Microsomal Heme Oxygenase

CHARACTERIZATION OF THE ENZYME*

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SUMMARY

This study characterizes microsomal heme oxygenase, a previously undescribed enzyme which catalyzes the oxidation of heme at the α-methene bridge to form biliverdin. This step is then coupled with soluble NADPH-dependent biliverdin reductase to form bilirubin; microsomal heme oxygenase is rate-limiting in this pathway. By all analytical criteria, the product of this reaction is bilirubin. Most, if not all, of the bilirubin is of the IX α configuration, which is the sole isomeric form of bilirubin occurring physiologically. Heme oxygenase is localized specifically to the microsomal fraction, has an absolute and stoichiometric requirement for NADPH and molecular oxygen, generates carbon monoxide in amounts equimolar to bilirubin, and is inhibited by carbon monoxide. These and other data suggest that this enzyme is a mixed function oxygenase. The enzyme is most active with protohemin IX or methemalbumin; substrates with less activity are methemoglobin, the α and β chains of hemoglobin, deuterohemin IX, coprohemin I, and the hemoglobin-haptoglobin complex, in this order. Oxyhemoglobin, carboxyhemoglobin, myoglobin, and free porphyrins are not acted upon by the enzyme. The apparent K_m for protohemin IX is 5.0 μM, and for the other substrates ranges from 4.5 to 5.1 μM. Sodium dodecyl sulfate, lipase, phospholipase, trypsin, potassium cyanide, sodium azide, and p-hydroxymercuribenzoate inhibit the enzyme. The kinetics and tissue distribution of this enzyme suggest that it is of major importance in the physiological degradation of hemoglobin and other hemoproteins to bile pigment.

In the intact organism, the hemoglobin of sequestered erythrocytes and also parenterally administered hemoglobin (1), hematin (2), and myoglobin (3) are converted almost quantitatively to bilirubin. The exact mechanism and the anatomic site of this conversion are unknown. The over-all reaction involves cleavage of the ferriprotoporphyrin ring at the α-methene bridge (4), resulting in formation of a linear tetrapyrrole of the α configuration (4). The α bridge carbon atom is oxidized to carbon monoxide (5), which is formed in equimolar amounts to bilirubin (6). The presence of a central di- or trivalent metal ion appears essential for the fission of the heme ring in vivo as free protoporphyrin is not converted to bile pigment (7). This absolute requirement for a metal chelate and the exclusive formation of the α isomer of bile pigment suggest that the reaction is catalyzed enzymatically (8), although alternate explanations for the strict isomer specificity have been offered (8). Moreover, the insertion of fission of two hydroxy groups into the α positions of the two terminal pyrroles indicates that the cleavage of the porphyrin ring is an oxidative step. These requirements would be fulfilled adequately by a mixed function oxidation similar to that involved in the oxidation of drugs and steroids (9) with cytochrome P-450 as the terminal oxidase (10). In the following paper such an enzyme system localized in the microsomes of several mammalian tissues is described and characterized (11).

EXPERIMENTAL PROCEDURE

Recrystallized hemin was purchased from Sigma. On spectroscopic analysis of the pyridine hemochromogen prepared with this material, the ratio of the maximal absorption in the α band to the minimal absorption between the α and β bands was 3.46 (12, 13). Hemin-14C was crystallized (14) from rat erythrocytes incubated with glycine-2-14C (15); for quantitative determination it was converted to its pyridine hemochromogen (12, 13). Specific activity was determined in a Packard Tri-Carb liquid scintillation spectrometer with toluene-14C as an internal standard (1); the efficiency was 71 to 83%. Protoporphyrin IX dimethylester, coproporphyrin I tetramethylester, and uroporphyrin I and III octamethylesters were obtained from Sigma: deuteroporphyrin IX dimethylester from K and K Laboratories; and mesoporphyrin IX dimethylester from Pierce Chemical. The free porphyrins were prepared by hydrolysis of these esters, followed by extraction (16). Individual hemins were obtained by inserting iron into the respective porphyrins according to the method of Lemberg et al. (17) as modified by Falk (18). The purity of the free porphyrins and of the hemins prepared therefrom was tested spectroscopically and by paper
chromatography (19). Hemoglobin A solutions were prepared as described by Rosi-Fanelli, Antonini, and Caputo (20). Hemoglobin was measured as cyanmethemoglobin (21). Methemoglobin was determined by the method of Evelyn and Malloy (22). Methemalbumin was prepared as described previously (11). Crystallized sperm whale myoglobin was purchased from Mann. When subjected to starch gel electrophoresis, this preparation moved as a single band (23, 24). α and β chains of hemoglobin were prepared by cleaving human hemoglobin A with p-hydroxynitritemylmercuribenzoate (25) followed by regeneration of the separated chains with 1-dodecanethiol (26). The spectra of the regenerated chains were identical with those previously reported (27). Moreover, on starch gel electrophoresis, carried out at 4°C in the discontinuous buffer system of Poulik (23) with the horizontal technique of Smithies (24), the individual chains appeared homogeneous. When mixed in equimolar amounts, the two chains rapidly formed a compound indistinguishable from hemoglobin A both by starch gel electrophoresis and by its reaction with oxygen and carbon monoxide (24). The amount of ferri-
methionyl derivatives formed under these circumstances was always less than 5% (28). Haptoglobin 1-1 and 1-2 were gifts of Dr. M. E. Raffelson, Department of Biochemistry, Presbyterian-St. Luke's Hospital, Chicago, Illinois. Pure hemoglobin-haptoglobin complexes were prepared by Sephadex G-200 gel filtration (29) and concentrated to a desired volume in dry filtration (29) and concentrated to a desired volume in dry Aquacide 1 (Calbiochem, molecular weight 70,000). In some experiments haptoglobin was added to the reaction mixture in an amount calculated to bind all hemoglobin present; this was confirmed by starch gel electrophoresis (23, 24). Hemopexin was a gift of Dr. U. Muller-Eberhard, Department of Biochemistry, Scripps Clinic and Research Foundation, La Jolla, California. The hemopexin-hemin complex was prepared according to Muller-Eberhard, Lemic, and Gunsalus (30). Lipase (type II) and phospholipase (Naja naja) were obtained from Sigma, and sterile trypsin (crystallized 3 times) from Worthington. All other chemicals were reagent grade. Deionized, distilled water was used in all studies.

Fasted male Sprague-Dawley rats weighing 200 to 350 g were decapitated and the livers were immediately perfused through the portal vein with cold isotonic saline. Liver, spleen, and other tissues were homogenized in 2 to 3 volumes (w/v) of 0.25 M sucrose and fractionated by the method of Schneider (31). Cytochrome oxidase was determined by the procedure of Wharton and Tzagoloff (32). Acid phosphatase was assayed using β-glyce-
obroposphate as substrate (33). Phosphorus was measured by the method of Chen et al. (34). Cytochrome P-450 was determined by the method of Omura and Sato (35). Protein was determined by the method of Lowry et al. (36).

Purified biliverdin reductase was prepared according to Ten-
hunen, Marver, and Schmid (37). Methemalbumin-dependent oxygen consumption was measured with a Clark-type oxygen electrode obtained from the Yellow Springs Instrument Company and attached to an Oxigraph model KM, manufactured by Gilson. Heme dependent NADPH consumption in the presence and absence of methemalbumin was estimated by the decrease in optical density at 340 μm: ΔE₃₄₀ = 10.8 (37). Production of ¹⁴CO from hemin-¹³C was measured by the method of White (38).

For the standard assay of microsomal heme oxygenase, a

¹ P. White, personal communication.

RESULTS

When methemalbumin was incubated in the standard assay procedure with liver or spleen microsomes, purified biliverdin reductase, NADPH, and molecular oxygen, a new absorption band appeared with a peak at 468 μm (11). That this absorption band was due to bilirubin formed from hemin was indicated by the following criteria. (a) Crystalline bilirubin, when added to the incubation mixture used, gave rise to a similar absorption band at 400 to 470 μm. (b) When a methanol solution of diazotized sulfanilic acid (38) was added directly to an 18,000 × g supernatant fraction of liver or spleen containing NADPH, a red azoderivative characteristic of bilirubin (43) was formed only after incubation with hemin in air. (c) After incubation with hemin a yellow pigment was extractable that had the solubility and spectrophotometric properties of bilirubin and gave a positive diazo reaction (39, 40). (d) On chromatography with silica gel this yellow chloroform-soluble pigment had an Rₚ of 0.70, identical with that of authentic bilirubin (44, 45). (e) When hemin was incubated in the 10,000 × g supernatant fraction of rat spleen containing NADPH, an average of 77% of the hemin that disappeared during incubation was accounted for by recovered bilirubin (11). (f) When hemin-¹³C with a specific activity of 406 dpm per μg (8 labeled carbon atoms per molecule) was used as substrate, radioactive material having the properties of bilirubin and exhibiting a specific activity of 358 dpm per μg (7
The 18,000 x g supernatant of rat spleen (2 to 4 mg of protein) was assayed for enzymatic activity in the presence of indicated compounds under otherwise standard conditions.

<table>
<thead>
<tr>
<th>Substance added to incubation mixture</th>
<th>Final concentration</th>
<th>Enzyme activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonium sulfate</td>
<td>200</td>
<td>89</td>
</tr>
<tr>
<td>Ammonium sulfate</td>
<td>300</td>
<td>33</td>
</tr>
<tr>
<td>Dodecyl sodium sulfate</td>
<td>0.3</td>
<td>9</td>
</tr>
<tr>
<td>Dodecyl sodium sulfate</td>
<td>0.6</td>
<td>4</td>
</tr>
<tr>
<td>Lipase</td>
<td>0.06</td>
<td>74</td>
</tr>
<tr>
<td>Lipase</td>
<td>0.2</td>
<td>17</td>
</tr>
<tr>
<td>Phospholipase (Naja naja)</td>
<td>0.2</td>
<td>66</td>
</tr>
<tr>
<td>Phospholipase (Naja naja)</td>
<td>0.7</td>
<td>39</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.06</td>
<td>56</td>
</tr>
<tr>
<td>Trypsin</td>
<td>0.25</td>
<td>4</td>
</tr>
</tbody>
</table>

labeled carbon atoms per molecule) was crystallized from the incubation mixture. (g) On mass spectrometry a mass to charge ratio of 584 (authentic bilirubin, molecular weight 584) was obtained for the recrystallized yellow pigment formed during incubation. Moreover, the degradation pattern (46) was similar for recrystallized bilirubin produced biosynthetically in vivo and for that obtained from bile.3 (h) When the yellow pigment, isolated and recrystallized from incubation mixture, was subjected to potassium permanganate oxidation (41, 42), pyrrolic acid I was the only oxidation product identified. This indicated that most if not all of the enzymatically formed bilirubin was of the IX (Y configuration and thus corresponded to the bile pigment produced in vivo (4).

The presence of microsomes in the incubation mixture was essential for the conversion of hemin to bilirubin. The microsomal fraction so prepared appeared relatively free of mitochondrial or lysosomal contamination. It contained 4.6% of the cytochrome oxidase activity, 14% of the acid phosphatase activity, and 65% of the cytochrome P-450 of the total spleen homogenate.

The complete system was enhanced by the presence of 105,000 x g supernatant or of purified biliverdin reductase (11). An enzyme preparation (18,000 x g supernatant of spleen), containing 15 to 25 mg of protein per ml, lost 30% of its activity when stored for 50 to 55 days at -20°, for 3 to 4 days at 0°, or for 1 to 2 days at 4°. All activity was lost in 5 min at 56°. Repeated rapid freezing and thawing did not significantly alter the activity of the enzyme. Enzyme activity remained membrane-bound after extensive dialysis or filtration through Sephadex G-25. Low concentrations of dodecyl sodium sulfate, lipase, phospholipase, and trypsin caused strong irreversible inhibition of activity (Table I).

The reaction required the presence of molecular oxygen and of NADPH or an operational NADPH-generating system (Table II). No bilirubin was formed under anaerobic conditions or in the absence of NADPH (Fig. 1). NADH in concentrations up to 200 µM, ascorbic acid up to 10 mM, and glutathione up to 10

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The reaction mixture (5.0 ml) consisted of 18,000 X g spleen supernatant (23.5 mg of protein); hemin-3H, 17 μM; NADP, 1.4 mM; glucose 6-phosphate, 4.0 mM; MgCl₂, 1.9 mM; and potassium phosphate buffer (pH 7.4), 90 mM. In the control flasks NADP and glucose 6-phosphate were replaced by 0.1 M potassium phosphate buffer, pH 7.4. The mixtures were incubated at 37° in closed 125-ml Erlenmeyer flasks filled with air for the time indicated. Bilirubin and carbon monoxide formed in each flask were determined immediately after completion of the incubation.

### Table III

Relationship between bilirubin formation and carbon monoxide production

The reaction mixture (5.0 ml) consisted of 18,000 X g spleen supernatant (23.5 mg of protein); hemin-3H, 17 μM; NADP, 1.4 mM; glucose 6-phosphate, 4.0 mM; MgCl₂, 1.9 mM; and potassium phosphate buffer (pH 7.4), 90 mM. In the control flasks NADP and glucose 6-phosphate were replaced by 0.1 M potassium phosphate buffer, pH 7.4. The mixtures were incubated at 37° in closed 125-ml Erlenmeyer flasks filled with air for the time indicated. Bilirubin and carbon monoxide formed in each flask were determined immediately after completion of the incubation.

<table>
<thead>
<tr>
<th>Incubation time (min)</th>
<th>NADP and glucose 6-phosphate present</th>
<th>Reaction products formed (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Bilirubin</td>
</tr>
<tr>
<td>5</td>
<td>−</td>
<td>0.3</td>
</tr>
<tr>
<td>20</td>
<td>−</td>
<td>0.4</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>23.6</td>
</tr>
<tr>
<td>5</td>
<td>+</td>
<td>25.0</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>44.7</td>
</tr>
<tr>
<td>10</td>
<td>+</td>
<td>41.2</td>
</tr>
<tr>
<td>20</td>
<td>+</td>
<td>63.8</td>
</tr>
<tr>
<td>30</td>
<td>+</td>
<td>76.7</td>
</tr>
</tbody>
</table>

* Not measurable.

Fig. 2. Reaction rate as a function of pH. Spleen 18,000 X g supernatant was assayed for enzymatic activity in 0.1 M potassium phosphate buffer (O-O) or in 0.1 M Tris-HCl buffer (○-○) under standard conditions as outlined under "Experimental Procedure."

The reaction rate could be increased slightly by addition of small amounts of human serum albumin to the incubation mixture (Fig. 3). In the standard assay system an albumin concentration of 0.3 mg per ml increased the reaction rate by 11%, and of 0.7 mg per ml of 5%. Under standard assay conditions higher albumin concentrations were inhibitory. Albumin concentrations of 1.1 mg per ml reduced the reaction rate by 37%.

With 18,000 X g supernatant of liver or spleen as the enzyme source, the reaction rate was maximal and constant for the initial 15 min of incubation (11). Under these conditions bilirubin production was a linear function of the enzyme concentration up to 3.5 mg of protein per ml of the incubation mixtures (Fig. 4).

In addition to protohemin IX (methemalbumin), several other hemins served as substrates for microsomal heme oxygenase (Table IV), but in all instances the reaction rates were lower. Free porphyrins were not acted upon by the enzyme. Methemoglobin, individual α and β chains of hemoglobin, and hemoglobin-haptoglobin complexes were active as substrates, but the reaction rates were much reduced as compared to protohemin IX (methemalbumin) (Table IV). Preliminary studies suggest that hemopexin-bound hemin may be a suitable substrate for the enzyme; activity in one experiment was 49% of that of protohemin IX. Oxyhemoglobin, carboxyhemoglobin, and myoglobin were inactive. Mesohemin IX, deuterohemin IX, and coprohemin I acted as competitive inhibitors of microsomal heme oxygenase when present in the incubation system together with protohemin IX (Fig. 5). The apparent Kₘ value, calcu-
The microsomal fraction of rat spleen (1.3 mg of protein) was assayed for enzyme activity in the presence of purified biliverdin reductase (Step 5, 15 to 19 pg of protein) under otherwise standard conditions.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activity</th>
<th>Apparent $K_m$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protohemin IX</td>
<td>100</td>
<td>5.0</td>
</tr>
<tr>
<td>Mesohemin IX</td>
<td>75-80</td>
<td>5.1</td>
</tr>
<tr>
<td>Deuterohem IX</td>
<td>24-29</td>
<td>4.7</td>
</tr>
<tr>
<td>Coprohemin I</td>
<td>11-15</td>
<td></td>
</tr>
<tr>
<td>Protoporphyrin IX</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Carboxyhemoglobin</td>
<td>0-2</td>
<td></td>
</tr>
<tr>
<td>Methemoglobin</td>
<td>31-44</td>
<td>4.0</td>
</tr>
<tr>
<td>a chains of hemoglobin</td>
<td>25-29</td>
<td>4.7</td>
</tr>
<tr>
<td>$\beta$ chains of hemoglobin</td>
<td>26-30</td>
<td>4.5</td>
</tr>
<tr>
<td>Hemoglobin-haptoglobin 1-1</td>
<td>6-10</td>
<td></td>
</tr>
<tr>
<td>Hemoglobin-haptoglobin 1-2</td>
<td>6-10</td>
<td></td>
</tr>
<tr>
<td>Myoglobin</td>
<td>0-2</td>
<td></td>
</tr>
<tr>
<td>Mesoporphyrin IX</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Deuteroporphyrin IX</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Coproporphyrin I</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Uroporphyrin I</td>
<td>0</td>
<td></td>
</tr>
<tr>
<td>Uroporphyrin III</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

**TABLE IV**

**Microsomal heme oxygenase: relative activity of various substrates**

Various compounds inhibited microsomal heme oxygenase (Table IV). In concentrations of $10^{-3} \, \text{M}$, the metal-chelating agents EDTA, $\alpha,\alpha'$-dipyridyl, and $\alpha$-phenanthroline inhibited only slightly, while KCN and NaN$_3$ were more powerful inhibitors. $p$-Hydroxymercurobenzoate inhibited strongly, whereas $\beta$-diethylaminoethyl diphenylpropyl acetate-HCl (SKF 525A), animpyrine, and hexobarbital, all of which are inhibitors of the microsomal oxidation of certain drugs, had little effect. Mg$^{++}$ (MgCl$_2$), Mn$^{++}$ (MnCl$_2$), Fe$^{++}$ (FeSO$_4$), Fe$^{++}$ (FeCl$_2$), and Zn$^{++}$ (ZnSO$_4$), when added to the standard incubation mixture in concentration from 1 to $2 \times 10^{-4} \, \text{M}$, had little or no inhibitory effect. Cu$^{++}$ (CuSO$_4$) inhibited 25% at $1 \times 10^{-4} \, \text{M}$ concentration and 91% at $2 \times 10^{-4} \, \text{M}$.

**DISCUSSION**

We have described an enzymatic system for the cleavage of heme at the $\alpha$-methene bridge that results in formation of a linear tetrapyrrole and CO. This enzymatic activity was identified in the microsomal fraction of several mammalian tissues, among which spleen and liver are the most active. The initial reaction product is biliverdin (11), which is rapidly reduced to bilirubin by NADPH-dependent biliverdin reductase (37), present in a 105,000 $\times$ g supernatant fraction of these tissues. In the complete system consisting of an 18,000 $\times$ g supernatant fraction as the enzyme source, biliverdin reductase is not rate-limiting (37).

The enzymatic mechanism presented presently differs in several regards from the model systems previously proposed for the fission of the porphyrin ring. Lemberg (48) suggested a coupled oxidation with ascorbate and molecular oxygen which in vitro was shown to convert pyridine hemochromogen to the bile pigments precursors choleglobin and verdohemochromogen; on acidification small amounts of biliverdin were obtained. Two
major arguments have been raised against this being the principal physiological mechanism of bile pigment formation. (a) Petryka, Nicholson, and Gray (42) reported that this enzymatic procedure results in a mixture of isomeric bile pigments whereas in vivo only the \( \alpha \) isomer is produced. (b) More recently, Nichol and Morrell (49) indicated that enzymatic cleavage of heme is remarkably inefficient and therefore is unlikely to be of physiological importance. Similarly, the system proposed by Nakajima et al. (50) results at least in part in formation of the IX \( \beta (\beta) \) isomer of biliverdin (49). The activity of this system initially was believed to be due to an enzyme, heme \( \alpha \)-methenyl oxygenase (50), but later was shown to be accounted for by the presence of a dialyzable, heat-stable factor functioning as a reducing agent (51, 52). In a preliminary communication, Wise and Dabkin (53) described a light mitochondrial system obtained from the hemogluous organ of the dog placenta that converted hemoglobin to biliverdin. This enzyme system resembled the microsomal heme oxygenase system presently described in being particulate in nature, but differed from it by requiring NAD and ATP. Moreover, because of its localization in an esoteric organ of unknown function, long known to contain biliverdin (54), the relevance of this system to the physiological mechanism of bile pigment formation in the intact organism was uncertain.

The microsomal enzyme system converting heme to bilirubin appears to be a mixed function oxygenase with cytochrome P-450 as the terminal oxidase (55). This conclusion is suggested by the findings that the system has an absolute requirement for NADPH and molecular oxygen (Fig. 1, Table II) and is inhibited by carbon monoxide (Fig. 1); the inhibition by CO is reversible by photodissociation of the cytochrome P-450-CO complex. Moreover, mass spectrometry showed incorporation of atmospheric oxygen into the products bilirubin and CO.\(^2\) The stoichiometry of the reaction is consistent with a mixed function oxidation (Table II). Three moles of oxygen are consumed per mole of bilirubin formed; 1.5 moles are used for the oxidation of the tetrapyrrole including the \( \alpha \)-methene carbon bridge, and 1.5 moles of oxygen are needed to oxidize the NADPH (55). With the complete system consisting of spleen microsomes and purified biliverdin reductase (37), a total of 5 to 6 moles of NADPH were consumed per mole of bilirubin formed (Table II). Four moles of NADPH are probably the minimum required for the formation of bilirubin; the additional 1 to 2 moles of NADPH may represent heme stimulated NADPH consumption via non-bilirubin pathways. Bilirubin and CO are formed in equimolar amounts (Table III). The apparent inhibitory effect of cyanide (Table V) is unrelated to the participation of cytochrome P-450 in the reaction and appears to reflect interaction of cyanide with the substrate, heme (56). This is further demonstrated by the fact that with cyanide competitive kinetics were obtained with heme, but not with microsomes.

Although these findings leave little doubt that the enzymatic mechanism responsible for the conversion of heme to bilirubin is a mixed function oxidation, the findings in Table V indicate distinct dissimilarities between this reaction and the mixed function oxygenase systems involved in the metabolism of drugs and endogenous steroids (9). Since hexobarbital, aminopyrine, and SKF 525A failed to inhibit heme oxygenase (Table V), it is apparent that, despite its microsomal nature, this enzyme system differs from that involved in the microsomal oxidations of many drugs. While such diversity has been observed previously in the metabolism of certain xenobiotics and steroids (9, 57), it is also possible that these differences reflect localization of these enzymatic mechanisms in different cell types. It has long been assumed that conversion of hemoglobin to bile pigment takes place in cells of the reticuloendothelial system (58). Consistent with this concept are the observations that spleen has the highest heme oxygenase activity, that the enzyme is present in macrophages harvested from the peritoneal cavity or the lung, and that the rate of bilirubin formation is enhanced with microsomes obtained from liver, the reticuloendothelial function of which had been stimulated.\(^4\) Thus, it is possible that in the liver, oxidation of drugs and steroids may be predominantly a function of the oxygenase systems of the parenchymal cells, whereas the heme oxygenase system may be localized in the Kupffer cells.

With the crude enzyme preparation used in the standard assay, activity is limited to substrates that permit easy dissociation of the heme group. Thus, in addition to methemalbumin, methemoglobin and isolated \( \alpha \) and \( \beta \) chains of hemoglobin readily serve as substrates (Table IV). By contrast, oxyhemoglobin, carboxyhemoglobin, and myoglobin, in which the heme group is more firmly bound (59), are nearly inactive. As would be expected from its strong interaction (60), haptoglobin-bound hemoglobin is a poor substrate for the isolated heme oxygenase system (Table IV). Since, in vivo, the circulating hemoglobin-haptoglobin complex is removed intact (61), it is apparent that after its sequestration the protein moiety must be altered before its heme may be cleaved to bile pigment.

The paradoxical effects of low and high albumin concentrations on the reaction rate (Fig. 3) probably reflect the binding interaction between heme and albumin at the different ratios used.

Although highest enzyme activity is obtained with protohemin IX, limited substrate activity is observed with other hemes of the IX isomer series as well as with coprohemin I (Table IV). This indicates that heme oxygenase activity is not critically dependent on the nature or sequence of the side chains of the pyrrole rings. On the other hand, no free porphyrin is attacked by the enzyme, suggesting that the central iron atom is indispensable for heme oxygenase activity.

The activity of microsomal heme oxygenase in the spleen and liver, as determined in the present assay system, is in good agreement with the kinetic requirements for hemoglobin turnover in the whole rat organism (11). Moreover, the enzyme is capable of responding with adaptive changes in substrate loads (62). These kinetic properties, the anatomic localization of the enzyme, and the exclusive formation of the \( \alpha \) isomer of bilirubin suggest that microsomal heme oxygenase plays a major role in heme turnover in the intact organism.

**Acknowledgments**—We are indebted to Ruth Poppenhausen for excellent technical assistance, to Peggy Clark for expert secretarial work, to Lydia Hammaker for many helpful suggestions, and to Dr. Neville Pimstone for help in the isomer analyses. Dr. Z. Petryka, University of Minnesota, was kind enough to perform preliminary analysis of the bilirubin isomers. We thank Dr. R. W. Estabrook for his helpful comments and Dr. T. P. Singer for critical review of the manuscript.

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