Destruction of Heme and Hemoproteins Mediated by Liver Microsomal Reduced Nicotinamide Adenine Dinucleotide Phosphate–Cytochrome P-450 Reductase†

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ABSTRACT: The purified microsomal flavoprotein reduced nicotinamide adenine dinucleotide phosphate–cytochrome P-450 reductase (NADPH:ferricytochrome oxidoreductase, reducing; EC 1.6.2.4) mediated the destruction of free ferriprotoporphyrin IX and the same heme bound in liver microsomal cytochromes P-450, P-420, and b_5 and other hemoproteins in the presence of NADPH and O_2. Other microsomal flavoproteins did not mediate such reactions. The mechanism of destruction involves the attack of ferrous (as opposed to ferric) heme by H_2O_2 or derived oxygen species. Most of the H_2O_2 was shown to be generated at the site of the heme as opposed to the flavin. Hemoproteins present in microsomal preparations were also destroyed when endogenous catalase was inhibited by N_3^-. In microsomal or reconstituted heme destruction systems devoid of catalytic activity, only low levels of bile pigments (i.e., biliverdin and bilirubin) were detected; most of the products of free or cytochrome P-450 bound heme were water-soluble compounds and, in the case of cytochrome P-450, compounds covalently bound to protein. The major water-soluble product derived from either cytochrome P-450 or free heme was characterized as a dipyrrole of the propentdyopent type. In the presence of free heme, NADPH–cytochrome P-450 reductase reduced this compound to a characteristic pentdyopent having an absorption maximum at 525 nm. Spectral studies with microsomal preparations suggested that CO is also a product. This destructive mechanism appears to be distinct from that in which heme oxygenase [heme, hydrogen donor:oxygen oxidoreductase (α-methene-oxidizing, hydroxylating), EC 1.14.99.3] converts heme to biliverdin, and biliverdin does not appear to be an intermediate in the conversion of heme to propentdyopent.

During the course of studies on the destruction of cytochrome P-450 that occurs during its metabolism of vinyl chloride, the flavoprotein NADPH–cytochrome P-450 reductase (NADPH:ferricytochrome oxidoreductase, reducing; EC 1.6.2.4) was found to be capable of mediating the destruction of the heme of P-450[1] in the absence of added substrates (Guengerich and Strickland, 1977). The purified reductase also mediated the NADPH-dependent destruction of FePPIX (Guengerich and Strickland, 1977), as did a protease-derived segment of the enzyme (Masters and Schacter, 1976).

At least five physiological systems for oxidative heme destruction have now been identified. (1) Heme and hemoproteins are converted to bile pigments in coupled systems using ascorbate or other mild reducing agents (Lemberg, 1956). This mechanism is thought to account for the artifactual "heme α-methenyl oxygenase" activity reported by Nakajima (1963). (2) FePPIX and several hemoproteins are converted specifically to biliverdin IXα by the heme oxygenase system (Tenhunen et al., 1969; Yoshida et al., 1974; Yoshida and Kikuchi, 1977), a mixed-function oxidase which consists of NADPH–cytochrome P-450 reductase and a heme-binding protein termed heme oxygenase [heme, hydrogen donor:oxygen oxidoreductase (α-methene-oxidizing, hydroxylating), EC 1.14.99.3]. Since the majority of FePPIX administered to rats intravenously is recovered as bilirubin IXα, the reduction product of biliverdin IXα, this system is believed to be responsible for the bulk of in vivo heme metabolism. (3) P-450 is destroyed during microsomal lipid peroxidation (Nishibayashi et al., 1968; Levin et al., 1973), which is enhanced in the presence of CCl_4 and other compounds (Koch et al., 1974). (4) P-450 is also destroyed by products of its mixed-function oxidative metabolism of certain olefins, such as vinyl chloride (Guengerich and Strickland, 1977), secobarbital (Levin et al., 1973), and allylisopropylacetamide (DeMatteis, 1971). (5) P-450 and FePPIX are destroyed in the presence of NADPH–cytochrome P-450 reductase (Masters and Schacter, 1976; Guengerich and Strickland, 1977).

All of these systems appear to have distinct mechanisms, but the latter four processes all involve enzymes and utilize NADPH, O_2, and the microsomal flavoprotein NADPH–cytochrome P-450 reductase. Chemical mechanisms are obscure in all cases, and products have only been conclusively identified in the first two cases. In this study, attention was focused upon the mechanism of the last system, the least complex in terms of required enzyme components.

Experimental Procedure

Materials. FePPIX (hemin chloride) was purchased from Eastman; bilirubin and biliverdin were from Sigma.

Assays. Heme, P-450, P-420, and cytochrome b_5 were assayed according to Omura and Sato (1964). Protein was estimated using the procedure of Lowry et al. (1951). H_2O_2 was estimated fluorimetrically (Snyder and Hendley, 1971). Biliverdin (Tenhunen et al., 1970) and bilirubin (Schacter et al., 1972) were estimated spectrophotometrically; heme oxygenase activity was assayed in the presence of biliverdin reductase

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[1] Abbreviations used are: P-450, liver microsomal cytochrome P-450; P-420, liver microsomal cytochrome P-420; FePPIX, ferriprotoporphyrin IX; di-12-GPC, L-α-dilauroylglyceryl-3-phosphorylcholine; NaDOC, sodium deoxycholate; BHT, butylated hydroxytoluene; Hepes, *N*-2-hydroxyethylpiperazine-*N*'-2-ethanesulfonate; TLC, thin-layer chromatography; Tris, 2-amino-2-hydroxymethyl-1,3-propanediol; EDTA, (ethylenedinitrilo)tetraacetic acid.

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(Yoshida et al., 1974). Radioactivity measurements were made according to Guengerich and Broquist (1973). Statistical analyses used Student's t test.

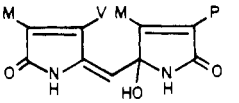
Polyacrylamide gel electrophoresis was carried out in the presence of sodium dodecyl sulfate according to Laemmli (1970). Gels were sliced into 2-mm segments; each section was mixed with 2.0 mL of 15% H_2O_2 in a capped polypropylene vial (with a plastic liner) and heated at 50 °C overnight. The vials were cooled, 10 mL of Triton X-100 cocktail was added to each vial, and vials were counted after standing in darkness overnight.

Preparation of Enzymes. Microsomes were prepared (van der Hoeven and Coon, 1974) from livers of male rats (100–150 g) that drank a 0.1% phenobarbital solution for 5 days. Rats were treated with [4-^{14}C]- or [3,5-^{3}H]aminolevulinic acid as described by Levin et al. (1973) to prepare microsomes containing labeled P-450.

NADPH–cytochrome P-450 reductase was purified by octylamino-Sepharose 4B and 2′,5′-ADP–agarose chromatography as previously described (Guengerich, 1977a,b) with slight modification. The reductase was eluted from the former column with 50 mM potassium phosphate (pH 7.25) buffer containing 0.35% sodium cholate and 0.15% NaDOC (Imai, 1976) (all buffers used in reductase purification included 1 mM EDTA, 2 μM FMN, and 20% glycerol). The reductase was applied directly to the 2′,5′-ADP column (10 mL) which had been equilibrated with 10 mM potassium phosphate (pH 7.25) buffer. The column was washed with 100 mL of 150 mM potassium phosphate (pH 7.7) buffer containing 0.2% Emulgen 913. After washing the column with 150 mL of 30 mM potassium phosphate (pH 7.25) buffer containing 0.1% NaDOC, the reductase was eluted with the same buffer (70 mL) to which 5 mM 2′-AMP had been added. Peak fractions were pooled, dialyzed twice against 50 volumes of 10 mM Tris–acetate (pH 7.4) buffer, and stored at −20 °C. All preparations migrated as electrophoretically homogeneous species (M_r 74 000) (Laemmli, 1970; Guengerich, 1977b) and had A_{455}/A_{380} ratios of at least 1.10; preparations used here had specific activities of 38–46 μmol of cytochrome c reduced min^{-1} (mg of protein)$^{-1}$.

P-450 was used after elution from octylamino-Sepharose 4B and treatment with calcium phosphate gel (Guengerich, 1977a). P-420 was prepared from P-450 by the addition of 0.1% sodium dodecyl sulfate followed by two dialyses against 500 volumes of distilled water (24-h each). Porcine spleen heme oxygenase was carried through the DEAE step of Yoshida and Kikuchi (1977). Rat liver cytochrome b_5 was prepared by octylamino-Sepharose 4B (Imai, 1976), DEAE-cellulose, and Sephadex G-75 and G-25 chromatography (Spatz and Strittmatter, 1971). NADH–cytochrome b_5 reductase was prepared according to Spatz and Strittmatter (1972) after recovery in the 33 mM phosphate wash from the P-450 hydroxylapatite column (Guengerich, 1977a). Rat liver biliverdin reductase was carried through step 4 of the procedure of Tenhunen et al. (1969). Porcine liver microsomal flavoprotein oxidase [dimethylaniline monooxygenase (*N*-oxide-forming, EC 1.14.13.8] (Ziegler and Mitchell, 1972) was a gift of Dr. D. M. Ziegler, University of Texas, Austin. Bromelain-solubilized liver microsomal NADPH–cytochrome c reductase was donated by Dr. S. D. Aust, Michigan State University, East Lansing.

Preparation of Propentdyopent. An aqueous FePPIX solution (1%, w/v, adjusted to pH 8 with NH_4OH) was heated at 60 °C in the presence of 5% (v/v) H_2O_2 (Fischer and Müller, 1937). The concentrated filtrate was taken up in CH_3OH and esterified with ethereal CH_2N_2. The concentrated esters were dissolved in $CHCl_3$, washed with water, and separated on layers of silica gel G ($CHCl_3$–CH_3OH–CH_3CO_2H, 95:5:0.5, v/v). $Na_2S_2O_4$–NaOH treatment of TLC sections revealed the presence of two pentdyopent-positive (red) bands at R_f 0.5 and 0.7. The material in the upper band was assigned structure I based on previous studies (Bonnet and



I

Stewart, 1975; Lightner, 1978) and the following data: MS M$^+$ m/e 332 (expected for $C_{17}H_{20}N_2O_5$, 332); uv λ_{max} 275 nm (ϵ 25.9 mM^{-1} cm^{-1}; CH_3OH); IR 3430, 3330, 3060, 2950, 2920, 1760, 1730, 1690, 1460, 1375, 1260, 1175, 1075, 1035, and 740 cm^{-1} (M = $-CH_3$, V = $-CH{=}CH_2$, and P = $-CH_2CH_2CO_2CH_3$ in I).

Fractionation of Incubates. The following procedure, based on that of Schwartz et al. (1960), was used to separate heme metabolites. Each incubation was mixed vigorously with an equal volume of acetone; one volume of ether was then added with vigorous mixing. The protein and the two layers were separated by centrifugation. The aqueous phase (fraction 1) was transferred and saved. The organic (upper) phase was transferred and mixed with an equal volume of 2 N HCl to obtain an aqueous phase and an organic phase (fraction 2). That aqueous phase was mixed with 0.5 volume of $CHCl_3$, centrifuged, and divided into the resulting organic (fraction 3) and aqueous (fraction 4) phases. The protein precipitate from the first extraction was mixed vigorously with a 4:1 mixture of ethyl acetate–acetic acid (ca. 2 mL/mg of protein) and centrifuged—the supernatant (fraction 5) was saved and the apoprotein precipitate was washed successively with 15 mL each of 0.2% HCl in acetone, 10% Cl_3CCO_2H, and 95% ethanol (three mixes with each solvent for 60 s in a Sorvall Omni-Mixer followed by centrifugation) to give fraction 6. In this scheme, heme is found in fraction 5, biliverdin is in fraction 3, and bilirubin is in fraction 2. Overall recovery of radioactivity was greater than 90% in all cases.

Results and Discussion

Specificity of Destruction of Heme and Hemoproteins. Conditions were optimized for the destruction of FePPIX in the presence of NADPH and NADPH–cytochrome P-450 reductase; both phospholipid and ionic detergent were necessary for the maximal rate of heme destruction.[2] Under these conditions, the reaction was linear for 10 min, and the observed rates of destruction were identical when the loss of heme was assayed kinetically at 400 nm or with the pyridine hemochrome assay. The apparent K_m for FePPIX was 7.5 μM and the V_{max} was 13.3 nmol of FePPIX destroyed min^{-1} (mg of reductase)$^{-1}$ at 37 °C; however, substrate inhibition was observed at FePPIX concentrations above 15 μM. FePPIX enhanced the fluorescence emission (525 nm) of NADPH–cytochrome P-450 reductase (excitation at 455 nm);[3] a double-reciprocal plot of the fluorescence enhancement vs. FePPIX concentration was linear and yielded a dissociation constant of 6 μM, suggesting that the fluorescence change is related to FePPIX binding that leads to destruction.

NADPH–cytochrome P-450 reductase and NADPH also mediate the destruction of a number of important hemopro-

[2] Di-12-GPC and NaDOC are necessary for optimal interaction of NADPH–cytochrome P-450 reductase and P-450 (Guengerich, 1977).

[3] FePPIX did not fluoresce at these wavelengths.

TABLE I: NADPH-Dependent Destruction of Heme and Hemoproteins Mediated by NADPH-Cytochrome P-450 Reductase.[a]

substrate	rate of heme destruction (nmol/min^{-1} (mg reductase)$^{-1}$)
FePPIX	5.30 ± 0.45
methemalbumin (bovine)	3.63 ± 0.15
hemoglobin (equine)	5.30 ± 0.25
myoglobin (whale skeletal muscle)	4.26 ± 0.31
P-450	2.21 ± 0.33
P-420	1.31 ± 0.27
catalase (bovine erythrocyte)	0.67 ± 0.18
cytochrome b_5	1.56 ± 0.16
cytochrome c (equine heart)	1.35 ± 0.16

[a] Each incubation contained NADPH-cytochrome P-450 reductase (45 μg/mL), KHepes buffer (50 mM, pH 7.7), $MgCl_2$ (15 mM), FePPIX or the indicated hemoprotein (5 μM), di-12-GPC (30 μM), NaDOC (0.25 mM), glucose-6-phosphate dehydrogenase (1.0 IU/mL), and $NADP^+$ (0.2 mM) in a final volume of 1.5 mL. After equilibration for 5 min at 30 °C, reactions were initiated by the addition of glucose 6-phosphate (3 mM). Incubations proceeded at 30 °C in darkness for 10 min and were stopped by freezing in a dry ice-acetone bath, after which pyridine and NaOH were added for the heme determinations. All values are means of triplicate experiments ± SD.

TABLE II: Destruction of Heme and Individual Hemoproteins in Rat Liver Microsomes.[a]

component	heme or hemoproteins remain. after incubat (nmol/mg of protein) −NADPH	+NADPH	difference nmol/mg (of protein)	% change
heme	3.60 ± 0.30	2.53 ± 0.02[b]	−1.05	−28
Cyt P-450	2.17 ± 0.14	1.36 ± 0.14[c]	−0.81	−37
Cyt P-420	0.72 ± 0.10	0.58 ± 0.09[d]	−0.14	−17
Cyt b_5	0.58 ± 0.04	0.41 ± 0.01[c]	−0.17	−29
total	3.47	2.35	−1.12	−32

[a] Microsomes (2 mg/mL) were incubated in darkness for 10 min at 37 °C in 50 mM KHepes buffer (pH 7.7) with 15 mM $MgCl_2$, 1 mM NaN_3, 1 mM EDTA, and 20 μM BHT present; when noted, a NADPH-generating system consisting of 0.5 mM $NADP^+$, 10 mM glucose 6-phosphate, and 1.0 IU glucose-6-phosphate dehydrogenase/mL was present. The final sample volume was 3.5 mL. Microsomes were chilled on ice and precipitated by the addition of $CaCl_2$ to 10 mM followed by centrifugation at 20 000g for 20 min at 4 °C; the pellets were resuspended in buffer, and heme, P-450, P-420, cytochrome b_5, and protein were determined. All assays are means of triplicate experiments ± SD. [b] Significantly different from −NADPH control ($p < 0.025$). [c] Significantly different from −NADPH control ($p < 0.01$). [d] Not significantly different from −NADPH control ($p > 0.10$).

TABLE III: Destruction of Oxidized and Reduced P-450 and FePPIX by H_2O_2.[a]

system	heme remain. after incubat (μM)
Fe^{3+}PPIX	8.10 ± 0.44
Fe^{3+}PPIX, H_2O_2	7.83 ± 0.09[b]
Fe^{2+}PPIX, H_2O_2	6.92 ± 0.33[c]
Fe^{3+}PPIX, reductase, NADPH	4.19 ± 0.88[d]
P-450^{3+}	4.21 ± 0.10
P-450^{3+}, H_2O_2	4.24 ± 0.15[b]
P-450^{2+}, H_2O_2	3.38 ± 0.07[d]
P-450^{3+}, reductase, NADPH	2.59 ± 0.18[d]

[a] Incubations were carried out using the buffer system of Table I and 68 μg/mL of the reductase. H_2O_2, 125 (in the Fe^{3+}PPIX experiment) or 20 μM (in the P-450^{3+} experiment), was added to oxidized FePPIX (Fe^{3+}PPIX) or oxidized P-450 (P-450^{3+})—this concentration is equivalent to the NADPH oxidized in each reductase plus NADPH experiment. In the experiments with Fe^{2+}PPix)reduced FePPIX) and P-450^{2+} (reduced P-450), the concentrations of FePPIX or P-450 were the same—each sample was deaerated under N_2 in a Thunberg tube (Guengerich and Strickland, 1977) and an amount of $Na_2S_2O_4$ required for stoichiometric reduction was tipped from an anaerobic sidearm into the main well of the vessel (total volume = 5.0 mL). After mixing and allowing reduction to proceed for 30 min at 25 °C, H_2O_2 (amounts as above) was tipped anaerobically from a second sidearm and incubation was allowed to proceed for 10 min at 37 °C. Reactions were stopped by freezing in a dry ice-acetone bath prior to heme assays; all results are means (±SD) of three experiments. [b] Not significantly less than control (first line of series) ($p > 0.10$). [c] Significantly less than first line of series ($p < 0.05$). [d] Significantly less than first line of series ($p < 0.01$).

teins (Table I). Protoporphyrin IXα dimethyl ester was not destroyed under these conditions.

Bromelain-solubilized reductase, which is devoid of a peptide of about 8000 daltons (Welton et al., 1973) was also able to bring about the destruction of FePPIX, but the rate was 60% that of the detergent-solubilized enzyme under identical conditions. Neither of the two major flavoproteins found in the endoplasmic reticulum, NADH-cytochrome b_5 reductase or dimethylaniline monooxygenase, destroyed either FePPIX or P-450.[4]

Liver microsomes did not lose heme when incubated with NADPH in the presence of EDTA and BHT, potent inhibitors of lipid peroxidation (Welton and Aust, 1972); however, when NaN_3 was added to inhibit catalase, both endogenous hemoproteins (Table II) and added FePPIX (results not shown) were destroyed. While the loss of P-450 in a reconstituted system could be blocked by the addition of the substrate benzphetamine (Guengerich and Strickland, 1977), the loss of P-450 heme in microsomes was not inhibited by benzphetamine (1 mM), testosterone (0.1 mM), cyclohexane (10 mM), laurate (0.1 mM), or *N,N*-dimethylaniline (1 mM). In repeated experiments, approximately 25–30% of the total microsomal heme resisted destruction, even after 40 min. When this level of heme was used as the end point, heme destruction was first order (for ca. 4 half-lives) with a half-life of 7 min.

The Role of H_2O_2 in Heme Destruction. Previous studies indicated that the destructions of P-450 and FePPIX are inhibited by catalase (Guengerich and Strickland, 1977). H_2O_2 is produced during NADPH-cytochrome P-450 reductase catalyzed NADPH oxidation, in the absence or presence of either P-450 (Nordblom and Coon, 1977) or FePPIX. The ratios of H_2O_2 recovered to NADPH consumed were 0.77, 0.07, and 0.49 for the reductase-coupled systems containing P-450, FePPIX, and no heme, respectively. (The discrepancy in the case of FePPIX could be attributed to the measured catalatic activity of the FePPIX itself.) Concentrations of H_2O_2 equivalent to the levels of NADPH consumed in the previous experiments were added to either oxidized or reduced FePPIX or P-450 (Table III). No significant losses were observed with the ferric hemes. With the reduced hemes, both

[4] NADH replaced NADPH when NADH-cytochrome b_5 reductase was used. With dimethylaniline hydroxylase, incubations were started by the addition of enzyme, in order to prevent destruction of the enzyme observed in the absence of NADPH (Guengerich and Aust, 1977).

TABLE IV: Effects of CO on NADPH-Dependent Heme Destruction.[a]

system	heme remain. after incubat. (μM)
microsomes	3.42 ± 0.13
microsomes, NADPH, 100% air	2.58 ± 0.05
microsomes, NADPH, 80% air–20% CO	2.61 ± 0.12[b]
microsomes, NADPH, 90% CO–10% O_2	3.45 ± 0.21[c,d]
reductase, P-450	3.57 ± 0.66
reductase, P-450, NADPH, 100% air	1.81 ± 0.03
reductase, P-450, NADPH, 80% air–20% CO	2.34 ± 0.24[e]
reductase, P-450, NADPH, 90% CO–10% air	3.22 ± 0.13[c,d]
reductase, FePPIX	2.94 ± 0.15
reductase, FePPIX, NADPH, 100% air	1.06 ± 0.07
reductase, FePPIX, NADPH, 80% air–20% CO	2.55 ± 0.10[c]
reductase, FePPIX, NADPH, 90% CO–10% air	2.74 ± 0.15[c,d]

[a] Incubations were carried out essentially as described under Table II (with microsomes) or I (with P-450 or FePPIX). In all cases, the components were mixed in a Thunberg tube and equilibrated with the gas mixtures by ten alternate cycles of vacuum and mixing with gas. After incubation for 10 min, reactions were stopped by freezing in a dry ice–acetone bath and heme was determined; all values are means of three experiments ± SD. [b] Not significantly greater than the result of 100% air experiment ($p > 0.10$). [c] Significantly greater than the result of 100% air experiment ($p < 0.01$). [d] Not significantly less than the value obtained in the absence of NADPH ($p > 0.10$). [e] Significantly greater than the result of 100% air experiment ($p < 0.05$).

FePPIX and P-450 were destroyed under anaerobic conditions (no attempts were made to correct for heme oxidized nondestructively by the H_2O_2). Ferric FePPIX and P-450 can be destroyed by H_2O_2 (Guengerich and Strickland 1977; Nordblom et al., 1976), but required concentrations of H_2O_2 are much higher than produced in the reductase-coupled systems. Oxygen species derived from H_2O_2 may actually be responsible for the destruction; however, ethanol (a hydroxyl radical trap) did not inhibit destruction (Guengerich and Strickland, 1977). Singlet oxygen has been postulated to be the active species in the photooxidation of bilirubin to propentdyopents (Bonnett and Stewart, 1975), although production of this species from H_2O_2 and ferrous heme under anaerobic conditions (Table III) would seem unlikely. Superoxide anion ($O_2^{-}\cdot$) may be formed as an intermediate in the formation of H_2O_2: superoxide dismutase did not inhibit the destruction of FePPIX (Masters and Schacter, 1976; Guengerich and Strickland, 1977) but epinephrine inhibited the conversion of methemalbumin to green pigments by protease-solubilized reductase (Masters and Schacter, 1976).

Since H_2O_2 can be produced by the autoxidation of either the reductase or heme (FePPIX or P-450), the effect of adding CO (which binds to reduced P-450 or FePPIX to specifically block heme, but not flavin, autoxidation) was studied in hopes of attributing the source of H_2O_2. The destruction of FePPIX or P-450 in a reconstituted system, but not that of P-450 in microsomes, was inhibited by the presence of 20% CO (Table IV). Since others have found considerable variation in the amount of CO required to inhibit P-450-dependent reactions (Cooper et al., 1977), the CO/O_2 ratio was raised from 1.25 (20% CO) to 9.0 (90% CO) (while decreasing the O_2 concentration from 16 to 10%); under these conditions, all of the heme destruction systems were strongly inhibited, implying O_2 reduction by the heme as opposed to the flavin. (Incubation of any of the systems under anaerobic conditions [100% N_2] also completely abolished all destructive activity.) However, some of the H_2O_2 responsible for the destruction can be produced by the reductase: the bromelain-solubilized reductase, which does not reduce P-450 because of an inability to bind the cytochrome (Coon et al., 1975), destroyed P-450 at a rate about 20% that of the detergent-solubilized reductase.

Products of Heme Destruction. During the metabolism of methemalbumin by the heme oxygenase system (Tenhunen et al., 1969) and perhaps in the destruction of P-450 by microsomal lipid peroxidation (Nishibayashi et al., 1968; DeMatteis et al., 1977), one of the heme methene bridges is converted to CO. When microsomes were incubated with and without NADPH in the presence of EDTA and BHT, a difference spectrum was obtained with a shoulder in the 440–450-nm region attributed to a low steady-state level of oxygenated P-450 (Estabrook et al., 1971; Guengerich et al., 1975). Repetition of the experiment in the presence of NaN_3 indicated a depression in the Soret region due to heme loss and the formation of a large peak at 450 nm, consistent with the production of CO which becomes bound to ferrous P-450 (Nishibayashi et al., 1968).

No such heme-CO peaks were observed with NADPH-cytochrome P-450 reductase in reconstituted systems (Figure 1), where the kinetics of individual reaction steps have probably been changed appreciably and reduced heme might not accumulate. The changes observed in the methemalbumin difference spectrum resemble those reported by Masters and Schacter (1976) with a protease-treated reductase, except that the peak attributed to bilirubin was not seen here (Figure 1A). When FePPIX was incubated with the reductase, a strong peak centered at 525 nm formed (Figure 1B). The spectral changes observed with P-450 resembled those seen with methemalbumin (Figure 1C); i.e., the Soret band decreased and a weak broad green band (600–800 nm) appeared.

Most of the ^3H or ^{14}C from the heme of P-450 was recovered in fractions 1 (44%), 5 (33%), and 6 (20%) after incubation of a reconstituted system (or microsomes and NaN_3) and fractionation as described under Experimental Procedure.[5] In all cases, all (>95%) of the radioactivity in fraction 5 chromatographed with FePPIX (TLC: activated silica gel G; benzene–CH_3OH–CH_3CO_2H, 90:10:2, v/v). Label bound to protein (fraction 6) could not be removed by extensive washing or by electrophoresis (vide infra), and the binding is assumed to be covalent.

Although some green compounds were observed spectrally in the incubation of P-450 and NADPH-cytochrome P-450 reductase (Figure 1), no more than 3–5% of the destroyed heme could be accounted for as biliverdin production in spectral or radioisotopic experiments. The small amount of radioactivity found in fraction 2 was judged not to be bilirubin IXα upon TLC (silica gel G; benzene–CH_3OH–CH_3CO_2H, 90:10:2, v/v). Radiometric experiments were repeated in the presence of 100 nmol each of biliverdin IXα and bilirubin IXα, and ascorbic acid (20 mg/mL) was added to the acetone prior to extraction (Maines and Kappas, 1977). With the microsomal system, 0.9% of the label was recovered as biliverdin, while 1.6% was recovered with the reconstituted system; in both cases, recovery of label as bilirubin was less than 0.1%. When the reconstituted system was incubated in the presence of biliverdin reductase and bilirubin (100 nmol) was added immediately before fractionation, no label was detected in the bilirubin IXα isolated by TLC; moreover, no spectral formation of bilirubin was observed during the reaction. In a control experiment, biliverdin was identified as the only colored me-

[5] In control experiments (minus NADPH), no more than 5% of the label was recovered in fraction 1 or 6 and at least 92% was recovered in fraction 5.

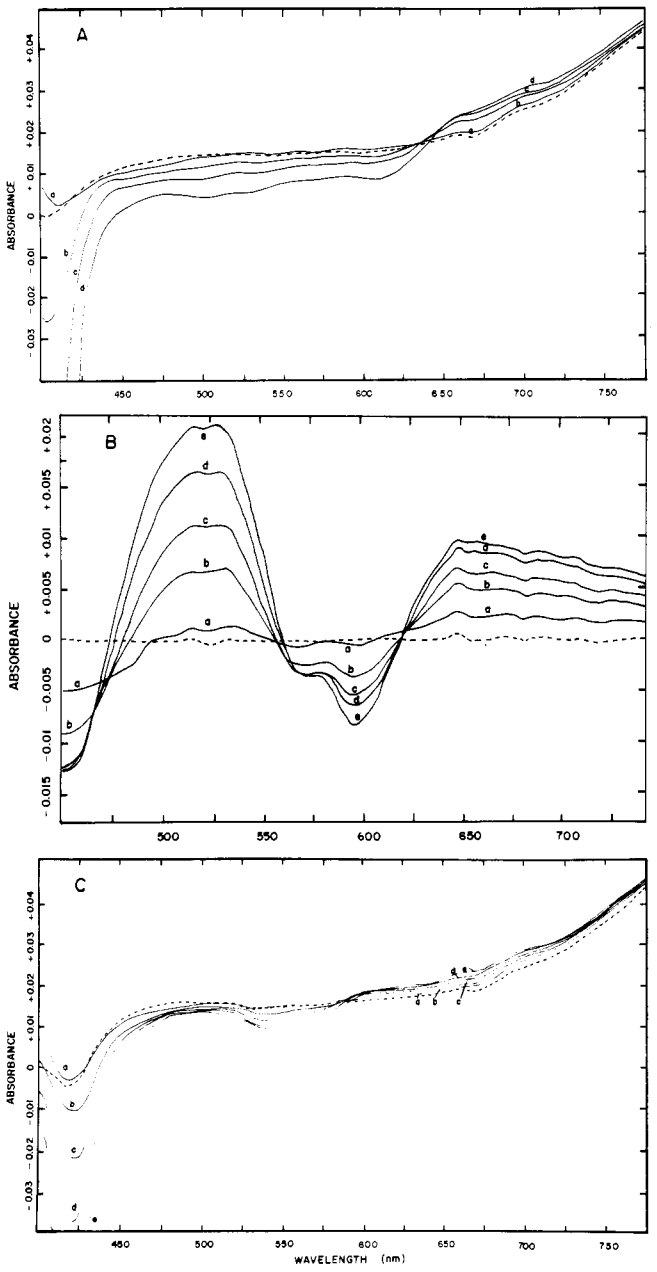


FIGURE 1: Spectral changes accompanying the NADPH-cytochrome P-450 reductase-mediated destruction of methemalbumin, free FePPIX, and P-450. In each case, a mixture of NADPH-cytochrome P-450 reductase (20 μg/mL), di-12-GPC (38 μM), NaDOC (0.25 mM), KHepes (50 mM, pH 7.7), $MgCl_2$ (15 mM), $NADP^+$ (0.1 mM), glucose 6-phosphate (10 mM), and FePPIX or hemoprotein was divided into two 3-mL cuvettes, and the indicated baseline (- - -) was recorded using an Aminco DW-2 spectrophotometer equilibrated at 25 °C. Glucose-6-phosphate dehydrogenase (1 IU/mL) was added (in 6 μL) to the sample cuvette and spectra (labeled a, b, c, etc., in consecutive order) were recorded at 10-min intervals: (A) methemalbumin (11.4 μM), (B) FePPIX (11.4 μM), and (C) P-450 (5.6 μM).

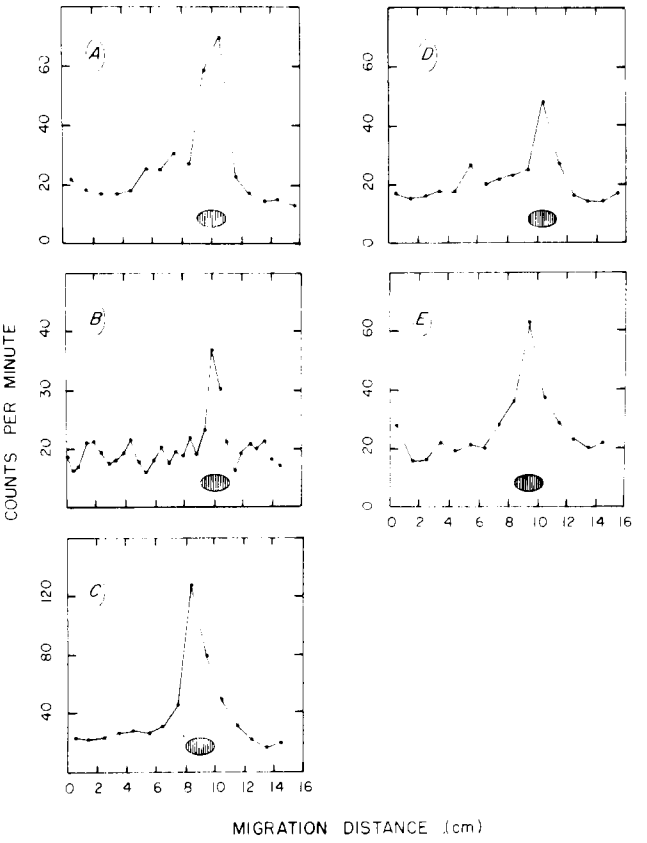


FIGURE 2: TLC of incubates with standard propentdyopent. The shaded area indicates the migration of the standard. All chromatography was of methyl esters on 0.25-mm layers of activated silica gel G. Incubations were carried out using the general conditions described in the tables. (A) Incubation: ^{3}H-labeled microsomes, NaN_3, NADPH; solvent (TLC) $CHCl_3$-CH_3OH-CH_3CO_2H, 90:10:0.5, v/v. (B) Incubation: ^{14}C-labeled microsomes, NaN_3, NADPH; solvent $CHCl_3$-CH_3OH-CH_3CO_2H, 90:10:0.5. (C) Incubation: ^{3}H-labeled microsomes, NaN_3, NADPH; solvent benzene-CH_3OH-CH_3CO_2H, 75:25:0.5, v/v. (D) Incubation: ^{3}H-labeled P-450, NADPH-cytochrome P-450 reductase, di-12-GPC, NaDOC, NADPH; solvent $CHCl_3$-CH_3OH-CH_3CO_2H, 90:10:0.5. (E) Incubation as under D; solvent as under C.

tabolite of FePPIX using liver microsomes isolated from rats in which heme oxygenase was induced by $CoCl_2$ (Maines and Kappas, 1975); more than 90% of the biliverdin was the IXα isomer (O'Carra and Colleran, 1970). However, incubation of a mixture of equal amounts of $CoCl_2$-induced rat microsomes and ^{3}H-labeled microsomes from phenobarbital-induced rats (with NADPH in the absence of NaN_3) did not yield labeled biliverdin.

The above experiments suggested that metabolism of P-450 heme may not be carried out by heme oxygenase to the usual bile pigments. The accumulation of a peak at 525 nm (Figure 1B) during FePPIX destruction first suggested that propentdyopent structures were being formed and reduced during the reaction. Fraction 1 was washed with $CHCl_3$, dried in vacuo, and esterified with CH_2N_2; TLC indicated that most of the label recovered in this fraction (from labeled P-450 heme) migrated with standard propentdyopent methyl ester (Figure 2). In addition, the extract prepared from a reductase-FePPIX-NADPH incubation gave a single I_2- and pentdyopent-positive spot of the same R_f as the propentdyopent ester (experiment not shown). Further evidence for the formation of propentdyopent is presented in Figure 3, as shown by the characteristic spectra obtained with extracts of microsome-NADPH-NaN_3 and reductase-FePPIX-NADPH incubations. These extracts failed to yield adducts with Ehrlich's reagent (Mattocks and White, 1969), which reacts with pyrroles containing an unsubstituted α position.

It is interesting that propentdyopent is not reduced during P-450 or methemalbumin destruction (Figure 1A,C). Propentdyopent methyl ester was saponified (1 N KOH, 100 °C, 10 min) and incubated with NADPH and NADPH-cytochrome P-450 reductase, but no spectral changes were observed. Thus, FePPIX may be required for electron transfer to propentdyopent.

The nature of the P-450 heme product(s) bound covalently to protein (fraction 6) was pursued. Biliverdin is known to bind

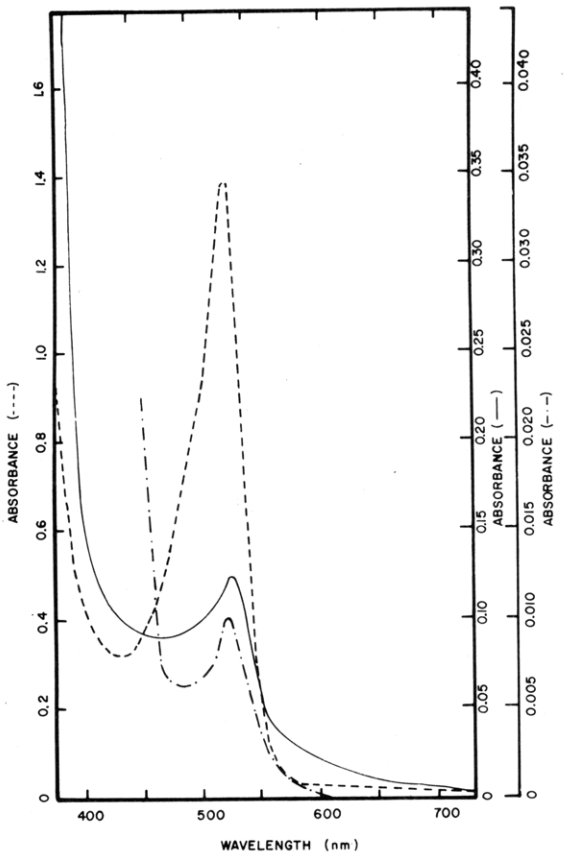


FIGURE 3: Pentdyopent spectra of heme derivatives. An aliquot of each incubation was dissolved in 1 N NaOH and a baseline was recorded. Several crystals of solid $Na_2S_2O_4$ were added to the sample and the contents of each cuvette were transferred to test tubes, heated 2 min at 100 °C, and cooled on ice; the resulting spectra were recorded: FePPIX plus H_2O_2 in alkaline H_2O (see Experimental Procedure) (···); NADPH–cytochrome P-450 reductase plus NADPH and Fe PPIX (see Figure 1B) (—); and liver microsomes plus NADPH and NaN_3 (- • -).

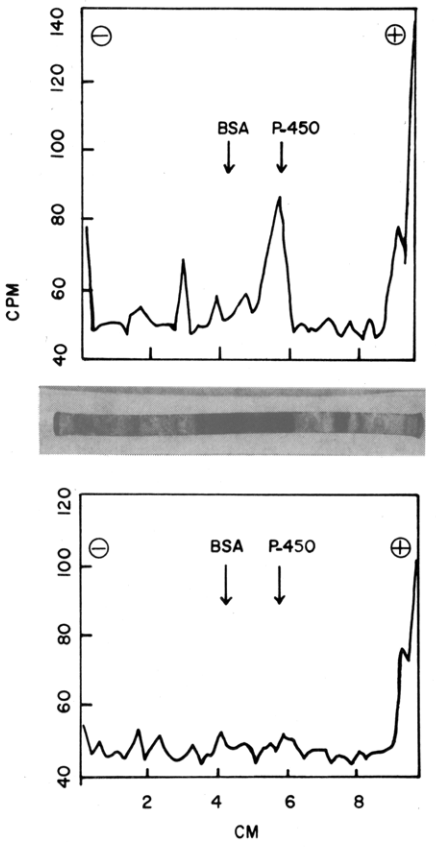


FIGURE 4: Electrophoresis of incubations containing ^3H-labeled P-450, NADPH–cytochrome P-450 reductase, and bovine serum albumin carried out in the presence and absence of NADPH. Incubations were carried out in parallel with ^3H-labeled P-450 (0.5 mg/mL, 3.2 μM, 6.1 × 10^3 dpm/mL), NADPH–cytochrome P-450 reductase (60 μg/mL), bovine serum albumin (1.0 mg/mL), di-12-GPC (75 μM), NaDOC (0.25 mM), KHepes (30 mM), and $MgCl_2$ (10 mM); the final volume was 0.80 mL in each case. One incubation (upper frame) contained the NADPH-generating system as described under Table II, while the other (lower frame) did not. Both incubates were shaken at 37 °C in the dark for 90 min, washed (fraction 6 procedure), and electrophoresed. The positions of the bovine serum albumin (BSA) and P-450 are indicated with arrows.

to protein sulfhydryls via its vinyl side chains (Lemberg, 1956), but the addition of reduced glutathione (5 mM) during incubation did not significantly reduce the level of radioactivity bound to protein. Incubation of the reconstituted system was carried out in the presence of a twofold excess of bovine serum albumin; proteins were separated by electrophoresis. Figure 4 shows that essentially all of the label was bound to apo-P-450 and not to albumin, consistent with the hypothesis that the heme product was rather reactive and did not migrate from apo-P-450 before becoming covalently bound. [^3H]Propentdyopent was isolated and incubated with unlabeled P-450, but no label was covalently bound to the protein.

Relationship of FePPIX Catabolism by NADPH–Cytochrome P-450 Reductase in the Presence and Absence of Heme Oxygenase. A reconstituted heme oxygenase system was insensitive to catalase (800 IU/mL), in contrast to the destruction carried out in the absence of heme oxygenase (Masters and Schacter, 1976; Guengerich and Strickland, 1977). Neither NADH–cytochrome b_5 reductase nor dimethylaniline monooxygenase would replace NADPH–cytochrome P-450 reductase in this heme oxygenase system. The possibility was considered that, in the heme oxygenase system, the reductase produces a propentdyopent which is converted by heme oxygenase to biliverdin IXα. However, after the 525-nm metabolite accumulated in the experiment of Figure 1B and heme oxygenase was then added, the level of the 525-nm species remained constant, while the broad green absorbance centered at 670 nm increased. The alternative was considered, namely, that NADPH–cytochrome P-450 reductase converts biliverdin to the 525-nm species. NADPH–cytochrome P-450 reductase was found to destroy biliverdin IXα at the rate of 3.5 nmol min^{-1} (mg of reductase)$^{-1}$ in the presence of NADPH ([biliverdin IXα] = 10 μM), but no new peaks appeared in the visible spectrum (400–800 nm). Neither biliverdin IXα nor bilirubin IXα gave rise to propentdyopents when incubated with NADPH and NADPH–cytochrome P-450 reductase.

The possibility should be considered that the heme of P-450 may be degraded in vivo via the mechanism described in this report rather than with heme oxygenase: (1) NADPH–cytochrome P-450 reductase has a high affinity for both FePPIX (vide supra) and P-450 (Coon et al., 1975). (2) Heme oxygenase is localized in reticuloendothelial cells and P-450 in parenchymal cells (Hupka and Karler, 1973). P-450 has to be denatured to P-420 to release heme (Maines and Anders, 1973), which would require transport to another cell for most efficient processing. (3) Although certain divalent metals induce heme oxygenase and depress P-450 levels, the latter is not necessarily a result of the former response (Krasny and Holbrook, 1977; Paine and Legg, 1978). (4) The degradation of the heme of microsomal P-450 (or P-420) by heme oxygenase to biliverdin has never been demonstrated, and the work reported here argues against such metabolism. (5) Propentdyopents are present in gallstones and in the urine of patients with

jaundice and certain other liver diseases (Lightner, 1978). The possibility exists that such compounds may be formed in normal metabolism at low levels and excreted undetected or perhaps metabolized first. A less than total catalatic inhibition of the reductase-coupled process might allow this mechanism to play a significant role in the in vivo catabolism of heme and hemoproteins.

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