The Carbon Monoxide-binding Pigment of Liver Microsomes

I. EVIDENCE FOR ITS HEMOPROTEIN NATURE

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The presence in mammalian liver microsomes of a carbon monoxide-binding pigment has been reported by Klingenberg (1) and by Garfinkel (2). The CO compound of the reduced pigment has an intense absorption band at 450 nm and thus can be readily detected in dithionite-treated microsomes by difference spectrophotometry. The CO difference spectrum of reduced microsomes is, however, unusual in that it shows no peak other than that at 450 nm and, therefore, provides no clue to the nature of the pigment. The elucidation of its nature has further been hampered by the reported lability of the microsomal pigment to detergents, low pH, and enzymatic digestion (1, 2). In addition, the CO compound has been reported as not photodissociable (1).

In preliminary communications (3, 4), we have reported evidence for the hemoprotein nature of the microsomal CO-binding pigment, provisionally called “P-450,” and shown that it can be converted into a solubilized form, which we term “P-420,” by treatment of microsomes anaerobically with snake venom or deoxycholate. The solubilization is accompanied by an unusual change in the spectral properties of the pigment. Further, the solubilized pigment has been partly purified, free from cytochrome b₅, and shown to possess absorption spectra characteristic of hemoproteins (5). The present paper gives a detailed account of the investigations on rabbit liver microsomes and crude microsomal digests, which have led us to postulate the hemoprotein nature of the pigment. Purification and properties of the solubilized hemoprotein will be reported in the accompanying paper (6).

EXPERIMENTAL PROCEDURE

Microsomal Preparations—The microsomes used were prepared from rabbit liver by a modification of the method of Mitoma et al. (7). Male rabbits (2.5 to 3 kg) were starved for 24 hours and killed by injecting air into the ear vein. The thoracic cavity was opened, and the liver was thoroughly perfused in situ with more than 200 ml of 0.9% NaCl solution. The liver was excised, finely chopped with a razor, and homogenized with 4 volumes of isotonic (1.15%) KCl solution in a Potter glass homogenizer equipped with a Teflon pestle. The homogenate was centrifuged at 12,000 \( \times g \) for 25 minutes in a refrigerated centrifuge, and the precipitate was discarded. The microsomes were sedimented by centrifugation at 78,000 \( \times g \) for 90 minutes in a Hitachi model 40P preparative ultracentrifuge. The firmly packed pellet of microsomes was resuspended in isotonic KCl solution with the Potter homogenizer and again centrifuged as above. The washed microsomes were finally suspended in isotonic KCl, usually at a concentration of 10 mg of protein per ml. The resultant microsomal suspensions were stored at 4° and used within 2 to 3 days. In these preparations isotonic KCl (ionic strength, about 0.15) was employed, instead of the more usual 0.25 M sucrose, so as to minimize the adsorption of hemoglobin on microsomes (8). The microsomal preparations thus obtained were found to be practically free of adsorbed hemoglobin, when examined by zone electrophoresis as described by Paigen (8). As shown below, the absence of hemoglobin could be confirmed further spectrophotometrically.

Measurements of Difference Spectra—Difference spectra of microsomal preparations were measured in a Cary model 14 spectrophotometer with cuvettes of 1-cm optical path. Microsomal preparations, usually containing 2 mg of protein per ml of 0.1 M phosphate buffer (pH 7.0), were placed in both the sample and reference cells. After recording the baseline, the content of sample cell was treated with various reagents, and the spectral difference thereby induced was measured. When CO was used, it was carefully bubbled through the sample for about 20 seconds; this was sufficient to saturate the sample with the gas. Reduction of samples with dithionite was effected with a few milligrams of solid Na₂S₂O₄. Thunberg-type cuvettes, also of 1-cm optical path, were employed when it was necessary to keep the sample anaerobic, or under a gas mixture of definite composition. Anaerobic conditions were established simply by evacuating the cell, or by replacing the gas phase with oxygen-free nitrogen. In some experiments the sample and reference cells were treated differently, and the difference spectrum was measured. All spectrophotometric measurements were made at room temperature (20°-25°). Further details are given in legends of figures.

Photodissociation of CO Compound of P-450—Microsomes suspended in 0.1 M phosphate buffer, pH 7.0, were placed in a 1-cm square cell and reduced by a few milligrams of solid sodium dithionite. A suitable amount of water saturated with CO was added to the suspension to convert reduced P-450 to the CO compound. The final concentration of CO was calculated from the solubility of CO in water at 20°. The sample cell was then placed in the sample compartment of a Cary model 14 spectrophotometer and illuminated from the side with a 100-watt tungsten lamp through a set of a condenser lens and a red glass filter (Toshiba R-52). The filter transmitted only red light of wave length longer than 520 nm. An interference filter with a sharp transmittance maximum at about 450 nm was inserted between the sample cell and the measuring phototube to absorb the red light and to pass the light from the monochromater (450 nm). The reference cell containing the same suspension was placed in the reference cell compartment without illumination. The photodissociation was measured by following the
absorbance change at 450 μm caused by illumination of the sample with the red light. Control experiments using reduced microsomes were carried out without CO addition to eliminate the effect of any red light leaking through the blue interference filter or blue light leaking through the red glass filter.

Solubilization Treatments—Solubilization of the microsomal CO-binding pigment and cytochrome bs was usually effected with venom of the snake, Trimeresurus flavoviridis, treated with heat as described previously (9). A mixture containing microsomes (3 to 6 mg of protein per ml), 0.1 M Tris buffer (pH 8.5), and 0.1% (in terms of unheated venom) of heated venom was incubated anaerobically in a Thunberg tube at 4° for 15 to 20 hours. Prolonged incubation up to 48 hours was also carried out, with results similar to those obtained on shorter incubation. Unheated snake venom was equally effective in solubilizing the pigments. The hemoproteins could also be effectively solubilized by addition of 0.5% (final concentration) of sodium deoxycholate to the microsomal suspensions in 0.1 M Tris or phosphate buffer under anaerobic conditions (pH 8.0); solubilization was maximal within 10 to 20 minutes at room temperature. Both solubilization treatments resulted in liberation of 70 to 90% of the microsomal protein in a form not sedimentable by centrifugation at 105,000 × g for 60 minutes. Both the CO-binding pigment and cytochrome bs appeared almost quantitatively in the supernatant. As described below, the CO-binding pigment thus solubilized (P-420) possessed spectral properties considerably different from those of the microsomal bound form (P-450).

Analytical Procedures—The protoheme content of microsomes was measured spectrophotometrically after converting the heme into pyridine-hemochromogen in the presence of 0.1 N NaOH and 20% pyridine. In computing the concentration, a value of 32.4 cm⁻¹ mm⁻¹ was used for the difference in molar extinction between 557 and 575 μm in the dithionite-reduced minus oxidized difference spectrum of the hemochromogen. This value was obtained from the molar extinction coefficient of reduced pyridine protohemochromogen (E₅₇₅ mₜ = 34.7 cm⁻¹ mm⁻¹) reported by Paul, Theorell, and Akesson (10) and the dithionite-reduced minus oxidized difference spectrum of pyridine-hemochromogen prepared from crystalline hemin chloride. Cytochrome bs was determined from the difference spectrum between NADH-reduced and air-saturated microsomes (Fig. 7, Curve A). As will be shown, in NADH-reduced microsomes P-450 was mostly in the oxidized form whereas cytochrome bs was in the reduced form. The increment of molar extinction between 424 and 400 μm in this difference spectrum was assumed to be 185 cm⁻¹ mm⁻¹. This value was obtained with a purified preparation of cytochrome bs isolated in this laboratory1 and is different from those reported previously (1). Total iron was analyzed colorimetrically with α-phenanthroline as the reagent after digesting the samples with boiling sulfuric acid (11). Protein was determined by the method of Lowry et al. (12) with bovine serum albumin as the standard.

Chemicals—NAD and NADP were purchased from the Sigma Chemical Company. NADH was prepared from NAD by the procedure described by Bonneisen (13), but yeast alcohol dehydrogenase, purified according to Racker (14), was used instead of the liver enzyme. NADPH was prepared from NADP by the isocitrate dehydrogenase method (15). CO was prepared from formic acid and concentrated sulfuric acid by the conventional method, and purified by bubbling through a KOH solution. Ethyl isocyanide was synthesized from silver cyanide and ethyl iodide and redistilled twice (16). Lyophilized venom of Trimeresurus flavoviridis collected in 1960 on Amami Oshima Island, Kyushu, Japan was used.

RESULTS

Carbon Monoxide Difference Spectra of Microsomes—The addition of dithionite to liver microsomes produced, as previously observed (1, 2), a spectral change that could be mostly, if not entirely, accounted for by the reduction of cytochrome bs and possibly also of flavins. There was scarcely any sign of the presence of any other reducible pigment. In confirmation of previous work (1, 2), however, a difference spectrum with a peak at 450 μm appeared when CO was bubbled into the dithionite-treated microsomes (Fig. 1, Curve A) indicating the presence of P-450, the microsomal CO-binding pigment. Unlike usual hemoprotein compounds, this pigment showed no peaks in the CO difference spectrum between 500 and 650 μm; the only feature in this region was a broad and shallow trough around 500 μm. The addition of CO to air-saturated microsomes, on the other hand, induced no significant change in spectrum (Fig. 1, Curve B) indicating that P-450 was a reducible pigment and only the reduced form could bind CO (cf. (2)).

The spectra shown in Fig. 1 indicated further that the microsomes used were virtually free from hemoglobin and methemoglobin, because neither peak nor shoulder could be seen at 420 μm where a sharp absorption maximum of carboxyhemoglobin was to be expected. Because cytochrome bs does not combine with CO (17), it was evident that P-450 was not this cytochrome.

The peak at 450 μm was lowered gradually when the dithionite-treated sample in a CO-filled cuvette was evacuated, but resumed the original height on readmission of CO, which indicated reversibility of CO binding. The reaction of CO with reduced microsomes was slow (1); a preliminary value for the first order velocity constant was determined to be 5 min⁻¹. This may be compared with a t₅₀ value of 10 seconds (corresponding to a velocity constant of 4.2 min⁻¹) reported for the CO-binding reaction of rat liver microsomes (1).

As shown in Fig. 2, the CO 450-μm band intensity diminished

![Fig. 1. Carbon monoxide difference spectra of liver microsomes. Curve A, dithionite-reduced microsomes. Both the sample and reference cells contained a microsomal suspension (2 mg of protein per ml, 0.1 M phosphate, pH 7.0) that had been treated with dithionite. The content of the sample cell was then saturated with CO, and the difference spectrum was measured after several minutes. Curve B, aerobic microsomes. The difference spectrum was measured in the absence of dithionite.](image-url)

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1 K. Hirota and T. Omura, unpublished observations.
ethyl isocyanide. In addition to typical \(\alpha, \beta\), and Soret peaks at 560, 530, and 430 nm, respectively. As seen in Fig. 4, however, the bands at 430 and 455 nm diminished in the same proportion when the concentration of ethyl isocyanide was decreased. It was therefore concluded that one hemoprotein was responsible for the two absorption peaks.

Fig. 4 also permitted an estimation of the affinity of ethyl isocyanide for P-450. Both the sample and reference cells contained a microsomal suspension (2 mg of protein per ml, 0.1 M phosphate, pH 7.0) that had been reduced by dithionite. The content of the sample cell was saturated with CO by bubbling the gas for 2 minutes, and then treated with ethyl isocyanide. The difference spectrum was measured after about 2 minutes. During this equilibration time, CO was occasionally bubbled into the sample cell to secure the saturation of the gas. The final concentration of ethyl isocyanide used was as follows: Curve A, 0 (CO difference spectrum); Curve B, 0.3 mM; Curve C, 3 mM; Curve D, 1 mM (ethyl isocyanide difference spectrum). Curve D was measured in the absence of CO. The concentration of CO in the saturated solution was assumed to be 1 mM.
isocyanide for the microsomal pigment. Thus, the concentra-
tion of unbound isocyanide in the presence of half-saturated pigment was determined to be 1 μM, a value smaller than that reported for hemoglobin (19).

**Competition between Carbon Monoxide and Ethyl Isocyanide**—Fig. 5 and Table I indicate that there was competition between CO and ethyl isocyanide for dithionite-reduced microsomes. Hence, it was concluded that the microsomal CO- and ethyl-isocyanide-binding pigments were identical with each other. This, in turn, suggested that P-450 was a hemoprotein, although the spectrum of its CO compound was unusual for a hemoprotein.

The competition data, coupled with the dissociation constant of the isocyanide compound, permitted further an estimation of the affinity of CO for reduced P-450. The dissociation constant of the CO compound of P-450 was thus determined to be 2 μM. This value was in agreement with that obtained above from the effect of CO concentration on the development of the 450-mu band (Fig. 2).

**Reduction and Oxidation of P-450**—Microsomal P-450 was reported to be reduced incompletely by NADH, based on partial appearance of the 450-mu band of CO (1, 2). In a CO-O₂ atmosphere (80:20, by volume), the 450-mu band of NADH-treated microsomes was in fact only 10 to 30% as high as that attainable by dithionite treatment. As seen in Fig. 6, however, the band could be intensified to the dithionite level if the gas phase was replaced by pure CO (or a CO-N₂ mixture). When the gas phase was again replaced by the CO-O₂ mixture, the intensified band returned quickly to the low level. Similar changes were also observed in the presence of NADPH. These findings suggested that P-450 was in fact fully reducible by both NADH and NADPH under anaerobic conditions, but was rapidly reoxidizable on exposure to oxygen.

Experiments with CO-O₂ mixtures of varying composition further indicated that at a given partial pressure of oxygen a steady state was established with regard to the reduction and reoxidation of P-450. However, the extent of reduction in a steady state could not be determined accurately, because the reduction of P-450 was detectable only indirectly by the absorption of its CO compound which was in equilibrium with the reduced pigment. In view of the very high affinity of CO for reduced P-450, the true extent of reduction may not be very large as compared with the value estimated from the CO band, e.g. 10 to 30% in the presence of 20 volume % of oxygen.

In contrast to P-450, cytochrome b₅ in microsomes could be reduced almost fully by both NADH and NADPH and maintained in the reduced state even in the presence of dissolved oxygen (20). Cytochrome b₅ was also reduced partly by ascorbate and cysteine, whereas these reagents failed to reduce P-450 even in the absence of oxygen. This was in contrast to Garfinkel's finding (2) and suggested an oxidation potential for P-450 lower than that of cytochrome b₅.

**Spectral Difference between Dithionite- and NADH-reduced Microsomes**—As stated above, the addition of NADH to microsomes under aerobic conditions resulted in almost full reduction of cytochrome b₅, but left P-450 mostly in the oxidized state. Accordingly, the spectral change thereby induced (Fig. 7, Curve...
A) was nearly identical with the reduced minus oxidized difference spectrum of purified cytochrome b$_5$. On addition of dithionite, on the other hand, both cytochrome b$_5$ and P-450 were fully reduced. The spectral difference between dithionite- and NADH-reduced microsomes, therefore, should have produced a spectrum attributable to the difference between the reduced and oxidized forms of P-450. Curve B in Fig. 7 shows the difference spectrum thus obtained. This spectrum closely resembled that reported for rat liver microsomes (1) and showed two broad peaks around 550 and 440 nm. Although Klingenberg (1) attributed these peaks to the presence of methemoglobin, the microsomes used here were free from both hemoglobin and methemoglobin as already mentioned. It was more likely that this spectrum was equivalent, at least partly, to the spectral difference between the reduced and oxidized forms of P-450. It was, however, rather ambiguous in shape and therefore provided little information concerning the nature of the pigment.

**Spectral Changes Induced by Solubilization Treatments**—It was reported that treatment of microsomes with bile salts, digitonin, or pancreatin either destroyed or greatly decreased the CO-binding capacity (1, 2). Klingenberg (1) further observed a very slow formation of a "CO-protophemochromogen" on addition of CO to cholate-treated microsomes. Such lability of the CO-binding capacity to solubilization treatments was confirmed in the present study. Thus, no peak appeared at 450 nm in the CO-difference spectrum of microsomes after they had been treated with 0.5% sodium deoxycholate (pH 8.0, 10 minutes, 30°) or with 0.1% heated venom of T. flavoviridis (pH 8.5, 15 hours, 4°). However, it was noticed that the treated microsomes showed a feeble CO difference spectrum with a small peak at 420 nm and a trough at 435 nm. This CO spectrum might have been the same one which Klingenberg had attributed to a "CO-protophemochromogen," although its formation was not very slow.

It was found that this feeble CO spectrum was greatly intensified by carrying out the solubilization treatments under anaerobic conditions. A still further intensification of the spectrum occurred when anaerobically treated microsomes were first saturated with CO and then reduced by dithionite, as compared with the sample which had been first reduced by dithionite and then subjected to CO bubbling. Curve B in Fig. 8 shows the CO difference spectrum, measured with the precautions cited, of the microsomes digested anaerobically with heated snake venom. The corresponding spectrum of untreated microsomes (Curve A) is included in the figure to emphasize the profound spectral change induced by the solubilization treatment. As can be seen, the CO spectrum of digested microsomes showed, in addition to the intense peak at 420 nm and the deep trough at 435 nm, two small peaks at 540 and 575 nm and a trough at 560 nm. It was clear from the shape of spectrum that a hemoprotein was responsible for the CO binding in digested microsomes. This hemoprotein was called "P-420." P-420 as a Solubilized Form of P-450—Although the CO spectrum of digested microsomes resembled closely that of hemoglobin, it was unlikely that the microsomes employed were contaminated by detectable amounts of the blood pigment, as repeatedly mentioned. The possibility was also excluded that P-420 was a denatured product of cytochrome b$_5$, because a purified preparation of the cytochrome acquired no CO-binding capacity even after prolonged treatment with heated venom. Instead, it seemed very likely that P-420 had been formed at the expense of P-450, because the emergence of the 420-nm peak in the CO spectrum was accompanied by the disappearance of the 450-nm peak. This possibility was in fact confirmed by a quantitative experiment shown in Fig. 9 and Table II. It may be seen that the amount of P-450 which disappeared in the course of the venom treatment was always proportional to that of P-420 which appeared. This could be explained by assuming that P-420 was a product of P-450, although their spectral properties differed considerably from each other. Similar parallelism was also observed with deoxycholate as the solubilizing agent.
When the microsomal digests were centrifuged at 105,000 × g for 60 minutes, both P-420 and cytochrome b₅ were recovered in the supernatant almost quantitatively. This indicated further that P-420 was in fact a solubilized form of P-450.

Properties of P-420—As suggested by the intensification of the 420-mu band of CO by solubilisation under anaerobic conditions, the microsomal CO-binding pigment was labile when solubilized in the presence of oxygen. But once solubilized anaerobically and converted to P-420, it became more stable to aeration. P-420 was, however, extremely labile to oxygen in the presence of dithionite. Anaerobic conditions were, therefore, required for the reduction of P-420. For this reason, the CO spectrum of anaerobically digested microsomes had been intensified by first saturation the sample with CO and then reducing by dithionite.

P-420, when anaerobically reduced by dithionite, combined also with ethyl isocyanide to give a difference spectrum illustrated in Fig. 10. This spectrum with an intense Soret peak at 433 mu again differed substantially from the corresponding spectrum of P-450 (Fig. 3). The solubilized pigment in microsomal digests could be reduced slowly by both NADH and NADPH under anaerobic conditions, as evidenced by the appearance of the 420-mu band in the presence of CO. Like P-450, however, the reduced pigment could be very rapidly reoxidized by molecular oxygen. Only a small portion of P-420 seemed to be in the reduced form in the presence of air. Cytochrome b₅ in the digests, on the other hand, could be rapidly and fully reduced by reduced pyridine nucleotides and maintained in the reduced state even in the presence of oxygen.

Cytochrome Nature of P-420—As in the case of P-450, the difference in the oxidation-reduction behaviors of P-420 and cytochrome b₅ in microsomal digests permitted the measurement of a reduced minus oxidized difference spectrum of P-420 itself. When digested microsomes were kept anaerobic in the presence of dithionite, both P-420 and cytochrome b₅ were fully reduced. When NADH was added to the preparation under aerobic conditions, only cytochrome b₅ was fully reduced and P-420 remained mostly oxidized. The spectral difference between these two samples should, therefore, have been equivalent to a reduced minus oxidized difference spectrum of P-420, ignoring the contribution of flavins to the spectrum. The resultant difference spectrum, shown in Fig. 11, was that of a typical hemochromogen with α, β, and Soret peaks at 500, 530, and 427 mu, respectively, and closely similar to that of bacterial cytochrome b₅ (21, 22). This finding, together with the fact that all the heme in microsomes could be extracted with acetic-acetone, strongly suggested that this pigment was a cytochrome of the b type.

Conversion of P-450 into P-420 by treatment of microsomes with heated snake venom

The experimental conditions were the same as specified in the legend of Fig. 9. Four out of the six experiments recorded correspond to Curves A, B, C, and D in Fig. 9. The percentages of CO-binding pigment in the forms of P-450 and P-420 were calculated from the absorbance increments at 450 and 420 mu, respectively.

<table>
<thead>
<tr>
<th>Incubation time</th>
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<tr>
<td></td>
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can be ascribed to the difference between the reduced and oxidized forms of P-420 (Fig. 11) is not like those of hemoglobin and methemoglobin in the microsomes used can be ruled out, because purified cytochrome bs acquired no CO-binding capacity even after prolonged treatment with heated venom, and the microsomal content of NADH-reducible cytochrome b6 did not change detectably during the solubilization treatment.* Therefore, it may be concluded that P-420 (hence also P-450) is a new hemoprotein.

Table III
Composition of rabbit liver microsomes

<table>
<thead>
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<th>Content</th>
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<td>Protoheme</td>
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<tr>
<td>Cytochrome b6</td>
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</tr>
<tr>
<td>P-450</td>
<td>1.55</td>
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<tr>
<td>Total iron</td>
<td>140*</td>
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</table>

* Millimicromoles per mg of protein.

It is probable that most of the nonheme iron is due to the presence of ferritin in the microsomal preparations (24). The content of P-450 was roughly associated with cytochrome b6, and P-450 in different preparations were fairly constant. Finally, the microsomes contained large amounts of nonheme iron, the content of which varied considerably from one preparation to the other. It is of interest to note that the content of P-450 was roughly equal to that of protoheme which was present in excess of the amount associated with cytochrome b6. It was also noticed that the contents of total heme, cytochrome b6, and P-450 in different preparations were fairly constant.

The absorption spectra of P-450 are uncommon for a hemoprotein.

Table III
Composition of rabbit liver microsomes

Average of 40 determinations on microsomes obtained from different rabbits. Figures indicate the contents in millimicromoles per mg of microsomal protein.

The absorption spectra of P-450 are uncommon for a hemo- protein and differ considerably from those of P-420. The fact that the conversion of P-450 into P-420 is effected by solubilizing agents capable of attacking phospholipids suggests that microsomal phospholipids may be responsible for the peculiar spectral properties of P-450. It seems that P-450 is embedded in microsomal lipids. However, further studies are needed before one can be sure of the state of P-450 in microsomes. Conversely, such unusual spectra of P-450 seem to provide an interesting problem in the physical chemistry of hemoproteins. No hemoproteins have so far been shown to change their spectra considerably on liberation from the structures to which they are bound in vivo.

Other variant hemoproteins are known. An example is the Rhodospirillum rubrum hemoprotein (25), which contains 2 moles of heme per mole and is related to a cytochrome of the c type (26, 27). Unlike usual c-type cytochromes, reduced RHP shows a spectrum with only a broad band in the α- and β-band region, and the Soret band of the oxidized form is not markedly intensified on reduction. In the reduced form it binds CO and this binding results in a marked intensification of the Soret peak. Under alkaline conditions, this protein shows profound changes in its spectra and the reduced form shows a spectrum characteristic of c-type cytochromes. Cryptocytochrome c, recently purified and crystallized from Pseudomonas denitrificans, also has properties similar to those of RHP (28).

Klingenberg (1) reported the failure of his attempts to induce photodissociation of the microsomal CO addition compound. In the present study it was also unsuccessful to observe the photodissociation of the CO compound of P-450 under conditions in which light showed a detectable effect on CO-hemoglobin. Since all the CO compounds of hemoproteins so far known, including that of RHP, are dissociable in the light, these findings might be regarded as evidence against the hemoprotein nature of P-450. However, all the other evidence reported in this paper seems to indicate rather decisively that the pigment is in fact a hemoprotein and, as will be reported later (6), a highly purified preparation of its solubilized form (P-420) did yield protoheme on treatment with acid-acetone. It seems, therefore, possible that the photodissociation can be detected by improving the experimental conditions such as light intensity. Recently, Estabrook, Cooper, and Rosenthal (29) have reported that CO inhibits the steroid C-21 hydroxylation reaction in adrenal cortex microsomes and that this inhibition is reversed by light. They have further found that the photochemical action spectrum obtained by examining the effect of light of various wave lengths in reversing the CO inhibition shows a maximum at 450 μm. Since the presence of P-450 in adrenal cortex microsomes has been confirmed (29), these findings indicate the photodissociability of the CO compound of adrenal P-450, as well as the involvement of the pigment in the steroid C-21 hydroxylase system.

The microsomal CO-binding pigment is labile when solubilized under aerobic conditions, and the solubilized pigment (P-420) is extremely unstable to oxygen in the presence of dithionite. Such instability of the pigment appears to be related to aerobic peroxidation of microsomal unsaturated lipids (30). It is likely that the peroxidation is accompanied by oxidative decomposition of the heme moiety of P-420. It was in fact found that P-420, when further purified, becomes more stable to oxygen even in the presence of dithionite, probably owing to removal of an unknown contaminant by the purification procedure (31). The microsomal CO-binding pigment is labile when solubilized under aerobic conditions, and the solubilized pigment (P-420) is extremely unstable to oxygen in the presence of dithionite. Such instability of the pigment appears to be related to aerobic peroxidation of microsomal unsaturated lipids (30). It is likely that the peroxidation is accompanied by oxidative decomposition of the heme moiety of P-420. It was in fact found that P-420, when further purified, becomes more stable to oxygen even in the presence of dithionite, probably owing to removal of an unknown contaminant by the purification procedure (31).
associated lipid constituents (5). In contrast to P-420, cytochrome $b_5$ is not affected by oxygen under all the conditions examined. This appears to reflect the difference in the state of protoheme in the two microsomal hemoproteins.

It confirmation of previous work (1, 2, 23), it was found that liver microsomes contained protoporphine in excess of the amount associated with cytochrome $b_5$. As is evident from Table III, it seems that most of the extra heme is present in the form of the CO-binding pigment. There is no spectral evidence suggesting the occurrence of any hemoproteins other than cytochrome $b_5$ and P-450. The microsomal preparations used were, however, found to possess a weak activity of catalase, but no peroxidative activity could be detected.

Microsomal bound P-450 is reducible by both NADH and NADPH. As will be reported later (6), however, purified P-420 is not reducible by both NADH and NADPH. The addition of purified NADH-cytochrome $b_5$ reductase (31) in the presence of NADPH also fails to reduce the purified pigment. It is likely that the reduction of P-450 by NADH and NADPH is catalyzed by specific reductases. Reduced P-450, on the other hand, is rapidly reoxidizable by oxygen, and neither the reduction nor reoxidation are influenced by cyanide. These findings indicate that P-450 can mediate aerobic oxidation of both NADH and NADPH by liver microsomes. It has in fact been reported that liver microsomes catalyze slow cyanide-insensitive oxidation of reduced pyridine nucleotides by oxygen (32, 33).

In rabbit liver microsomes, the rate of NADH oxidation has been reported to be 1 to 2 mmoles per minute per mg of protein and that of NADPH oxidation to be 3 to 5 mmoles per minute per mg of protein at 20° and pH 6.5 (34, 35). It has further been shown that the slow autoxidation of microsomal bound cytochrome $b_5$ can account for only 30% or less of the microsomal oxidation of reduced pyridine nucleotides (36). The rest of oxidation, therefore, seems to be catalyzed by a mechanism involving P-450. It is, however, dubious to assign an important role to this slow oxidative activity of P-450. As mentioned above, Estabrook et al. (29) have reported evidence suggesting that P-450 in adrenal cortex microsomes functions in oxygen activation for the steroid C-21 hydroxylase reaction. It appears therefore likely that P-450 in liver microsomes is also functioning in similar mixed function oxidase reactions such as hydroxylation and N-demethylation of foreign aromatic compounds (7, 9, 37).

Hashimoto, Yamano, and Mason (38) have further found by electron spin resonance spectroscopy that liver microsomes contain an entity called "microsomal Fe", which is capable of alternate reduction and oxidation. The properties reported for this entity, which has not yet been solubilized, seem to be similar to those of P-450.

The spectrum representing the difference between the reduced and oxidized forms of P-420 (Fig. 11) is similar to that of bacterial cytochrome $b_5$ (21, 22) and suggests that this pigment is a cytochrome of the $b$ type. In agreement with this, protoporphine is the only heme present in microsomes and the microsomal heme can be extracted with acid-acetone. Moreover, the fact that P-420 is capable of undergoing reversible reduction and oxidation qualifies the pigment as a cytochrome. However, no $b$-type cytochromes so far known have been reported to combine with CO.

The occurrence in liver microsomes of two hemoproteins, P-450 and cytochrome $b_5$, may suggest that there is an interaction between them in the microsomal electron transfer mechanism. Two observations, however, appear to provide evidence against this possibility. First, microsomal bound cytochrome $b_5$ is partly reducible by ascorbate, whereas this reagent is completely incapable of reducing P-450 even in the absence of oxygen. The flow of electrons from cytochrome $b_5$ to P-450 seems to be improbable from these findings. Secondly, the oxidation of reduced P-450 by molecular oxygen proceeds much faster than that of cytochrome $b_5$. This latter fact seems to exclude the possibility that oxidation of reduced P-450 takes place by way of cytochrome $b_5$. Thus the two hemoproteins appear to behave independently in oxidative functions, though they are present in the same subcellular fraction of liver.

**SUMMARY**

Spectrophotometric evidence is presented that "P-450," the CO-binding pigment, in rabbit liver microsomes is a new hemoprotein with unusual properties. Microsomal bound P-450, when reduced, binds CO and ethyl isocyanide. Although the CO difference spectrum of the reduced pigment is unusual for a hemoprotein, the isocyanide difference spectrum is characteristic of a hemoprotein compound. In microsomes P-450 is reducible by both NADH and NADPH under anaerobic conditions, but the reduced form is very rapidly reoxidizable in the presence of molecular oxygen.

When microsomes are incubated anaerobically with heated snake venom or with deoxycholate, the pigment is converted to a solubilized form called "P-420." The solubilization is, however, accompanied by profound changes in the spectral properties of the pigment. The CO compound of reduced P-420 shows a difference spectrum characterized by an intense Soret peak at 420 mμ. P-430 is labile to aeration, especially in the presence of dithionite. In microsomal digests it can be reduced by NADH and NADPH in the absence of oxygen, but most of the pigment is maintained in the oxidized form under aerobic conditions even in the presence of NADH or NADPH. Cytochrome $b_5$ in the digests, on the other hand, is fully reduced under comparable conditions. Taking advantage of such difference in oxidation-reduction properties of P-420 and cytochrome $b_5$, it is possible to measure a reduced minus oxidized difference spectrum of P-420 alone. The spectrum thus obtained suggests that P-420 is a cytochrome of the $b$ type.

Liver microsomes contain protoporphine in excess of the amount associated with cytochrome $b_5$. Most of the extra heme seems to be associated with the CO-binding pigment.

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Microsomal CO-binding Pigments. I