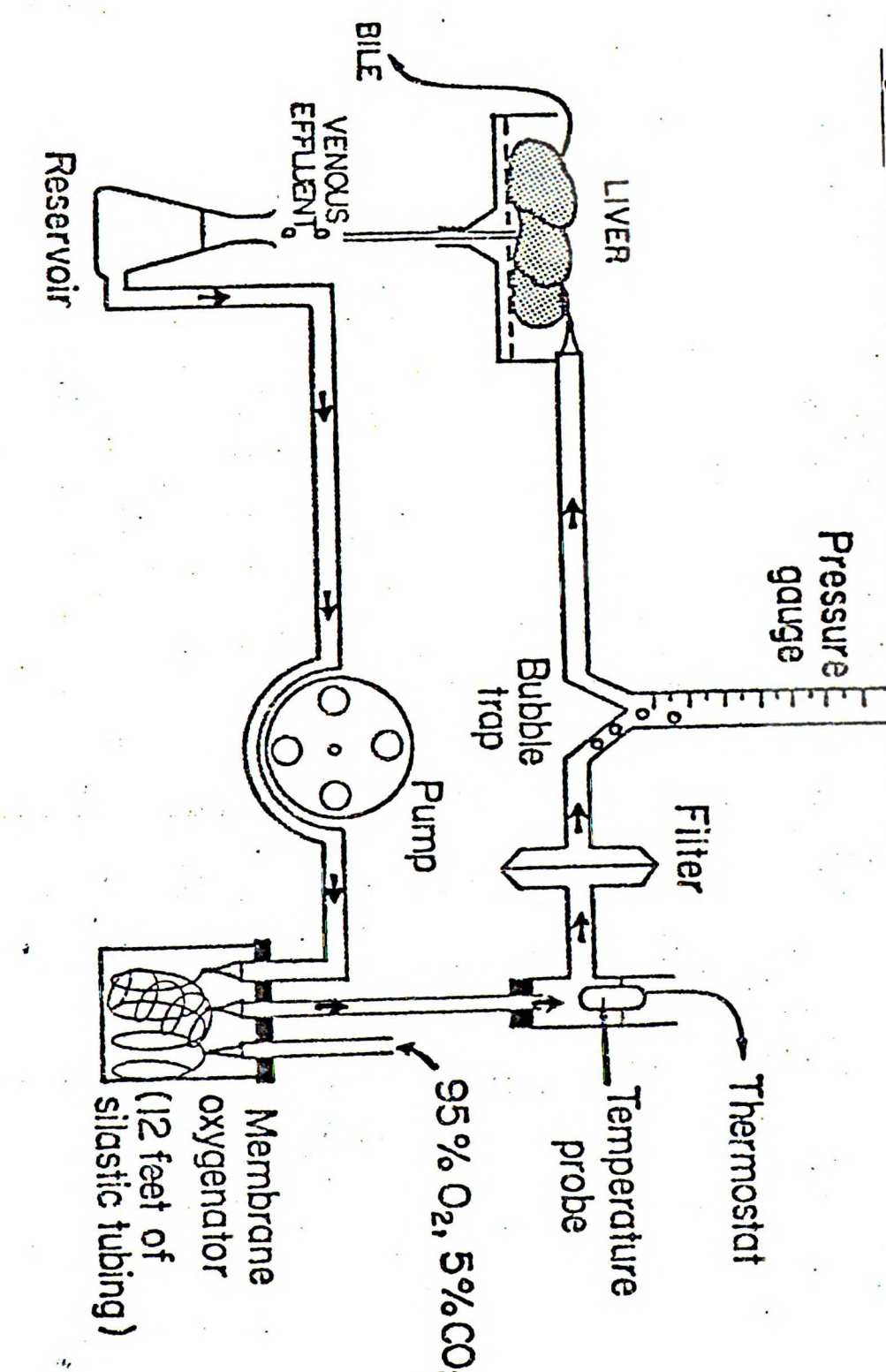


Figure 7. Recirculating Isolated Rat Liver Perfusion System.

because, (1) the distribution of administered hemoglobin and AIA was limited to the liver, (2) large doses of hemoglobin could be administered since normal elimination of hemoglobin by renal filtration was circumvented, and (3) confounding effects of endogenous sources of hemoglobin were removed by using a hemoglobin free perfusion medium.

Perfusion medium: Livers were perfused with Fluosol^{R}-43 (Alpha Therapeutics Corp., Los Angeles, Ca.), a hemoglobin-free medium containing a Krebs-Ringer bicarbonate solution and a perfluoro-chemical emulsion as an oxygen carrier (75). This mixture was made up fresh and 100 units penicillin added. After thorough mixing, the solution was filtered through a 10 μ filter (Millipore Filter Corp., Bedford, Ma.) and the pH adjusted to 7.4 with $NaHCO_3$ (0.1 M).

Perfusion apparatus: The perfusion circuit (Figure 7) was housed in a thermostatically controlled lucite cabinet. Perfusate (50 ml) was recirculated by means of a peristaltic pump (LKB Multiperpex 2115, Bromma, Sweden). The sequence of circulation was the following: Perfusate was stirred in a glass reservoir, passed through the pump to a membrane "lung" (90) comprised of coiled Silastic tubing (4 m, 0.147 cm i.d., 0.196 cm o.d., Dow Corning Corp., Midland, Mi.), to a temperature sensitive probe (connected to a thermostat maintained at 37°C), through a stainless steel filter screen (Millipore), to a bubble trap and pressure gauge, and finally into the portal vein through a teflon cannula. Before the isolated liver was placed in the circuit, the perfusate was oxygenated with a mixture of 95% O_2 and 5% CO_2 (gas flow rate = 0.4 l/min). After the liver was connected to the circuit, 100% O_2 was substituted.

Liver isolation: All rats used in perfusion experiments were pretreated with phenobarbital (80 mg/kg, i.p.) daily for five days. Livers were removed and prepared for perfusion using the operative technique described by Hems et al., 1966 (93). Under ether anesthesia, the bile duct

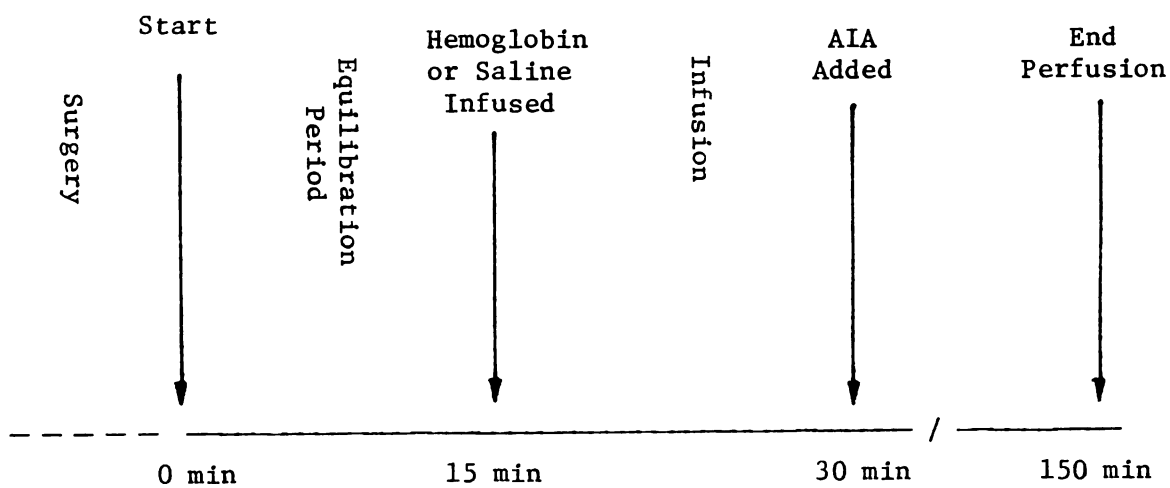
was cannulated with PE-10 tubing (Clay Adams, Div. of Becton, Dickinson and Co., Parsippany, N.J.) The portal vein was cannulated with a 16-gauge catheter (Angiocath, Deveret Pharmaceuticals, Sandy, Ut.), the liver flushed with 1.5 ml of heparin in 0.15 M NaCl, and then perfused continuously (approximately 8 ml/min) throughout the remaining surgery with an oxygenated Krebs-Henseleit-Ringer bicarbonate solution (54) containing glucose (11.5 mM). The inferior vena cava was cannulated above the diaphragm. The liver was then dissected from the animal, placed on a nylon mesh, rinsed with warm saline and transferred to the perfusion cabinet. Total time required for liver isolation was 20-22 min.

Before placing in the cabinet, the liver was held at an incline so that perfusate would flow in a retrograde direction while the portal vein cannula was disconnected from the Krebs-Henseleit-Ringer solution and reconnected to the Fluosol^{R}-43 perfusion medium. The liver was then positioned on a draining dish above the perfusion reservoir and covered with a closed plexiglass cylinder which contained a moistened gauze pad. The perfusion flow rate was adjusted to 25 ml/min, resulting in a stable perfusion pressure of 14 cm water. Sodium taurocholate (0.33M) was infused into the perfusate (1 ml/h) to stabilize bile flow.

An equilibration period of 15-30 min was routinely observed before starting individual experiments. In those experiments in which AIA was administered, the liver acquired a green-brown discoloration, but otherwise appeared normal at the end of the perfusion period. Viability of perfused livers was satisfactory after 2.5 h perfusion as reflected by a normal appearance on electron microscopy, stable perfusate lactate dehydrogenase and transaminase activity, and normal hepatic oxygen consumption (85).

Incorporation of Hemoglobin-Heme into Hepatic Cytochrome P-450:

Experimental design: In separate experiments, the isolated rat liver perfusion system was employed to study: (1) if administration of hemoglobin increased the metabolism of ^{14}C-AIA and (2) whether hemoglobin-heme reconstitutes apo-cytochrome P-450 stripped of its heme by AIA. The design of liver perfusion for these experiments was the same. The concentration of AIA (1.8 mM) employed was previously shown (71) to be optimal for destruction of cytochrome P-450 in the isolated rat liver perfusion system. Male rats (235-275 g) were paired on the basis of body weight and two perfusions (hemoglobin-treated and control) were performed the same day as schematically diagrammed below. After 15 min equilibration period, hemoglobin (570-580 nmol) or saline (control) was added to the perfusion medium by slowly infusing with a multispeed-infusion pump (Harvard Apparatus Co., Millis, Mass.) ^{14}C-AIA (89 μmol, specific activity 55-70 dpm/nmol), dissolved in 5 ml of Fluosol ^{R}-43 containing bovine serum albumin (fraction V, 8.3 μmol), was then added to the perfusion reservoir. Control experiments for the reconstitution of cytochrome P-450 were performed in an identical manner, except hemoglobin or AIA was omitted.



(1), ^{14}C-AIA elimination after hemoglobin addition: During liver perfusions, the perfusate was sampled (0.25 ml) at 5 min intervals for 2 h. Aliquots of samples (0.1 ml) were combined with 0.4 ml of phosphate buffer (0.1 M) and extracted with 5 ml of diethyl ether by vortexing for 60 sec. Ether was separated from the aqueous phase by brief centrifugation, the aqueous layer frozen in a dry ice/ethanol bath, and the ether decanted into scintillation vials. Employing this protocol, the AIA extraction efficiency was 99% (71). Ten ml of Aquasol (New England Nuclear, Boston, Mass.) was added to vials and radioactivity determined by liquid scintillation spectrometry.

(2), Structural and functional reconstitution of hepatic cytochrome P-450: Following the 2.5 h perfusion, livers were flushed with 60 ml of ice-cold KCl (0.15 M) injected through the portal vein cannula. Microsomes were prepared, as previously described (49), by homogenizing livers (50% v/v) in phosphate buffer (0.1 M). Cytochrome P-450 content was measured by (CO + $Na_2S_2O_4$) - CO difference spectroscopy (67), in an Aminco DW-2 spectrophotometer, using an extinction coefficient of 100 $mM^{-1}cm^{-1}$. Cytochrome P-450 content was expressed as nmol/mg protein. Protein concentration was determined by the method of Lowry, et al, 1951 (117).

The functional reconstitution of cytochrome P-450 dependent mixed function oxidase was assayed by measuring formaldehyde formed (134) from microsomal N-demethylation of ethylmorphine (47) and p-chloro-N-methylaniline (PCNMA) (48). Reaction mixtures (final volume 5 ml) contained ethylmorphine (2 mM) or PCNMA (1 mM), an NADPH-generating system [NADP (0.4 mM), glucose-6-phosphate (4 mM), and glucose-6-phosphate dehydrogenase (2 units)], KCl (77 mM), $MgCl_2 \cdot 6H_2O$ (2 mM), semicarbazide HCl (7.5 mM), Na_2HPO_4 (32 mM), KH_2PO_4 (8 mM), and microsomes (1-4 mg protein). Incubations were conducted in air in a shaking water bath (37°C) for 15 min and reactions

terminated by addition of $ZnSO_4$ $7H_2O$ (0.4 mM). Excess sulfate was precipitated by adding $Ba(OH)_2$ $8H_2O$ (0.35 mM). After brief centrifugation, supernatant (5 ml) was mixed with 3 ml of Nash's reagent (0.39 mol ammonium acetate, 0.2 ml 2,4-pentanedione, and 0.3 ml glacial acetic acid made up to 100 ml volume with distilled water (134). The mixture was heated at 60°C for 20 min. After cooling, the formaldehyde content was determined at 412 nm using an extinction coefficient = 0.88 mM^{-1} cm^{-1}, (in a Beckman, model B Spectrophotometer). Activity was expressed as nmol formaldehyde/mg protein/15 min.

Incorporation of Radiolabeled Hemoglobin-Heme into AIA-Porphyrin Adducts:

Liver Perfusion Experiments with Isotopic Hemoglobin: In preliminary experiments, ^{3}H-Hemoglobin and AIA were administered to isolated perfused rat livers to demonstrate formation of radiolabeled AIA-porphyrin adducts. These experiments were conducted in the same manner as the perfusion studies described above, except that ^{3}H-hemoglobin was substituted for non-radiolabeled, native hemoglobin. At the end of the perfusion (2.5 h), livers were homogenized in 5 ml of phosphate buffer (0.1 M) and AIA-porphyrin adducts extracted, isolated and purified as described below.

Perfusion isolated livers with ^{3}H-hemoglobin and AIA resulted in the formation of radiolabeled AIA-porphyrin adducts, thereby demonstrating incorporation of radiolabeled hemoglobin-heme into hepatic cytochrome P-450. However, since the isotopic hemoglobin added to the perfusate retained a small fraction of methemoglobin that had escaped sodium dithionite-reduction (see Quantitation of Methemoglobin Contamination), it was necessary to examine whether radiolabeled heme, incorporated into the adducts, was derived from methemoglobin. This was possible in view of the report that, under physiological conditions, methemoglobin-heme is less tightly bound and readily dissociates from its globin chain, exchanging with other

hemoglobin molecules, as well as with methemalbumin (36). Ligands of heme (O_2, CO, or CN^-) or binding of hemoglobin to haptoglobin prevent such dissociation and exchange of hemoglobin-heme. Similar dissociation of ^{3}H-heme from methemoglobin and its subsequent incorporation into cytochrome P-450, could largely, if not solely, account for the labeled AIA-heme adducts obtained in isolated livers perfused with isotopic hemoglobin. To resolve this ambiguity, separate in vivo experiments were performed.

In Vivo Experiments with Isotopic Hemoglobin: To determine the relative extent to which radiolabeled heme, incorporated into cytochrome P-450, was derived from hemoglobin and/or contaminating methemoglobin, it was necessary to prepare hemoglobin that was quantitatively identical (in terms of heme content) to isotopic hemoglobin, but contained radioactive heme in amounts equivalent to that in the methemoglobin present in isotopic hemoglobin. Since methemoglobin-heme, because of decreased binding affinity, readily dissociates from globin, it was possible to simulate the methemoglobin content of isotopic hemoglobin by adding radiolabeled heme (isolated from isotopic hemoglobin) to native non-radiolabeled hemoglobin. Indeed, heme added in this manner was spectrally indistinguishable from methemoglobin and was quantitated by the standard spectrophotometric assay for methemoglobin (68) (see Quantitation of Methemoglobin). The quantity of ^{3}H-heme (33 nmol) that was added to native hemoglobin to represent isotopic methemoglobin-heme contamination was calculated on the basis of the combined methemoglobin contents of native and isotopic hemoglobins.

Animal treatment: Male rats (230-280 g) were pretreated daily with phenobarbital (80 mg/kg, i.p.) for 5 days. The experimental protocol employed eight rats; four rats were injected with ^{3}H-hemoglobin, and the other four received native hemoglobin + ^{3}H-hemin. Within each 4-rat group

two rats received AIA, and two served as controls, receiving saline. The schedule was as follows: One h prior to administration of AIA or saline rats were anesthetized with diethyl ether, and kidneys were surgically ligated to prevent renal filtration of hemoglobin (35), thus maximizing its hepatic uptake. Surgeries were completed within 25-30 min, and an additional 30 min was observed for animals to recover from anesthesia. AIA (200 mg/kg), dissolved in distilled water, was injected subcutaneously; control rats received subcutaneous injections of an equal volume of saline. Thirty min later, hemoglobin (^{3}H-hemoglobin or native hemoglobin + ^{3}H-heme) was injected (1 ml) via the penile vein; respective concentrations, expressed in terms of heme, were 471 and 475 μM.

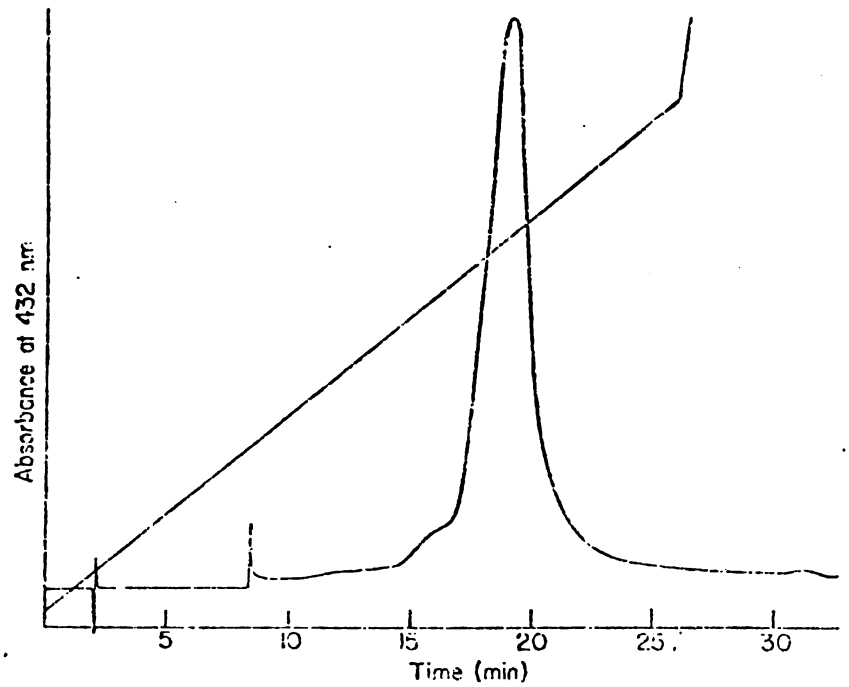
Two hours after injecting hemoglobin, animals were killed by decapitation. Livers were removed, weighed before and after perfusion with ice-cold KCl (0.15 M), and minced and homogenized individually without buffer. Aliquots (2 g for AIA-treated livers and 5 g for control livers) were suspended (1:10, v/v) in phosphate buffer and used for preparation of microsomes and CO-binding particles. The remainder of the AIA-treated livers was used for isolation of AIA-porphyrin adducts. Cytochrome P-450 and heme content, and heme-specific radioactivity were assayed in homogenates, microsomes, and CO-binding particles. Cytochrome b_5 was measured in microscomes and CO-binding particles (described below).

Isolation and purification of AIA-porphyrin adducts: AIA-porphyrin adducts were isolated, as previously described (45), by extracting (methylating) overnight in cold sulfuric acid/methanol (1:20 v/v, 125 ml/liver) at 4°C in the dark. The following morning, denatured protein was removed by filtration through Whatman filter paper. After washing the protein with dichloromethane (25 ml), the filtrate and distilled water (approximately 125 ml) were added to a separatory funnel and the crude methylated adducts

extracted three times with dichloromethane. Extracts were combined, washed three times with an equal volume of water, and dried over anhydrous sodium sulfate. Zinc acetate, saturated solution in methanol (1 ml), was then added to complex the porphyrin adducts with zinc. Reaction flasks were swirled intermittently for 15 min and the dichloromethane removed by roto-evaporation. The crude adducts were dissolved in a small volume of chloroform and isolated by TLC on Silica Gel GF TLC plates (1,000 μ Analtech, Inc., Newark, Del.) in chloroform/acetone (3:1 v/v). The zinc complex of the porphyrin adducts migrated as a green band which fluoresced red under UV light (R_f = 0.4-0.5). This band was scraped from TLC plates and the zinc complex extracted with acetone. Acetone was removed by roto-evaporation, and a second TLC isolation was performed under the same conditions using 500 μ plates. The AIA-porphyrin adducts were extracted with chloroform (5-10 ml) and quantitated spectrophotometrically (432 nm) using an Aminco DW-2 spectrophotometer, extinction coefficient = 125,000 $M^{-1}cm^{-1}$ (139); aliquots (0.1 ml) were removed for scintillation counting.

Further purification of zinc-complexed-porphyrin adducts was accomplished by repetitive high pressure liquid chromatography (HPLC), using a Hitachi 100-40 variable wavelength UV-VIS detector equipped with an analytical flow-cell module (200 μl volume), two Altex (110 and 110A) analytical solvent-metering pumps, a Laboratory Data Control solvent programmer (model 410) and a Linear dual pen recorder, model 585. The detector was set to a wavelength of 432 nm, and the adducts were eluted from a Whatman Particil PAC column (10 μ, 4.6 x 250 mm) using a 30 min linear gradient (0-100%) of methanol into hexane/tetrahydrofuran (1:1 v/v). A typical elution profile is shown in Figure 8. Sequential effluents were analyzed for radioactivity. The eluent fraction containing the zinc complex was dried under nitrogen, quantitated as before, and the specific activity determined. HPLC analyses were repeated (2-3 times) to eliminate all labeled

Figure 8. HPLC Elution Profile of Zinc-Complexted AIA-Porphyrin Adducts.



contaminants which might have tailed into the zinc complex fraction during the first elution. The porphyrin adducts were considered pure when a constant specific activity was attained. The identity of the zinc complex was established by its characteristic absorption spectrum (143) shown in Figure 9. The spectrum exhibits a Soret band at 432 nm and three absorption peaks at 547, 589, and 638 nm.

Recovery of AIA-porphyrin adducts: Recoveries of zinc-complexed adducts after HPLC analyses varied among individual rats and from one HPLC purification step to the next, thus making it difficult to quantitate the total formation of AIA-porphyrin adducts. Total adducts were therefore quantitated, after TLC isolation, from extraction efficiencies determined in separate but identical experiments. Purified, radiolabeled AIA-porphyrin adducts (zinc complex) were added to liver homogenates obtained from AIA-treated rats, and the adducts were extracted and isolated in the same manner as described above. On the basis of the total radioactivity extracted, the recovery of adducts after TLC was 35 $\pm$ 17% of that initially added (mean values $\pm$ S.D., n=5. The total formation of adducts *in vivo* studies was calculated by dividing the amount of adducts recovered following TLC isolation by the extraction efficiency. This value was then multiplied by the specific activity of purified adducts to yield the total radioactivity attributable to AIA-porphyrin adducts. The specific activities employed in the calculation of total adduct formation represented an average of activities obtained in the two final HPLC purifications.

Preparation of tissue fractions and analytical determinations: Aliquots of liver homogenate suspended in the phosphate buffer containing glutathione (2 mM) and nicotinamide (10 mM) were used for preparation of microsomes, as described above. Carbon monoxide-binding particles were

prepared from an aliquot of microsomes as follows: After determining the protein concentration, the microsomal suspension was supplemented with 20% (v/v) glycerol and incubated with and without protease (10 μg/mg microsomal protein, (44). Digestion with protease removed cytochrome b_5 with minimal removal of cytochrome P-450 and therefore, cytochrome P-450 was left as the only hemoprotein in the preparation; the heme content of these particles largely represented cytochrome P-450-heme.

Quantitation of cytochrome P-450 and heme was performed as described above. Total radioactivity was determined from aliquots (0.2 ml) of tissue fractions as described above. Heme-specific radioactivity was determined in the same manner, following its isolation and crystalization (described above). Cytochrome b_5 was determined by oxidized-reduced difference spectrophotometry using NADH to reduce the cytochrome (424 - 409 nm, extinction coefficient = 185 $mM^{-1}cm^{-1}$) (137).

Statistical Analysis of Results:

Differences between groups in isolated rat liver perfusion experiments, conducted to demonstrate structural and functional reconstitution of cytochrome P-450, were assessed for statistical significance employing the Student's paired "t" test. All other experiments were statistically analyzed using the unpaired "t" test.

Preliminary Experiments

(1), Intravascular Formation of Methemoglobin Following Injection of ^{3}H-Hemoglobin:

Previous investigators have demonstrated that hemoglobin, released intravascularly following hemolysis, is autooxidized to methemoglobin (78). Therefore, it was possible that the methemoglobin content of ^{3}H-hemoglobin would be elevated above that initially measured, following its injection.

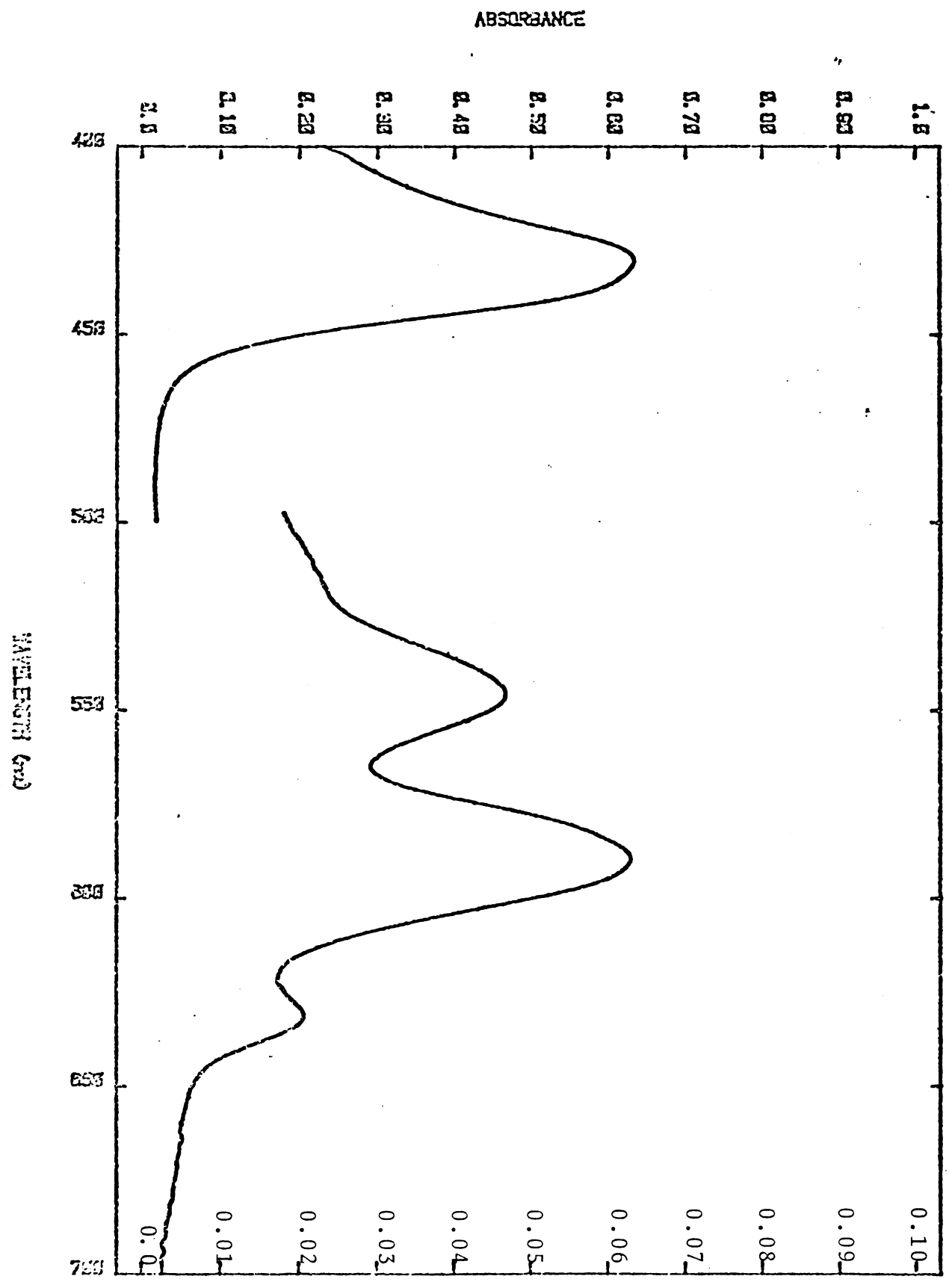


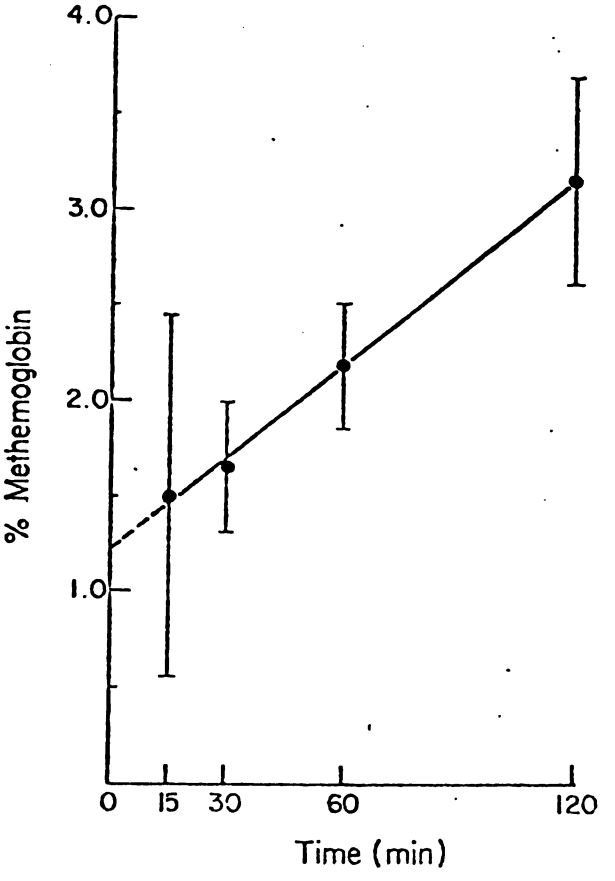
Figure 9. Characteristic Absorption Spectrum of Zinc-Complexed AIA-Porphyrin Adducts.

If ^{3}H-hemoglobin was autooxidized, intravascularly, to an appreciable extent, the amount of ^{3}H-methemoglobin-heme available for incorporation into cytochrome P-450 would be significantly increased. In solution, the degree to which hemoglobin is oxidized is dependent upon the oxygen tension of the solution (78). The rate of methemoglobin formation was therefore examined in an in vitro experiment which was performed under conditions simulating an intravascular environment

Assays were conducted in Ehrlenmeyer flasks, containing 10 ml of heparinized, whole blood. Flasks were placed in a 37°C waterbath and equilibrated for 2 h under an atmosphere of 9.2% O_2, 5.0% CO_2, and 85.8% N_2. Based on the assumption that the volume of distribution of venous and arterial blood are equal (78), gas flow meters were adjusted to provide an atmospheric pO_2 of 70 mm Hg, which represented an average of venous and arterial pO_2 (venous pO_2 = 45 mm Hg, arterial pO_2 = 95 mm Hg). Prior to the addition of native hemoglobin, the initial pO_2, as measured by blood gas analysis, was 72.9 $\pm$ 1.5 mm Hg (mean value $\pm$ S.D. of 3 determinations). One ml of freshly prepared, native hemoglobin (350 nmol) was added to whole blood using a syringe. Solutions of blood and hemoglobin were incubated, with gentle agitation, for 15, 30, 60 and 120 min. Erythrocytes and plasma were then separated by brief centrifugation and the percent methemoglobin present in plasma was determined as described above. The concentration of hemoglobin (nmol/ml) was constant for all samples, indicating little, if any, hemolysis of erythrocytes. After 120 min incubation, the pO_2 determined in control flasks was 74.4 $\pm$ 3.7 mm Hg.

Methemoglobin formation, although limited, was linear with time (k=0.016, r=0.999) (Figure 10); approximately 2% of the added hemoglobin was converted to methemoglobin over the 2 h period.

Figure 10. Temporal Formation of Methemoglobin Under Conditions Simulating an Intravascular Environment.



See Methods for details.

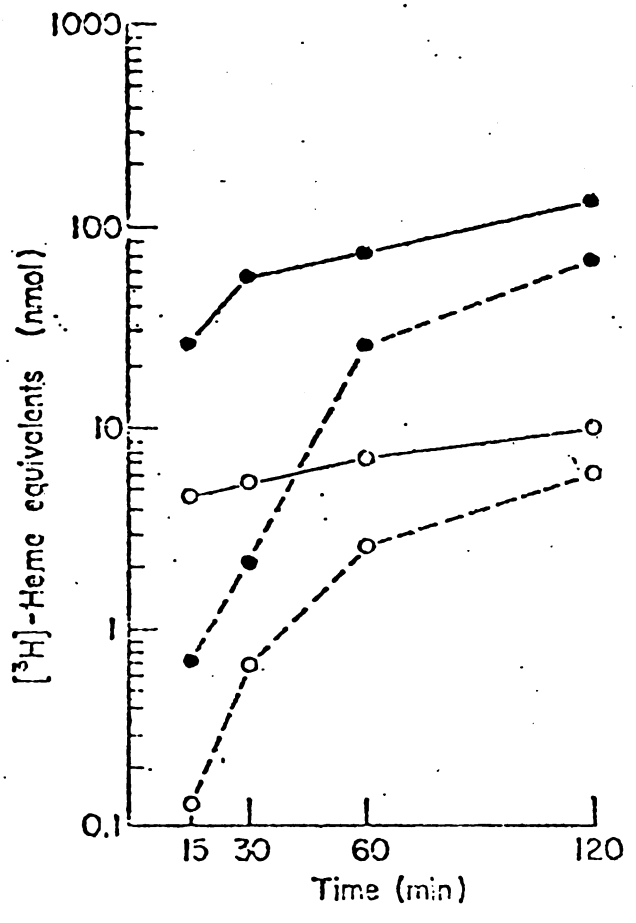
(2), Hepatic Uptake of ^{3}H-Heme Following Injection of ^{3}H-Hemoglobin or Native Hemoglobin + ^{3}H-Heme:

Heme derived from methemoglobin and heme prosthetically bound to hemoglobin are incorporated into hepatocytes by separate mechanisms. Methemoglobin-heme rapidly dissociates from globin, is bound by the carrier-protein, hemopexin, and is subsequently transported to the liver (36, 94). Hemoglobin, on the other hand, is bound by serum haptoglobin, and the hemoglobin-haptoglobin complex is taken up intact by the liver (21,34). Once inside the liver, heme is delivered, by mechanisms not yet clarified, to the free-heme pool. Thus, the incorporation of ^{3}H-heme into cytochrome P-450 is a function, not only of the amount of ^{3}H-heme injected, but also of the rate at which heme is taken up by liver parenchymal cells.

In vivo experiments were performed to determine the rate of hepatic uptake of methemoglobin-heme and heme prosthetically bound to hemoglobin. These experiments were carried out employing the same experimental design as used to determine the *in vivo* formation of radiolabeled AIA-porphyrin adducts (described above), except: (1), bile ducts were cannulated with PE-10 tubing and (2), rats were injected subcutaneously with saline rather than AIA. Subcutaneous injections of AIA were omitted because treatment with AIA would prevent the normal catabolism of ^{3}H-heme and therefore affect the disposition of radiolabeled metabolites in bile (110).

Animals were killed at 15, 30, 60, and 120 min following the injection of hemoglobin. Livers were excised, weighed, thoroughly perfused with ice-cold KCl (0.15 M), and homogenized in phosphate buffer. Homogenates and bile (0.2 ml) were added to scintillation vials and bleached overnight with 30% hydrogen peroxide (0.2 ml). Hydroxide of Hyamine (1.0 ml) was added to samples and radioactivity determined by scintillation spectrometry.

Figure 11. Temporal Hepatic Uptake and Biliary Secretion of Radioactivity Administered as ^{3}H-Hemoglobin and Native Hemoglobin + ^{3}H-Heme.



Radioactivity is expressed in heme equivalents. (●) and (○) represent ^{3}H-hemoglobin and native hemoglobin + ^{3}H-heme, respectively. Solid and dashed lines respectively represent radioactivity present in the liver and secreted in the bile. See Methods for details.

The hepatic uptake of ^{3}H-heme, administered as ^{3}H-hemoglobin or native hemoglobin + ^{3}H-heme (methemoglobin control), was quantitated on the basis of radioactivity recovered from liver homogenates and bile. The amount of ^{3}H-heme administered in the form of ^{3}H-hemoglobin to rats was approximately twenty times higher than that administered to rats which received native hemoglobin + ^{3}H-heme (470 nmol and 25.5 nmol, respectively). Thus, the amount of ^{3}H-heme which was taken up by livers, and subsequently transported to bile, was far greater in animals which received ^{3}H-hemoglobin (Figure 11).

Although the quantity of radiolabeled heme taken up by hepatocytes was greater in rats receiving ^{3}H-hemoglobin, the relative rate of uptake, expressed as percentage of radioactivity injected, was highest in rats receiving native hemoglobin + ^{3}H-heme (Table II). Fifteen minutes after injection, 19% of the ^{3}H-heme added to native hemoglobin was taken up by the liver, as compared to 5.8% for ^{3}H-hemoglobin-heme. The appearance of radioactivity in bile (possibly as bilirubin) reflects the rate of incorporation of isotopic heme into the free-heme pool and its subsequent metabolism. Again, on the basis of percentage of radioactivity injected, the intrahepatic transfer of isotopic heme to the free-heme pool was greater in rats which received native hemoglobin + ^{3}H-heme than in those which received ^{3}H-hemoglobin.

Examination of the ratio of bile to liver radioactivity with time, however, revealed that the initial rate of heme transfer to the free-heme pool was comparable for animals given either ^{3}H-hemoglobin or native hemoglobin +^{3}H-heme (at 15 min, ratios of bile to liver radioactivity were 0.03 and 0.02, respectively, Table II). This similarity may reflect the uptake of methemoglobin-heme present in ^{3}H-hemoglobin as contaminant. At 30 and 60 min, the ratio of bile to liver radioactivity was substantially increased in rats which received native hemoglobin + ^{3}H-heme, indicating a

TABLE II. PERCENTAGE OF RADIOACTIVITY DETERMINED IN LIVER AND BILE FOLLOWING INJECTION OF ISOTOPIC HEMOGLOBIN AND NATIVE HEMOGLOBIN + ISOTOPIC HEME.

	^{3}H-Hemoglobin (Percent of Radioactivity Injected)				Native Hemoglobin + ^{3}H-Heme (Percent of Radioactivity Injected)			
Preparation	15 Min	30 Min	60 Min	120 Min	15 Min	30 Min	60 Min	120 Min
Liver	5.8 ± 0.9	12.6 ± 1.7	16.7 ± 4.9	28.6 ± 10.9	19.0 ± 6.9	21.5 ± 12.4	27.9 ± 9.2	38.3 ± 6.6
Bile	0.2 ± 0.1	0.5 ± 0.3	2.9 ± 1.7	17.2 ± 2.7	0.5 ± 0.2	2.6 ± 1.4	10.1 ± 3.6	24.4 ± 7.5
Total	6.0	13.1	19.6	45.8	19.5	24.1	38.0	63.2
Ratio of Bile/Liver Radio-Activity	0.03	0.04	0.17	0.60	0.02	0.12	0.36	0.62

Values are mean ± S.D. from three individual experiments.

more rapid intrahepatic transfer of non-prosthetically bound heme to the free-heme pool. Two hours after injection, however, the transfer of isotopic hemoglobin-heme to the free-heme pool was equivalent to that of methemoglobin-heme, as reflected by comparable ratios of bile to liver radioactivity for rats administered ^{3}H-hemoglobin and native hemoglobin + ^{3}H-heme. The delay in the intracellular transfer of ^{3}H-hemoglobin-heme is expected, since hemoglobin-heme must first dissociate from the hemoglobin-haptoglobin complex.

The percentage uptake of isotopic hemoglobin observed in these experiments was lower than that previously reported. Bissell et al., 1972 (21), reported that 30% of isotopic hemoglobin-heme, administered in amounts not exceeding the haptoglobin-binding capacity, was removed by the liver in the first 30 minutes following injection. In the present study, 30 minutes after injection of hemoglobin in excess of the haptoglobin binding capacity, hepatic uptake was only 13%. These results suggest that hemoglobin, not bound to haptoglobin, may be taken up by hepatocytes at a much slower rate than is haptoglobin-bound hemoglobin, thus resulting in a decrease in the overall rate of hepatic uptake of hemoglobin. The rate of hepatic uptake of free hemoglobin is presently not known.

(3), Hemoglobin Catalyzed Alkylation of Hemoglobin-Heme by AIA:

Previous investigators have demonstrated that hemoglobin, under certain conditions, may function enzymatically in the metabolism of various substrates (7,22,39,128). In fact hemoglobin served as a terminal oxidase, in a reconstituted aniline hydroxylase system, at a level of activity comparable to that of cytochrome P-450 (128). Furthermore, hemoglobin catalyzed phenylhydrazine oxidation results in N-arylation of the heme of hemoglobin with the production of a green pigment (7). In the present study, it was therefore conceivable that hemoglobin might be partially re-

sponsible for the formation of radiolabeled AIA-porphyrin adducts in vivo or in isolated perfused livers.

To determine whether hemoglobin catalyzed AIA-mediated alkylation of its own prosthetic group, the mixed function oxidase activity of cytochrome P-450 was inhibited by administration of SKF-525-A. Use of SKF-525-A to inhibit the self-catalyzed destruction of cytochrome P-450 by AIA has been described previously (56), whereas oxidations catalyzed by hemoglobin are not inhibited by SKF-525-A (128). In addition, to further restrict the formation of AIA-porphyrin adducts, untreated rather than phenobarbital-treated rats were used.

Rats (325-375 g) were injected with SKF-525-A (60 mg/kg, i.p.) 1 h prior to subcutaneous administration of AIA (200 mg/kg). During the period between injections, kidneys were surgically ligated (described above). Thirty min following administration of AIA, 1.75 ml of ^{3}H-hemoglobin (610 uM) was injected via the penile vein. Analogous control experiments were performed with saline administered in place of hemoglobin, AIA, or SKF-525-A. Animals were killed 2 h after hemoglobin injection and livers were excised and examined for the formation of AIA-porphyrin adducts as described above.

Formation of AIA-adducts was decreased approximately four-fold in untreated rats, compared to phenobarbital pretreated animals (7.8 and 30.6 nmol/g liver, respectively) (Table III). In animals treated with SKF-525-A, the formation of adducts was further reduced from a control value of 7.8 to 1.8 nmol/g liver, independent of whether ^{3}H-hemoglobin was administered. These results confirm the inhibition of cytochrome P-450-dependent mixed function oxidation by SKF-525-A. Treatment of rats with SKF-525-A alone (without AIA) yielded no adducts. The fact that adducts were recovered from animals pretreated with SKF-525-A indicates that inhibition of cytochrome P-450-dependent mixed function oxidase activity might have been

Table III. The Contribution of Hemoglobin to the Formation of AIA Porphyrin Adducts Independent of Cytochrome P-450 Dependent Mixed Function Oxidations.

Treatment	N	Total AIA Adducts (nmol/g liver)	^{3}H-AIA Adducts Formed (nmol/g liver)
Phenobarbital + AIA + ^{3}H-Hb	4	30.6	1.04
AIA Only	2	7.8	----
SKF-525-A + AIA + ^{3}H-Hb	4	1.8	0.08
SKF-525-A + AIA	2	1.8	----
SKF-525-A Only	2	0	----

N = number of animals.
Values presented are means; for each determination, livers of two or more animals were pooled and extracted for AIA-porphyrin adducts (see Methods).

incomplete. In addition, radioactive adducts generated from animals receiving SKF-525-A may have been derived from ^{3}H-hemoglobin-heme incorporated into residual (unihibited) cytochrome P-450 and/or from direct oxidative alkylation of ^{3}H-hemoglobin by AIA. Thus, the possibility remains that a fraction of AIA-adducts may have been derived from ^{3}H-hemoglobin-directed catalysis of AIA (independent of cytochrome P-450). However, if this occurred, the contribution of adducts from hemoglobin-dependent catalysis was not more than 8% (0.08 nmol/g liver) of the total hepatic content of labeled AIA-porphyrin adducts generated by phenobarbital-pretreated rats given an equivalent dose of radiolabeled hemoglobin (Table III).

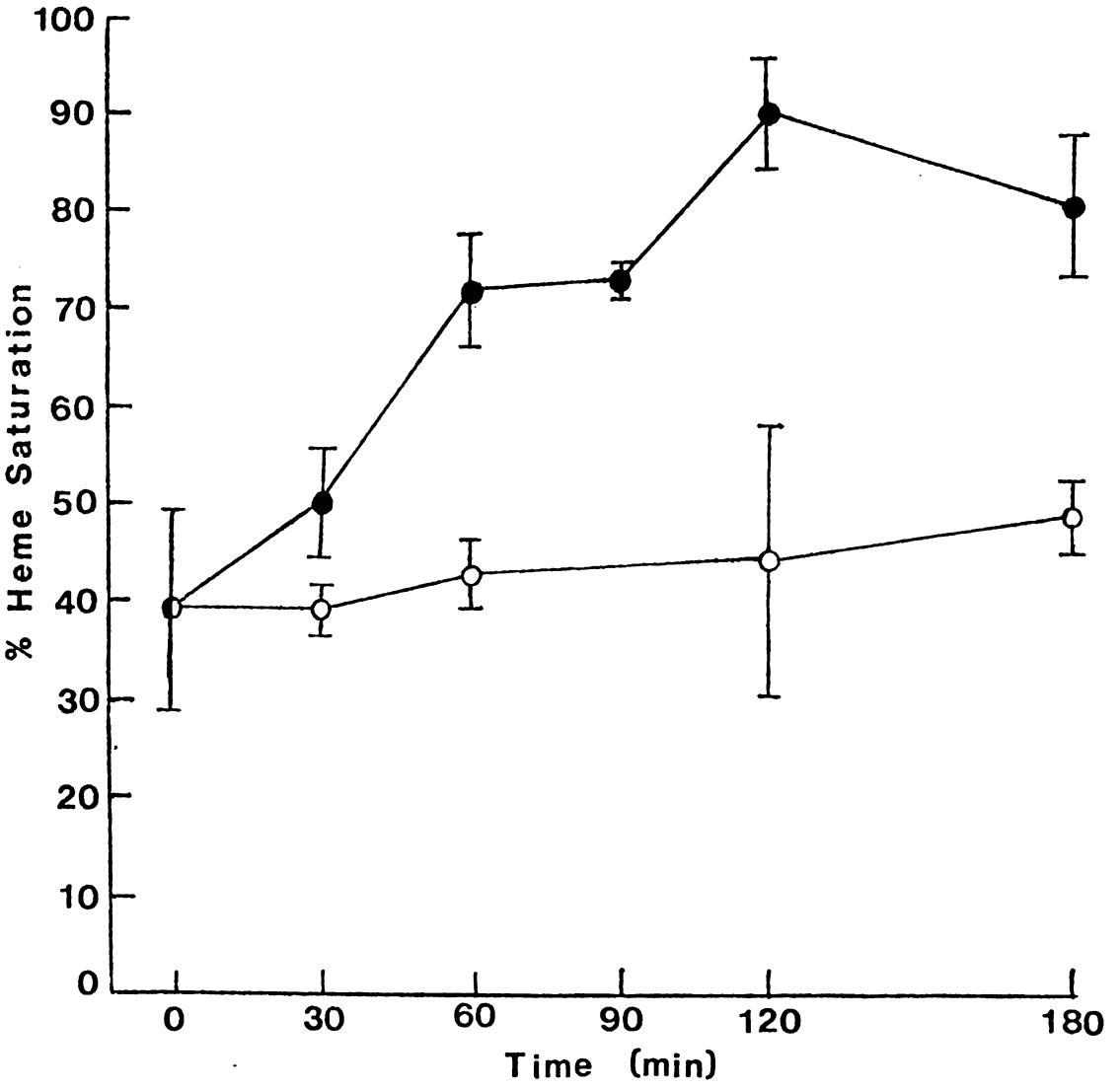
RESULTS AND DISCUSSION

Incorporation of Hemoglobin-Heme into Hepatic Tryptophan Pyrrolase:

Hemoglobin-heme mediated constitution of hepatic tryptophan pyrrolase, reflected by increased heme-saturation of the enzyme, was measured in rats during a three hour period following the intravenous injection of hemoglobin (see Methods). As early as 30 minutes following injection, heme saturation of tryptophan pyrrolase was significantly increased ($p<0.05$) above that of control (buffer treated) animals (Figure 12). Heme saturation increased steadily to a maximal level of 90%, dropping slightly, but not significantly ($p<0.2$), to a level of 80% at 180 min. The slight increase (10% over 180 min) in heme saturation of tryptophan pyrrolase observed in control animals was not significantly different ($p<0.2$) from that measured at 0 min.

Comparison of these results with those from similar experiments employing intravenously injected heme, suggest that heme from hemoglobin in incorporated into the hepatic free heme pool more slowly than is free heme. Thirty minutes after intravenous injection of approximately 140 nmol of heme to unfasted rats, hepatic tryptophan pyrrolase was 90% saturated with heme (189). This represented a 30% elevation of heme saturation above basal levels. In the present investigation, heme saturation of tryptophan pyrrolase, although significantly elevated, was increased only 11% above the basal level at 30 minutes following the injection of hemoglobin (350-400 nmol, equivalent to 1,400-1,600 nmol heme). This relatively high dose of hemoglobin-heme is, in fact, comparable to the 140 nmol heme dose (above) when one considers the mechanism of transport of hemoglobin to the liver. In this experiment, kidneys were not ligated, and therefore a portion of the hemoglobin injected might have been lost through renal filtration. However, it was shown previously that essentially all hemoglobin bound to

Figure 12. Reconstitution of Hepatic Tryptophan Pyrrolase Following Administration of Hemoglobin to Rats.



VALUES ARE MEAN ± S.D. FROM 3 INDIVIDUAL EXPERIMENTS.
(●) and (○) REPRESENT HEMOGLOBIN-TREATED AND CONTROL ANIMALS.
SEE METHODS FOR DETAILS.

serum haptoglobin was specifically incorporated into hepatocytes (21). Since both heme and globin moieties are incorporated into hepatocytes to the same extent, hemoglobin is thought to be taken up intact by the liver. Non-haptoglobin-bound-hemoglobin, which escapes renal filtration, is also apparently taken up by hepatocytes (21), but whether such free hemoglobin is taken up at a rate slower than that of haptoglobin-bound-hemoglobin is not known (Preliminary Experiments). Therefore, since the rat haptoglobin binding capacity for hemoglobin is 26.4 nmol/100 g (21), in the present study the minimum quantity of hemoglobin which might have been taken up by the hepatocytes thirty minutes following its injection, would be 40-55 nmol, which corresponds to 160-220 nmol of heme.

The fact that hemoglobin-heme constitutes tryptophan pyrrolase at a relatively slower rate than does intravenously administered heme can be partially attributed to the relatively slower rate of hepatic uptake of hemoglobin. When hemoglobin was administered in amounts not exceeding the haptoglobin binding capacity, it was found that 30% of the dose injected was incorporated into hepatocytes in the first 30 minutes (21). Intravenous heme, on the other hand, was shown to be 44% removed by hepatocytes in the same period of time ($t_{\frac{1}{2}}$ = 50 min) (72). Furthermore, once inside the liver, the transport of heme from hemoglobin to the free heme pool would be expected to proceed more slowly than that of heme, because hemoglobin-heme must first dissociate from the hemoglobin-haptoglobin complex.

Although hepatic uptake and intracellular transport of hemoglobin-heme may occur at a relatively slower rate, as compared with heme, these findings clearly demonstrate the functional constitution of tryptophan pyrrolase by hemoglobin-heme, and establish, therefore, that heme from hemoglobin gains access to the hepatic free heme pool. Since incorporation of hemoglobin-heme into tryptophan pyrrolase has not previously been demonstrated, this also constitutes the first report of such a finding.

Structural and Functional Reconstitution of Cytochrome P-450 Following Administration of Hemoglobin to Isolated Perfused Livers:

To determine whether hemoglobin-heme was similarly incorporated into a functional hepatic cytochrome P-450, a recirculating, isolated rat liver perfusion system was employed. Following perfusion, microsomes were prepared from homogenized livers and cytochrome P-450 content and N-demethylase activity were quantitated.

Addition of AIA destroyed approximately 35% of hepatic cytochrome P-450 content relative to that of non-AIA treated livers (Table IV). This is comparable to a previously reported level of destruction (42%) observed in experiments performed under identical conditions (71). Cytochrome P-450 levels in livers which received hemoglobin, however, were significantly elevated (20.7%) above those which received only AIA. When hemoglobin was added without AIA, cytochrome P-450 content was increased (13.9%) over control values. However, this increase was not significant as ascertained by the paired "t" test.

The destructive effect of AIA on cytochrome P-450 was selective for the phenobarbital-inducible form of the cytochrome. However, spectral quantitation of cytochrome P-450 does not selectively distinguish this specific isozyme, but is rather a measurement of the entire population of cytochrome P-450 isozymes. Since the AIA destruction of other isozymes is minimal (70,142), the full extent of destruction and reconstitution of the specific cytochrome P-450 observed in hepatic microsomes (Table IV) is undoubtedly shadowed by the remaining cytochrome P-450 species not affected by AIA. To determine whether the reconstituted cytochrome is functionally active, and also to examine the magnitude of destruction and reconstitution of the specific isozymes, the mixed-function oxidase activity of cytochrome P-450 was measured employing substrates known to be specifically metabo-

Table IV. Effects of AIA and Hemoglobin on Cytochrome P-450 Content and N-Demethylase Activity in Livers Isolated From Phenobarbital-Pretreated Rats.[a]

Preparation	Cytochrome P-450 (nmol/mg protein)	Increase (%)	Ethylmorphine (nmol/mg protein)	Increase (%)	PCNMA (nmol/mg protein)	Increase (%)
AIA Treated + Hb[b]	1.34 ± 0.24[c]	20.7 ($p<0.005$)[d]	194 + 48	40.1 ($p<0.05$)	89 ± 14	27.1 (NS)[e]
no Hb	1.11 ± 0.13 n=11[f]		138 ± 42 n=5		70 ± 25 n=5	
Untreated + Hb	1.97 ± 0.15	13.9 (NS)	387 = 23	9.3 (NS)	89 ± 16	1.1 (NS)
no Hb	1.73 ± 0.16 n=5		354 ± 70 n=3		88 ± 11 n=3	

(a) Freshly prepared rat hemoglobin (560-580 nmol) was infused into the perfusate over 15 min, followed by the addition of AIA (89 µmol). Livers were perfused for 2 h following addition of AIA (see Methods for details).
(b) HB=hemoglobin.
(c) Mean values ± S.D.
(d) Statistical significance ascertained by the paired t test.
(e) NS=not significant.
(f) n=number of observations.

lized by particular cytochrome P-450 isozymes. Microsomal N-demethylase activity was therefore determined using ethylmorphine and p-chloro-N-methylaniline (PCNMA), prototypic substrates of the phenobarbital- and 3-methylcholanthrene-inducible forms of cytochrome P-450, respectively (47,48).

AIA-mediated destruction of cytochrome P-450 significantly ($p<0.005$) reduced N-demethylation of ethylmorphine by 61%; N-demethylation of PCNMA was reduced (20%), but not significantly ($p>0.2$). In contrast to the latter finding, a significant reduction in PCNMA N-demethylation was previously reported for experiments performed *in vivo*, which employed a 3- to 4-fold higher dose of AIA than that used in this investigation (70). Addition of hemoglobin to AIA-treated perfused rat livers significantly reversed (40%) the effect of AIA on ethylmorphine N-demethylase activity, whereas, in the absence of AIA, the effect of hemoglobin on N-demethylation of ethylmorphine and PCNMA was not significant.

This finding confirms the selective destruction of the phenobarbital-inducible form of cytochrome P-450 by AIA, and indicates that hemoglobin-heme can structurally and functionally reconstitute this isozyme following such destruction. However, these findings could also be interpreted to indicate that hemoglobin prevents AIA-mediated destruction of cytochrome P-450, either by limiting hepatic uptake of AIA from the perfusion medium, or by intracellular entrapment of AIA prior to destruction of cytochrome P-450. If AIA was preferentially bound to circulating hemoglobin, the hepatic uptake of AIA might be blocked, and thus the destruction of cytochrome P-450 would be reduced because of decreased availability of AIA.

This possibility was excluded by examining hepatic elimination of ^{14}C-AIA in isolated rat liver perfusions in the presence and absence of hemoglobin. The fractional disappearance rates (k) of ^{14}C-AIA from the perfusate in hemoglobin-treated and control livers were $k = 0.0097 \pm 0.0024$ min^{-1} and $k = 0.0099 \pm 0.0016$ min^{-1}, respectively (mean values $\pm$ standard

deviation, n = 5). These results were not significantly different ($p>0.5$) as ascertained by the t test for two means. If hemoglobin prevented cytochrome P-450 destruction by reducing the hepatic uptake of AIA, the fractional disappearance rate of ^{14}C-AIA from the perfusate in hemoglobin-treated livers would have been less than that in non-hemoglobin-treated livers; such was not the case. Furthermore, the ^{14}C-AIA fractional disappearance rates remained comparable even following a 3.5-fold increase in hemoglobin concentration in the perfusate (single determination). Thus, it can be concluded that hemoglobin does not protect cytochrome P-450 from AIA-mediated destruction by limiting the hepatic uptake of AIA. Moreover, since AIA did not bind to hemoglobin extrahepatically, its intracellular entrapment by hemoglobin appeared unlikely and, therefore, was not experimentally addressed.

It has been previously shown that AIA-mediated destruction of cytochrome P-450 results in delayed plasma disappearance of ^{14}C-AIA in isolated perfused rat livers (73). Administration of heme 50 min after addition of AIA accelerated the plasma disappearance of AIA, indicating functional reconstitution of hepatic cytochrome P-450 by heme. In the present investigation, infusion of hemoglobin under identical conditions did not alter the plasma disappearance rate of ^{14}C-AIA. Failure of hemoglobin to significantly increase the disappearance rate of AIA during the 2 hour perfusion period is possibly due to the relatively slower rate of hemoglobin uptake by hepatocytes and subsequent transfer of hemoglobin-heme to the intracellular free heme pool, compared to that of free heme (Preliminary Experiments). Thus, under the experimental conditions described, the measurement of change in the plasma disappearance rate of ^{14}C-AIA did not provide a sufficiently sensitive assay to demonstrate the incorporation of hemoblogin-heme into cytochrome P-450.

To conclusively demonstrate the prosthetic incorporation of hemoglobin heme into hepatic cytochrome P-450, the following experiments were performed.

Incorporation of Radiolabeled Hemoglobin-Heme into AIA-Porphyrin Adducts In Vivo:

Phenobarbital-induced rats, treated with AIA, were injected with either isotopic hemoglobin or native hemoglobin containing radiolabeled heme (See Methods). AIA-mediated destruction of cytochrome P-450 and heme in liver homogenates, microsomes, and CO-binding particles, was comparable in rats treated with ^{3}H-hemoglobin to that in rats treated with native hemoglobin + ^{3}H-heme (Table V). However, the heme-specific radioactivity detected in various cell fractions from rats injected with ^{3}H-hemoglobin was considerably higher than that in cell fractions from rats which received native hemoglobin + ^{3}H-heme. Carbon monoxide binding particles, which essentially contained cytochrome P-450 as the only hemoprotein, had four to six times higher radioactivity in animals which received isotopic hemoglobin than in corresponding controls. These results were consistent with the findings of preliminary experiments (discussed above) which demonstrated that the amount of radiolabeled heme taken up by hepatocytes and subsequently incorporated into the free heme pool, was substantially higher in rats treated with ^{3}H-hemoglobin than in those receiving native hemoglobin + ^{3}H-heme (Figure 11).

Isolation and purification of AIA-porphyrin adducts: The heme-specific radioactivities of the liver preparations were decreased in AIA-treated rats, as compared to untreated animals, reflecting the formation of AIA-porphyrin adducts from cytochrome P-450-heme (Table V). Following isolation (see Methods), the specific activities of zinc-complexed adducts

Table V. Hepatic Cytochromes P-450, and b_5, heme content and heme-specific radioactivity following administration of isotopic hemoglobin (^{3}H-Hb) or native hemoglobin + isotopic heme (Hb + ^{3}H-Heme) to AIA-treated rats.

Liver Fraction	Cytochrome P-450 (nmol/g liver)		Cytochrome b_5 (nmol/g liver)		Heme (nmol/g liver)		Heme Specific Radioactivity (dpm/g liver)	
	Untreated	AIA	Untreated	AIA	Untreated	AIA	Untreated	AIA
Homogenate								
^{3}H-Hb	50.4	15.2	---	---	151.0	91.0	361,000	171,300
Hb + ^{3}H-Heme	39.4	13.0	---	---	143.8	85.3	42,700	18,300
Microsomes								
^{3}H-Hb	26.2	6.3	8.5	8.1	67.6	28.1	127,700	43,400
Hb + ^{3}H-Heme	27.2	6.5	8.2	7.0	54.8	26.1	16,200	10,600
CO-Particles								
^{3}H-Hb	23.3	5.8	1.6	1.1	45.6	15.1	105,400	26,400
Hb + ^{3}H-Heme	19.4	6.2	2.0	1.6	41.2	16.2	17,000	7,000

Values presented are from a typical experiment and represent the mean of two animals.

remained fairly constant with increasing purification by HPLC (Table VI). In the final purification steps, the adducts isolated from rats which had received ^{3}H-hemoglobin exhibited specific activities three to four times higher than those of adducts isolated from rats which were injected with native hemoglobin + ^{3}H-heme.

For the purpose of this discussion, it was assumed that all adducts formed following treatment with AIA were retained within the liver, although White, 1982, observed biliary excretion of green pigments formed following administration of the suicidal substrate, norethindrone. However, the excretion of pigments two hours after administration of norethindrone was minimal, being less than 2% of the amount excreted after 24 hours. DeMatteis, 1973, found that in rats sacrificed 1 hour after receiving AIA, the majority of heme degradation products (AIA-porphyrin adducts) were retained by the liver. In experiments reported here, rats were killed 2 hours following administration of AIA, and therefore it seems unlikely that substantial amounts of adducts would have been lost by biliary excretion during this time. Furthermore, since rats were matched for weight, and the doses of AIA administered were equivalent, the proportion of AIA-adducts excreted into bile, if any, should be constant from one rat to the next. Thus, for purposes of comparing the relative formation of radiolabeled adducts in rats treated with isotopic hemoglobin to that in rats treated with native hemoglobin + ^{3}H-heme, the biliary excretion of adducts was considered inconsequential, and was not determined.

The destruction of cytochrome P-450-heme and consequent formation of AIA-porphyrin adducts in rats given ^{3}H-hemoglobin or native hemoglobin + ^{3}H-heme is documented in Table VI. Consistent with the higher specific activities of AIA-porphyrin adducts isolated from rats treated with ^{3}H-hemoglobin (Table VI), the loss of isotopic heme and the formation of ^{3}H-

Table VI. Sequential Purification of AIA-Porphyrin Adducts (Zinc Complex) Isolated from Livers of AIA-Treated Rats Given Isotopic Hemoglobin (^{3}H-Hb) or Native Hemoglobin + Isotopic Heme (Hb + ^{3}H-Heme).

	TLC		HPLC-1		HPLC-2		HPLC-3	
	Amount (nmol)	Specific Activity (dpm/nmol)	Amount (nmol)	Specific Activity (dpm/nmol)	Amount (nmol)	Specific Activity (dpm/nmol)	Amount (nmol)	Specific Activity (dpm/nmol)
^{3}H-Hb								
Rat 1	36	1622	20	1023	0.4	1781	0.5	1415
Rat 2	133	1594	44	1607	12.2	1615	---	---
Hb + ^{3}H-Heme								
Rat 3	101	477	41	632	3.8	528	0.5	560
Rat 4	150	292	12	461	1.7	542	1.2	465

Adducts were quantitated spectrophotometrically using an extinction coefficient of 125mM^{-1}cm^{-1} at 432 nm.

adducts were substantitally higher in rats treated with isotopic hemoglobin as compared to corresponding controls. These data indicate that the ^{3}H-hemoglobin-heme incorporated into cytochrome P-450 originated largely from prosthetically bound heme of hemoglobin, rather than solely from the heme of contaminating methemoglobin.

The percent incorporation of isotopic heme into AIA-adducts, however, was lower in rats receiving ^{3}H-hemoglobin than in rats given native hemoglobin + ^{3}H-heme (Table VII). The decreased conversion of ^{3}H-hemoglobin-heme to AIA-porphyrin adducts is most likely due to the relatively slower rate of hepatic uptake and transfer of hemoglobin-heme to the intracellular free-heme pool (Table II). Conversely, animals treated with native hemoglobin + ^{3}H-heme appeared to rapidly incorporate isotopic heme into the free heme pool. This heme was therefore available for incorporation into cytochrome P-450 over a longer period of time, resulting in a higher percentage of isotopic heme being converted to AIA-adducts during the two hour period of treatment.

The relative formation of radiolabeled adducts from prosthetically-bound hemoglobin-heme and heme of methemoglobin: Having determined (1), the initial amount of methemoglobin-heme present in ^{3}H-hemoglobin (5.6%), (2), the amount of methemoglobin possibly formed intravascularly during the two hour period following injection of ^{3}H-hemoglobin (2%, Figure 11), and (3), the rate at which methemoglobin-heme is taken up by the liver and transported to the hepatic free-heme pool (Table II), it was possible to calculate the percentage of radiolabeled AIA-porphyrin adducts that was derived from radiolabeled methemoglobin-heme, and that from prosthetically bound heme, following the injection of ^{3}H-hemoglobin. The total amount of radiolabeled AIA-porphyrin adducts formed following the administration of ^{3}H-hemoglobin was 1.20 nmol/g liver (Table VIII, mean value from Table

Table VII. Conversion of Hepatic Cytochrome P-450 Heme to AIA-Porphyrin Adducts in Rats Treated with Isotopic Hemoglobin (^{3}H-Hb) or Native Hemoglobin + Isotopic Heme (Hb + ^{3}H-Heme).

Treatment	Loss of Cytochrome P-450 (nmol/g liver)	Loss of Heme (nmol/g liver)	Loss of Heme-Specific Radioactivity (dpm/g liver)	Loss of ^{3}H-Heme[a] (nmol/g liver)	AIA Adducts[b] Recovered (nmol/g liver)	Total Radio-activity of Adducts (dpm/g liver)	^{3}H-AIA Adducts[c] (nmol/g liver)	Recovery of Lost ^{3}H-Heme as AIA-Adducts (%)
^{3}H-Hb								
Rat 1	44.6	67.0	169,700	3.72	36.4	58,640	1.28	34.4
Rat 2	57.0	62.6	166,800	5.43	24.9	32,146	1.04	19.1
Rat 3	47.4	53.4	130,500	4.25	27.4	39,292	1.28	30.1
Hb + ^{3}H-Heme								
Rat 4	25.2	61.4	24,880	0.50	32.1	17,462	0.35	70.0
Rat 5	27.7	53.9	24,050	0.48	46.2	23,284	0.47	97.9

(a) Determined by dividing the heme-specific radioactivity by the specific activity of ^{3}H-hemoglobin-heme (Rat 1 = 45,630 dpm/nmol and Rats 2 and 3 = 30,700 dpm/nmol) or ^{3}H-heme isolated from ^{3}H-hemoglobin (Rats 4 and 5 = 49,810 dpm/nmol).

(b) Determined spectrophotometrically using a millimolar extinction coefficient of 125 cm^{-1} at 432 nm; values presented were corrected for 35% recovery of AIA-adducts after TLC, based on control experiments using known amounts of purified zinc-complex ^{3}H-adducts.

(c) Determined by dividing radioactivity of adducts by the specific activity of ^{3}H-hemoglobin-heme or ^{3}H-heme isolated from ^{3}H-hemoglobin, as in (a).

VII). Of this, the amount of radiolabeled adducts contributed by non-prosthetically bound heme could be calculated to be, at most, 0.5 nmol/ g liver. Thus, on a percentage basis, a maximum of 45% of the ^{3}H-AIA-porphyrin adducts formed following injection of isotopic hemoglobin may have been derived from methemoglobin-heme present in and/or formed from isotopic hemoglobin. The major fraction (55%) of ^{3}H-adducts apparently were formed from prosthetically-bound heme of isotopic hemoglobin. These results conclusively demonstrate that hemoglobin-heme which was incorporated into cytochrome P-450 was not limited to heme present as methemoglobin, but was also derived from prosthetically-bound hemoglobin-heme.

Hemoglobin-heme incorporated into hepatic cytochrome P-450 is therefore derived from two sources. Because heme from hemoglobin is taken up by hepatocytes as both free and prosthetically bound heme, the delivery of hemoglobin-heme to the hepatic free heme pool may be extended over a longer period of time than that observed for parenterally administered heme. Consequently, therapeutic use of hemoglobin, in place of heme, in the treatment of hepatic porphyrias may provide a relatively sustained correction of the underlying heme deficiency, compared to that achieved with parenteral heme administration.

Table VIII. Relative Formation of Radiolabeled AIA-Porphyrin Adducts in Rats from Heme Prosthetically-Bound to Hemoglobin and That Present as Contaminating Methemoglobin.

Source of ^{3}H-Heme	Amount of ^{3}H-Heme Injected (nmol/g liver)	Hepatic Uptake of ^{3}H-Heme (nmol/g liver)	^{3}H-AIA-Adducts Formed (nmol/g liver)	^{3}H-AIA-Adducts as % of Hepatic ^{3}H-Heme Uptake (%)	% Contribution of Radioactive Heme to ^{3}H-AIA Adducts (nmol/g liver)
^{3}H-Hb	38.7	19.2	1.20[d]	6.3	100 (Prosthetic + MetHb)
Hb + ^{3}H-Heme	2.4	1.53	0.41[d]	26.8	34.2 (Initial MetHb)
^{3}H-MetHb (calculated)	2.9[a]	2.02	0.54[e]	---	45.0

(a) Amount of ^{3}H-hemoglobin injected multiplied by the percent methemoglobin initially present plus that formed intravascularly after 2 h; total = 7.54%.
(b) Amount of ^{3}H-heme injected multiplied by the total (liver + bile) percent uptake (Table III).
(c) % total ^{3}H-adducts formed, divided by the total hepatic uptake of ^{3}H-heme.
(d) Mean values from Table VI.
(e) Value calculated from hepatic uptake of ^{3}H-heme (2.02 nmol/g liver) and its percent conversion (26.8) to radiolabeled AIA-adducts.

SUMMARY AND CONCLUSIONS

The findings of this investigation clearly establish that hemoglobin-heme gains access to the hepatic free-heme pool and is subsequently incorporated into hepatic hemoproteins. In summary:

(1) Hemoglobin-heme is incorporated into hepatic tryptophan pyrrolase as early as 30 minutes following its intravenous injection. Heme-saturation of tryptophan pyrrolase increased steadily to a level of 90% two hours following hemoglobin injection. The rate at which tryptophan pyrrolase was constituted with hemoglobin-heme was slower than that reported for exogenous heme administered intravenously.

(2) Cytochrome P-450, stripped of its prosthetic heme by treatment with AIA, was structually and functionally reconstituted in intact rats or in an isolated rat liver perfusion system by the administration of hemoglobin.

(3) Administration of ^{3}H-hemoglobin to AIA-treated rats resulted in the formation of radiolabeled AIA-porphyrin adducts, confirming the prosthetic incorporation of hemoglobin-heme into hepatic cytochrome P-450.

(4) Hemoglobin-heme incorporated into AIA-porphyrin adducts was derived not only from methemoglobin-heme, but also from heme prosthetically-bound to hemoglobin.

In conclusion, the results of this study indicate that, although hepatic uptake of parenteral hemoglobin is slower than that of heme, hemoglobin serves as an effective heme donor to the hepatic free heme pool. Since intravenous administration of stroma-free hemoglobin does not produce the vascular and renal toxicity that is reported to occur with heme administration, parenteral hemoglobin may be a safe and effective therapeutic alternative to heme in the treatment of heme deficient states, such as acute hepatic porphyrin.

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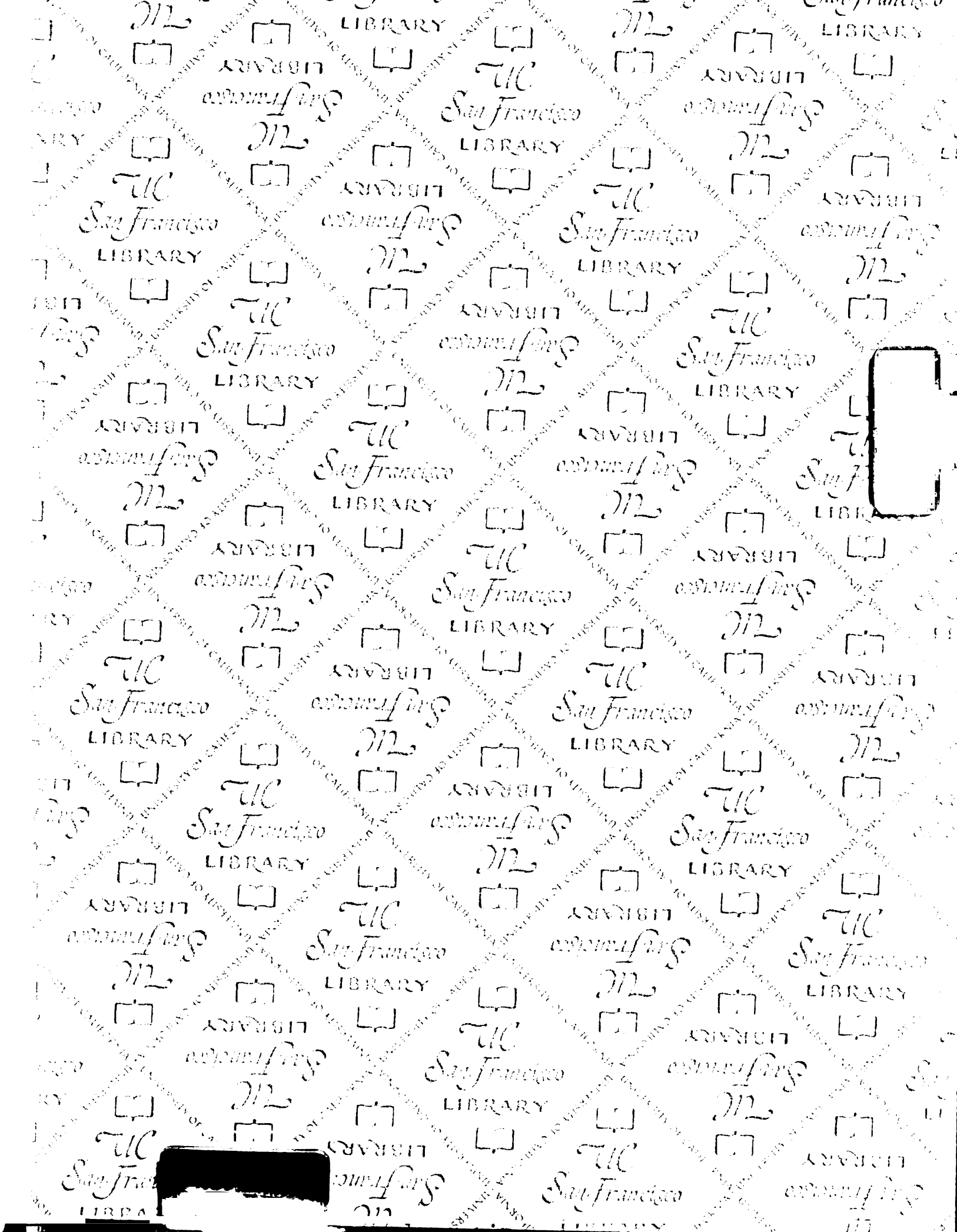
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