Enzymatic Degradation of Heme. Oxygenative Cleavage Requiring Cytochrome P-450†

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ABSTRACT: A microsomal enzyme system has been described that converts heme (ferriprotoporphyrin IX) to the linear tetrapyrrole biliverdin IXα. On the basis of its absolute requirement for NADPH and molecular oxygen, its sensitivity to inhibition by CO, and its appropriate stoichiometry, the enzyme system tentatively was classified as microsomal heme oxygenase. It now has been demonstrated that bilirubin formed enzymatically from heme in the presence of molecular $^{18}O_2$ contains 2 atoms of ^{18}O and an additional ^{18}O atom appears in the CO that originates from the α-methene bridge carbon of the heme. No ^{18}O is incorporated into the bile pigment when the enzymatic reaction is carried out in a medium containing $H_2{}^{18}O$ instead of the molecular $^{18}O_2$. In an atmosphere containing CO and O_2 in a ratio of 1:2, the enzyme reaction is inhibited by 47%. Monochromatic light in the 450- to 470-nm range effectively reverses this inhibition. The photochemical action spectrum for this reversal closely resembles the absorption spectrum of the CO derivative of reduced cytochrome P-450. These findings indicate that the enzymatic conversion of heme to bilirubin is a mixed-function oxidation and that the microsomal enzyme system includes a hemoprotein with the characteristics of cytochrome P-450 as a terminal oxidase. The reaction does not appear to involve formation of intermediates such as verdoheme, which would require hydrolysis for conversion to biliverdin.

Heme, whether free or as the prosthetic group of hemoproteins, is degraded in the intact organism almost completely to bile pigments (Ostrow *et al.*, 1962). This reaction involves oxidative cleavage of the ferriprotoporphyrin ring at the α-methene bridge (Fischer and Orth, 1937), resulting in formation of the linear tetrapyrrole, biliverdin IXα.

Recent studies have defined a microsomal system that *in vitro* converts hemin and other ferriporphyrins, including methemalbumin, and the heme moiety of methemoglobin or of the isolated α and β chains of hemoglobin to biliverdin IXα (Tenhunen *et al.*, 1969a). This reaction is coupled to soluble NADPH-dependent biliverdin reductase (Tenhunen *et al.*, 1970a) which selectively (Colleran and O'Carra, 1970) converts the IXα isomer of biliverdin to bilirubin. There is now substantial evidence that this enzyme complex constitutes the principal catabolic pathway for the physiologic formation of bile pigment (Tenhunen *et al.*, 1969b).

The microsomal oxidative step which catalyzes the cleavage of heme is of particular interest because of the following. (1) It appears to determine the rate at which heme is converted to bile pigments. (2) This enzyme system is appropriately active in tissues normally involved in heme catabolism, such as spleen, liver, and bone marrow. (3) Enzymatic activity may be augmented, seemingly by induction (Tenhunen *et al.*, 1970b), in tissues such as the kidney (Pimstone *et al.*, 1971a), macrophages (Pimstone *et al.*, 1971b), and arachnoid membrane (Roost *et al.*, 1971) by exposure of these tissues to extra-erythrocytic hemoproteins.

This oxidative system has tentatively been classified as "microsomal heme oxygenase" (Tenhunen *et al.*, 1968). This classification was based on the absolute requirement of the enzymatic reaction for NADPH and molecular oxygen, its inhibition by carbon monoxide and a stoichiometry that was consistent with mixed function oxidation (Tenhunen *et al.*, 1969a).

The present report describes additional studies employing mass spectrometry to characterize the oxidation of heme by microsomes and photochemical dissociation to elucidate the role of cytochrome P-450 in this reaction.

Experimental Section

Materials. NADPH, NADP, glucose 6-phosphate, glucose-6-phosphate dehydrogenase, and recrystallized hemin were purchased from the Sigma Chemical Co. The purity of the hemin was verified by spectrophotometric analysis of the pyridine hemochromogen prepared from this compound; the ratio of the maximal absorption in the α band to the minimal absorption between the α and β bands was 3.46 (Falk, 1964; Paul *et al.*, 1953). Methemalbumin was prepared from hemin as described previously (Tenhunen *et al.*, 1968). $^{18}O_2$ gas (93.4% enrichment) and $H_2{}^{18}O$ (10.68% enrichment) were obtained from Miles-yeda, Ltd. The gas mixtures of CO and O_2 in nitrogen (CP) were purchased from the Matheson Co., or prepared with a gas proportioner.

Methods. Male Sprague-Dawley or Holtzman rats, weighing 250–320 g were used as experimental animals. For the studies of microsomal heme oxygenase in liver, enzyme activity was increased by intraperitoneal administration of methemalbumin (4 μmoles/100 g body weight) twice daily for 2 days (Tenhunen *et al.*, 1970b). In all experiments the rats were fasted overnight. After decapitation, the liver was immediately perfused through the portal vein with ice-cold

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isotonic saline. Liver and spleen were homogenized in two to three volumes (w/v) of 0.25 M sucrose and fractionated by differential centrifugation (Schneider, 1948).

Photochemical action spectra were determined using the following final incubation mixture in a volume of 3.0 ml: liver or spleen microsomes (8–12 mg of protein); purified biliverdin reductase (Tenhunen *et al.*, 1970a) (step 5, 9 mg of protein); hemin (in the form of methemalbumin), 17 μM; NADP, 0.5 mM; glucose-6-phosphate dehydrogenase, 2 Kornberg units, and 90 mM potassium phosphate buffer (pH 7.4). Prior to the addition of glucose 6-phosphate and NADP, the other components of the reaction mixture were equilibrated with the specified gas mixtures in cuvets for 5 min at 30° in the dark. The reaction was then started by addition of the glucose 6-phosphate and NADP, and the incubation was continued at 30° for 15 min with continuous gassing with or without illumination as described previously (Rosenthal and Cooper, 1967). Formation of bilirubin during the incubation was determined in a Cary Model 14 split-beam recording spectrophotometer from the increase in optical density at 468 μm, the wavelength at which the bilirubin formed absorbs light maximally (Tenhunen *et al.*, 1968). In the reference cuvet glucose 6-phosphate and NADP were replaced by 0.1 M potassium phosphate buffer. The validity of this assay has been verified previously by a variety of criteria (Tenhunen *et al.*, 1968).

The photochemical action spectrum was obtained as described by Rosenthal and Cooper (1967). The reaction and measurements were carried out directly in optical cuvets sealed with serum stoppers. The appropriate gas mixtures were continuously circulated through the cuvets during the illumination and incubation period to ensure adequate equilibration and mixing. This was accomplished through needles inserted in the serum stoppers (Figure 1). After incubation the needles were carefully removed from the serum stopper, thus maintaining the same gas mixture during the spectrophotometric analysis.

The $^{18}O_2$ incorporation experiments were performed in 500-ml boiling bottles equipped with high-vacuum stopcocks. The incubation mixtures (30.0 ml) consisted of 18,000*g* supernatant of spleen (220–250 mg of protein), hemin (17 μM), NADP (0.5 mM), glucose 6-phosphate (2.6 mM), $MgCl_2$ (1.9 mM), and potassium phosphate buffer (pH 7.4, 90 mM) (Tenhunen *et al.*, 1968). Prior to the incubation, the $^{18}O_2$ was drawn into the boiling bottle through a three-way tap and diluted with air to about 1 atm (under the conditions of the incubation) so that the gas phase consisted of 33% oxygen containing 66% $^{18}O_2$. In control studies $^{16}O_2$ replaced $^{18}O_2$. For studies of the incorporation of oxygen from $H_2{}^{18}O$ into bilirubin an incubation mixture was used in which reagents were prepared in ^{18}O-enriched water.

The bilirubin formed during the incubation was extracted with chloroform until the chloroform layer was colorless. The combined chloroform extracts were washed three times with equal amounts of distilled water, filtered, and evaporated to a small volume. Bilirubin was crystallized and recrystallized as described previously (Ostrow *et al.*, 1961).

Mass spectra were recorded on an AEI MS-902 high-resolution mass spectrometer at a temperature of 280°, an ionizing voltage of 70 eV and an ionizing current of 485 μA. Crystalline bilirubin as well as the gasses in the incubation vessels following reaction were analyzed *via* the direct insertion probe, or cold inlet system, respectively. Exact masses were determined at a resolution of 20,000 by the electrical peak matching technique using perfluorotributylamine as a standard; these measurements are reproducible to within 2 ppm.

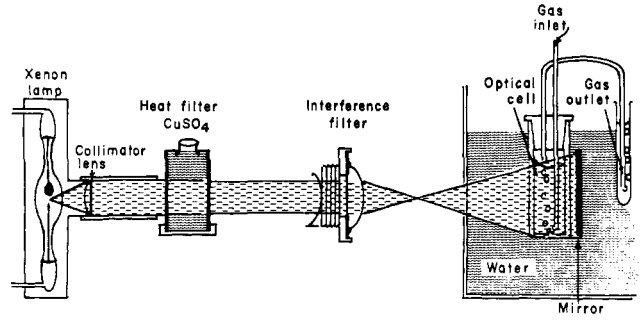

FIGURE 1: Arrangement for illumination of optical cells with monochromatic light.

Results

Effect of CO on Microsomal Heme Oxidation in Rat Liver and Spleen Microsomes. Carbon monoxide markedly inhibited the degradation of hemin to bilirubin by rat liver and spleen microsomes. A ratio of CO to O_2 of 0.5 inhibited the conversion by 47% in spleen microsomes; ratios of 1.0 and 2.25 inhibited the formation of bilirubin 74 and 87%, respectively. The partition constant K[1] (Warburg, 1949), *i.e.*, the ratio of CO to O_2 needed for 50% inhibition of the oxidation of hemin, was 0.33–0.34 in spleen microsomes and 0.54–0.59 in liver microsomes.

Effect of Monochromatic Light on CO Inhibition of Bilirubin Formation. Irradiation with monochromatic light decreased the inhibitory effect of CO on the degradation of hemin to bilirubin. With liver microsomes the effect of light on bilirubin formation from hemin was most marked at 450–460 nm (Figure 2) and with spleen microsomes, at 465–470 nm (Figure 3). Illumination (one-fourth maximum intensity) of the cuvets with light at 450–460 nm resulted in a 74% reversal of the CO inhibition with liver microsomes and illumination with light at 465–470 nm produced a 60% reversal of the CO inhibition with spleen microsomes. With full intensity light results were variable presumably because of the photodestruction of bilirubin by light in the 450- to 470-nm range.

Mass Spectrometry of Enzymatically Formed Bilirubin. The mass spectral fragmentation pattern of bilirubin has been reported (Jackson *et al.*, 1967) and is characterized by a strong parent ion at *m/e* 584, $C_{33}H_{36}N_4O_6$, and a base peak ion at *m/e* 299, $C_{17}H_{19}N_2O_3$, arising from cleavage at the central methene bridge of bilirubin. Bilirubin formed enzymatically by spleen microsomes from hemin in the presence of $^{18}O_2$ gave a prominent ion at *m/e* 588 (p + 4), $C_{33}H_{36}N_4{}^{16}O_4{}^{18}O_2$, and a base peak ion at 301 (p + 2), $C_{17}H_{19}N_2{}^{16}O_2{}^{18}O$ (Figure 4). In addition, on high resolution mass spectral analysis of the gas phase, it was found that $C^{18}O$ was formed during incubation. This pattern suggests that in the enzymatic conversion of heme to bilirubin, 2 atoms of ^{18}O are incorporated into the bilirubin and one atom into the CO. In the presence of $H_2{}^{18}O$, no ^{18}O enrichment was detectable in bilirubin or CO.

[1] $K = (n/1 - n)([CO]/[O_2])$, where n = (rate with CO)/(rate without CO).

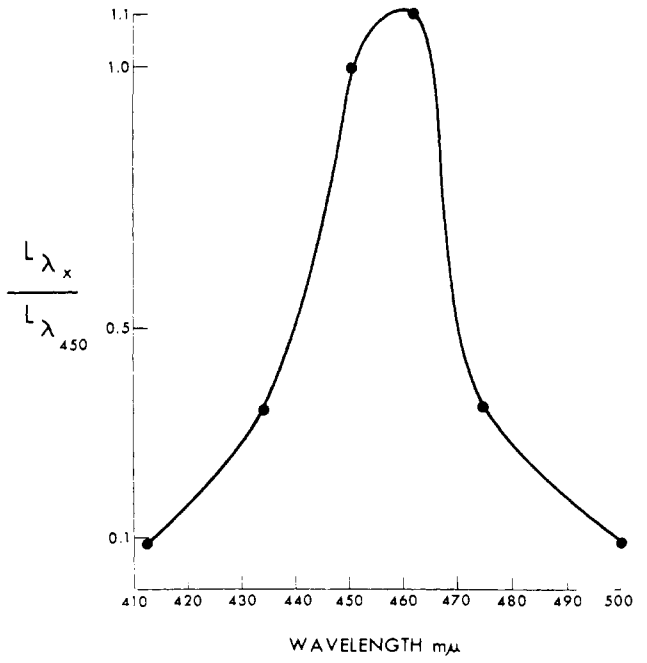

FIGURE 2: Photochemical action spectrum of hepatic microsomal heme oxygenase. The general procedure and the composition of the reaction system have been described under Experimental Section. Each flask contained 8 mg of liver microsomal protein. Incubation time was 15 min at 30°. The formation of bilirubin in 4% O_2, 96% N_2, in the absence of CO, was 0.077 nmole/min per mg of protein. The flasks for studying the CO inhibition were gassed with a mixture of 3.96% O_2 and 4.18% CO in nitrogen ($CO:O_2$ = 1.05). The results are plotted in terms of relative light sensitivity, $L_\lambda/L_{\lambda 450}$, versus wavelength of light applied. L was calculated from the equation: $L = (1/i)[(K_h - K_d)/K_d] = (1/i) \times (\Delta K/K_d)$, where i is the light intensity expressed as mole quanta cm^{-2} min^{-1} at wavelength λ, and K_h and K_d, the partition constants with and without illumination. The value of K_d was 0.536 (Rosenthal and Cooper, 1967; Warburg, 1949).

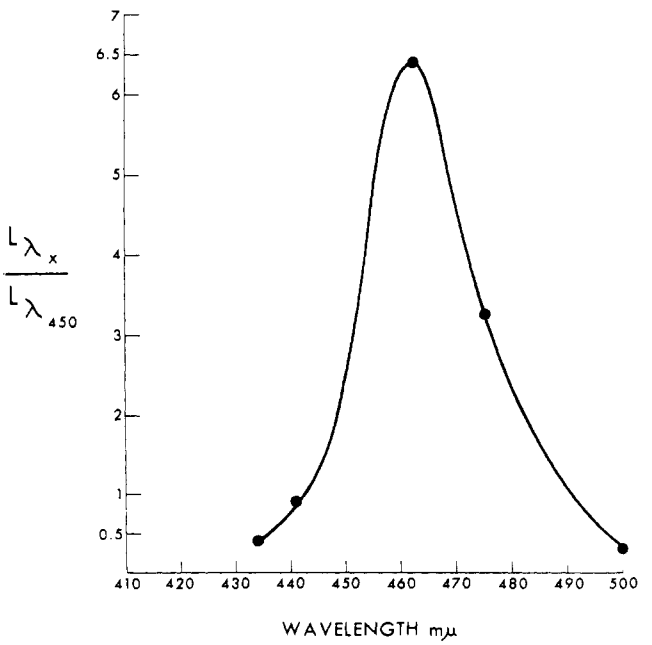

FIGURE 3: Photochemical action spectrum of splenic microsomal heme oxygenase. Experimental conditions are the same as those given in the legend to Figure 2. Each flask contained 12 mg of splenic microsomal protein. The formation of bilirubin in 4% O_2 and 96% N_2, in the absence of CO, was 0.078 nmole/min per mg of protein.

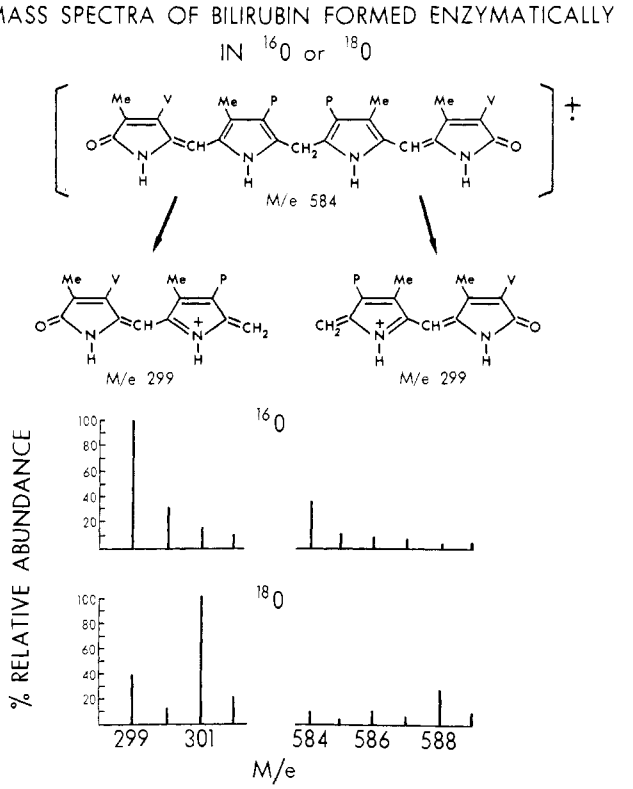

FIGURE 4: Mass spectra of bilirubin formed enzymatically in $^{16}O_2$ or $^{18}O_2$.

Discussion

It has been proposed that the microsomal enzyme system which converts heme to bilirubin (Tenhunen *et al.*, 1969a) is a mixed-function oxidase that resembles the type of oxygenase involved in the metabolism of many lipids, steroids, and foreign compounds (Mason, 1957; Omura *et al.*, 1965). The basic pattern of these reactions consists of the introduction of molecular oxygen into the substrate molecule with the concomitant oxidation of reduced triphosphopyridine nucleotide. The enzymatic conversion of heme to bilirubin by the microsomal enzyme system appears to fulfill these requirements (Tenhunen *et al.*, 1969a). Details of the mechanism responsible for the fissure of the porphyrin ring are poorly understood. The carbonyl function of the α-monohydroxyferriprotoporphyrin is lost as CO (Sjoestrand, 1952; Landaw *et al.*, 1970), the α-methene bridge carbon atom is replaced by two oxygen atoms and the iron is removed (Figure 5). However, the temporal sequence of these events is not clear nor is it known whether this occurs in a single step or whether it involves formation of one or more intermediate compounds.

We have found that when heme is incubated in the enzyme system in the presence of molecular $^{18}O_2$, two atoms of ^{18}O are incorporated into the product while an additional ^{18}O atom appears in the CO that originates from the α-methene bridge carbon of the heme (Figure 4). The oxygenase nature of the enzyme system is further demonstrated by the lack of ^{18}O incorporation into the bile pigment when the enzymatic reaction is carried out in a medium containing $H_2{}^{18}O$ instead of the molecular $^{18}O_2$.

A reaction scheme for the enzymatic conversion of heme to bilirubin which is consistent with these findings is proposed in Figure 5. The enzymatic system resembles the non-

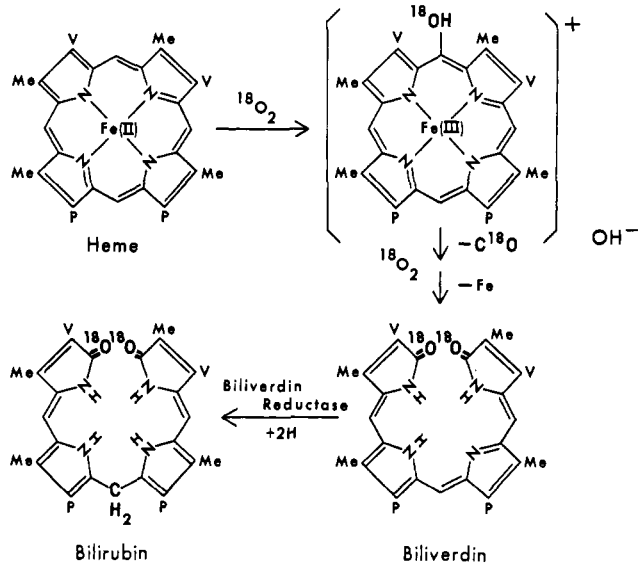

FIGURE 5: Proposed reaction scheme for the enzymatic conversion of heme to bilirubin.

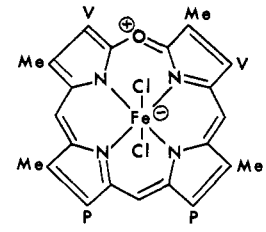

FIGURE 6: Structure of verdoheme.

enzymatic coupled oxidation of heme (Lemberg, 1956) in that the initial step probably involves formation of α-monohydroxyheme as an intermediate (Kondo *et al.*, 1971). It is unknown whether the enzyme can attack ferrous as well as ferric compounds, since only ferriporphyrins could be tested as substrates *in vitro;* the ferrous hemoproteins oxyhemoglobin, carboxyhemoglobin and myoglobin, in which the heme group is firmly bound to the protein moiety, are nearly inactive as substrates *in vitro* (Tenhunen *et al.*, 1969a). This does not exclude the possibility, however, that in the initial step of the enzymatic reaction the iron of the substrate may be reduced to the ferrous state as in the nonenzymatic coupled oxidation of heme with ascorbic acid and oxygen (Lemberg, 1956). It is evident and already has been pointed out by Kondo *et al.* (1971), that formation of intermediates such as verdoheme (Figure 6), which would require hydrolysis for conversion to biliverdin (Lemberg, 1956; Anan and Mason, 1961), can be excluded as incorporation of ^{18}O from $H_2\,^{18}O$ was not observed during the enzymatic formation of bilirubin. The formation of such intermediates, which occur in the nonenzymatic degradation of heme by coupled oxidation *in vitro* (Lemberg, 1956), has never been demonstrated conclusively in the intact organism nor is there evidence for their presence in the enzymatic reaction catalyzed by the microsomal system *in vitro* (Tenhunen *et al.*, 1968, 1969a).

The biliverdin IXα which is produced on fission of the α-monohydroxyheme is reduced to bilirubin by soluble biliverdin reductase (Figure 5) (Tenhunen *et al.*, 1970a). This is a soluble enzyme with a wide tissue distribution and it has an absolute requirement for reduced pyridine nucleotide. In the 20,000*g* supernatant fractions of rat liver, spleen and other tissues, which convert heme to bilirubin, biliverdin reductase usually is not rate limiting (Tenhunen *et al.*, 1970a; Pimstone *et al.*, 1971a).

Cytochrome P-450 serves as the oxygen-activating enzyme in many mixed-function oxidations (Omura *et al.*, 1965; Conney, 1967). The cytochrome has a strong affinity for CO, which leads to competition with O_2 for binding to the hemoprotein and consequently results in partial inhibition of enzymatic activity (Omura *et al.*, 1965; Ryan and Engel, 1957). The microsomal enzyme system involved in the cleavage of heme to bile pigment has a similar high degree of sensitivity to CO inhibition (Tenhunen *et al.*, 1969a) which is shown by the fact that a CO to O_2 ratio of 0.5 inhibits the formation of bilirubin by almost 50%. Moreover, the narrow range of the observed K values indicates that the transfer of reducing equivalents to the enzyme P-450 must be fast so as to maintain the enzyme in the Fe^{2+} state, the state in which it combines with carbon monoxide. This is a necessary condition for the measurement and evaluation of the photochemical action spectrum of cytochrome P-450 (Rosenthal and Cooper, 1967).

It was this high sensitivity of the system to CO, together with its absolute requirement for molecular oxygen and NADPH, that first suggested a possible role for cytochrome P-450 in the heme oxygenase system (Tenhunen *et al.*, 1969b). However, this evidence fails to establish that cytochrome P-450 is involved in the reaction as CO inhibition of the system could be caused by binding of the CO to the substrate heme (Lemberg and Legge, 1949), thereby making it unavailable for attack by the enzyme, as is the case with carboxyhemoglobin (Tenhunen *et al.*, 1969a). This possibility has now been excluded by the finding that light in the 450- to 470-nm range effectively reverses the CO inhibition of heme oxidation. The photochemical action spectrum for this reversal closely resembles the absorption spectrum of the CO derivative of the reduced cytochrome P-450, in that both exhibit a maximum at 450–460 nm with rat liver microsomes (Figure 2) (Tenhunen *et al.*, 1970b) and at 465–470 nm with spleen microsomes (Figure 3) (Tenhunen *et al.*, 1970b). Although for unknown reasons the spectra with liver and spleen microsomes showed slight differences, both were strikingly dissimilar from the absorption spectrum of the CO derivative of protoheme which absorbs maximally at 415 and 543 nm (Lemberg and Legge, 1949).

These observations strongly suggest that a hemoprotein with the characteristics of cytochrome P-450 is involved in the enzymatic conversion of heme to bilirubin. The light reversibility of the CO inhibition renders unlikely participation of other metalloproteins, such as copper-containing oxidases (Rosenthal and Cooper, 1967; Simpson and Boyd, 1962). It should be emphasized, however, and has been noted previously (Tenhunen *et al.*, 1969a, 1970a) that significant dissimilarities exist between the oxidative metabolism of many drugs and that of heme. These include the high specific activity of heme oxygenase in the spleen, the failure of enzyme inhibition by compounds such as hexobarbital, aminopyrene, and diethylaminoethyl 2,2-diphenylvalerate (SKF-525A) (Tenhunen *et al.*, 1969a) and the unusually high degree of sensitivity to CO inhibition. The suggestion that these differences may reflect localization of these enzymatic mechanisms in different cell types (Tenhunen *et al.*, 1970a) has lost force by the recent demonstration that in rat liver, most of the

native heme oxygenase activity is contained in the hepatic parenchymal cells rather than in sinusoidal cells (Bissell *et al.*, 1971), as had previously been assumed. It is evident, therefore, that the diversity in the properties of these microsomal mixed-function oxidase systems has other and as yet unknown explanations (Sladek and Mannering, 1969).

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