Biphasic Decrease of Radioactive Hemoprotein from Liver Microsomal CO-binding Particles

EFFECT OF 3-METHYLCHOLANTHRENE*

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SUMMARY

The administration of δ-aminolevulinic acid-3,5-3H to immature male rats leads to the incorporation of radioactivity into the hemoprotein contained in the "CO-binding particles" (steapsin-treated microsomes which do not contain cytochrome b5).

A biphasic decrease in the radioactivity incorporated into the hemoprotein in both control and 3-methylcholanthrene-treated rats was observed. Half-life determinations of the fast phase component (7 to 8 hours) and the slow phase component (46 to 48 hours) indicated that 3-methylcholanthrene treatment did not change the half-life of either hemoprotein fraction but did change the proportion of the fast phase component to the slow phase component from 3.8:1 to 1:1. Determination of the heme content of the CO-binding particles has indicated that treatment of rats with 3-methylcholanthrene leads to a 3- to 4-fold increase in the slow phase component, while not affecting the concentration of the fast phase component.

A microsomal hemoprotein has been implicated as the terminal oxidase of the enzyme system in the liver which hydroxylates chemical carcinogens, drugs, and steroids. This microsomal hemoprotein, in the reduced form, combines with CO to give a characteristic spectral peak at 450 nm and has been called cytochrome P448 or the CO-binding pigment (1,2). Early studies from Estabrook's laboratory (3) led to the proposal that this microsomal hemoprotein was the common oxygen-activating enzyme for a broad spectrum of monooxygenase-type reactions. However, studies on the hydroxylation in vitro of testosterone at three different positions on the steroid nucleus have provided evidence which suggests that one microsomal hemoprotein cannot alone impart specificity for all three hydroxylation reactions (4-10). In addition, when ethylisocyanide instead of carbon monoxide is used as the ligand for the reduced hemoprotein, two Soret peaks are observed at 430 nm and 455 nm (11). Sladek and Mani inger (12) have shown that the administration of phenobarbital to rats increased the absorption at 455 nm and 430 nm proportionately, while 3-methylcholanthrene treatment preferentially increased the absorption at 455 nm. These investigators, therefore, suggested that 3-MC caused the formation of a new hemoprotein which they called cytochrome P450. Recent studies have shown further spectral changes in the hemoprotein (or hemoproteins) after 3-MC treatment. The reduced hemoprotein in liver microsomes from 3-methylcholanthrene-treated rats, in combination with (3), has an absorption maximum at 448 nm instead of at 450 nm (6,13-15). Hildebrandt, Remmer, and Estabrook (16) obtained similar results in rabbits and found a different extinction coefficient for the microsomal hemoprotein after treatment in vivo with 3-MC. Evidence has been presented which suggests that protein synthesis is necessary both for the change in maximum absorption seen in the CO spectrum and for the change in the ratio of the ethylisocyanide spectral peaks following 3-MC treatment (6,13-15).

To learn more about the characteristics of the microsomal hemoprotein or hemoproteins which are responsible for the hydroxylation of drugs and steroids, 3H-δ-ALA has been used to label the heme portion of the cytochrome. The advantage of using 3H-δ-ALA to label hemoprotein is that the hemoprotein would be labeled in the heme moiety which is converted nearly quantitatively to bile pigment in the rat and excreted without reutilization (17,18). Studies by Levin and Kuntzman (19) demonstrated that the radioactivity incorporated into the heme moiety of microsomal hemoprotein after 3-MC treatment decreased at a slower rate than did the radioactivity incorporated into the hemoprotein of control or phenobarbital-treated rats. These studies have been extended and the results presented here show directly the existence of two hemoprotein fractions in the "CO-binding particles" of normal rats and indicate that treatment of rats with 3-MC alters the ratio of these two hemoprotein fractions.

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1 The abbreviations used are: 3-MC, 3-methylcholanthrene; 3H-δ-ALA, δ-aminolevulinic acid-3,5-3H.
Immature male rats (60 to 70 g) of the Long Evans strain were treated with a commercial diet and water ad libitum. 3-Methylcholanthrene was dissolved in corn oil and 25 mg of the polycyclic hydrocarbon per kg per day were administered intraperitoneally for 3 days. Control rats received corn oil.

δ-Aminolevulinic acid-3,5-3H (510 mCi per mmole; New England Nuclear) was injected intravenously in 0.9% NaCl solution, and the animals were killed at various times after the injection. Liver microsomes (microsome I) were prepared from 33% homogenates in 0.25 M sucrose as previously described (20). The microsomal pellets were washed and resuspended in 0.15 M KCl twice (microsomes II and III) to ensure complete removal of the soluble fraction. The final microsomal pellets were layered with 3 ml of 0.1 M KH2PO4-K2HPO4, pH 7.4, and stored at -15°C until used.

Immediately before use, the microsomal pellets were thawed at room temperature and suspended in potassium phosphate buffer to a concentration equivalent to 250 mg of liver, wet weight, per ml. In addition to the CO-binding pigment or pigments, liver microsomes contain another hemoprotein, cytochrome b5, and Omura and Sato (21, 22) have shown that this hemoprotein can be selectively solubilized by treatment of microsomes with steapsin. Microsomal suspensions (equivalent to 250 mg of liver, wet weight, per ml) were incubated with 0.2% steapsin (Nutritional Biochemicals) for 1 hour at 37°C under nitrogen and then centrifuged at 105,000 X g for 2 hours as described by Omura and Sato (22). The pellet thus obtained was termed the "CO-binding particles" by Omura and Sato (21, 22) because of its high content of the CO-binding hemoprotein and because this cytochrome was the only spectrally observed hemoprotein in these particles.

The CO-binding particles were resuspended in 0.1 M KH2PO4-K2HPO4 buffer, pH 7.4; the radioactivity in 0.2 ml of the resuspended particles was quantified in a liquid scintillation spectrometer with the scintillation mixture of Bray (23); and the protein content was determined by the method of Sutherland et al. (24). Microsomal hemoproteins were determined as described by Omura and Sato (21, 22), with an Amino-Chance dual wave length/split beam spectrophotometer.

The protoheme content of the CO-binding particles was measured after converting the heme into pyridine-hemochromagen as described by Omura and Sato (21). Cleavage of the heme-protein linkage was accomplished with the use of acid-acetone (23) or methyl ethyl ketone (20).

**RESULTS**

Subcellular Distribution of Radioactivity in Liver after Administration of 3H-δ-ALA to Rats—Immature male rats were treated by intravenous injection with 0.234 mg of 3H-δ-ALA per kg, and the animals were killed 2 hours later. Fig. 1 shows the distribution of total radioactivity in the various subcellular fractions of the liver. The percentage shown in parentheses indicates the subcellular distribution of the total radioactivity found in the liver. Recentrifugation of the 11,000 X g supernatant did not lead to any further removal of radioactivity. Of the radioactivity found in the 11,000 X g pellet, 84% could be removed at 600 X g, indicating that it was associated with nuclei and cell debris. As can be seen, the specific radioactivity in the microsomes increases through successive washes (microsomes I and II) until a constant specific activity is reached (microsome III).

Solubilization of Cytochrome b5—In addition to the CO-binding cytochrome, microsomes also contain another hemoprotein, cytochrome b5, and Omura and Sato (22) have shown that the incubation of rabbit liver microsomes with 0.2% steapsin at 37°C for 1 hour under nitrogen leads to the selective solubilization of cytochrome b5 along with 40 to 50% of the total microsomal protein. The pellet thus obtained on centrifugation for 2 hours at 105,000 X g contains only the CO-binding pigment, present mostly as cytochrome P420 (formed from P450 when microsomes are treated with detergents or lipases). Omura and Sato (22) termed this pellet "CO-binding particles" because of its high content of the CO-binding hemoprotein and because this cytochrome was the only spectrally observable hemoprotein in these particles.

FIG. 1. Subcellular distribution of radioactivity after the administration of 3H-δ-ALA. Male rats (60 to 70 g) were injected intravenously with 0.234 mg of δ-aminolevulinic acid-3,5-3H per kg and killed 2 hours later. Livers were homogenized and fractionated as described under "Methods." Percentage values in parentheses represent the distribution of the total radioactivity present in the whole homogenate. Values were obtained from the pooled livers of 12 rats.

The CO-binding particles were resuspended in 0.1 M KH2PO4-K2HPO4 buffer, pH 7.4; the radioactivity in 0.2 ml of the resuspended particles was quantified in a liquid scintillation spectrometer with the scintillation mixture of Bray (23); and the protein content was determined by the method of Sutherland et al. (24). Microsomal hemoproteins were determined as described by Omura and Sato (21, 22), with an Amino-Chance dual wave length/split beam spectrophotometer.

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**Incorporation of Radioactivity into Heme Moiety of Hemoprotein in CO-binding Particles**—The evidence which indicates that...
the radioactivity of the $^3$H-$\alpha$-ALA has been incorporated into the heme portion of a hemoprotein in both control and 3-MC-treated rats at both 2 and 72 hours after the administration of $^3$H-$\alpha$-ALA is shown in Table I. Addition of cold trichloracetic acid to the CO-binding particles led to the precipitation of the protein and 99% of the radioactivity, indicating that the radioactivity was associated with protein. Since treatment of a hemoprotein with cold acid-acetone is known to solubilize the heme moiety and precipitate the protein (25, 27, 28), the CO-binding particles were treated with acid-acetone. Over 99% of the radioactivity was solubilized, indicating that the radioactivity was in the heme moiety. Methyl ethyl ketone has also been used to split and extract the heme from a hemoprotein (26, 29), and this pro-

<table>
<thead>
<tr>
<th>Treatment of rats</th>
<th>Treatment of CO-binding particles</th>
<th>Radioactivity in supernatant or extracted</th>
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<tr>
<td></td>
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<td>2 hrs after $^3$H-$\alpha$-ALA</td>
</tr>
<tr>
<td>Corn oil</td>
<td>7% trichloracetic acid$^a$</td>
<td>1</td>
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<tr>
<td>3-MC</td>
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<td>1</td>
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<tr>
<td>Corn oil</td>
<td>Acid-acetone extraction$^b$</td>
<td>99</td>
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<td>3-MC</td>
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<tr>
<td>Corn oil</td>
<td>Methyl ethyl ketone extraction$^c$</td>
<td>93</td>
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<tr>
<td>3-MC</td>
<td></td>
<td>103</td>
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$^a$ An ice-cold 50% trichloracetic acid solution was added to CO-binding particles (500 mg of liver per ml) to give a final concentration of 7% trichloracetic acid; the samples were centrifuged at 1000 $\times$ g for 5 min, and the radioactivity in the supernatant was quantified.

$^b$ Ten volumes of ice-cold acid-acetone (25) were added slowly with stirring to CO-binding particles (equivalent to 500 mg of liver per ml). The samples were centrifuged at 1000 $\times$ g for 5 min, and the radioactivity in the supernatant was quantified.

$^c$ Two volumes of methyl ethyl ketone were added to CO-binding particles (equivalent to 500 mg of liver per ml), and the samples were mixed for a short period. After 1 hour the samples were centrifuged at 1000 $\times$ g for 5 min, and radioactivity in the organic phase was quantified.

Incorporation of Radioactivity into CO-binding Particle—The incorporation of $^3$H-$\alpha$-ALA into the CO-binding particles is shown in Fig. 4. 3-MC (25 mg per kg) or corn oil was administered to rats for 3 days. Twenty-four hours later 0.234 mg of $^3$H-$\alpha$-ALA per kg was injected intravenously, and the animals were killed at various times after the injection. Values represent the means obtained from three rats.

Incorporation of Radioactivity into CO-binding Particles—The incorporation of $^3$H-$\alpha$-ALA into the CO-binding particles is shown in Fig. 4. 3-MC (25 mg per kg) or corn oil was administered to rats for 3 days. Twenty-four hours later 0.234 mg of $^3$H-$\alpha$-ALA per kg was administered intravenously to the rats. Animals were killed at various times, and the radioactivity incorporated into the CO-binding particles was measured.
Radioactivity appears in the particles within 1 min after injection of \(^3H\) \(\delta\)-ALA and reaches a maximum within 30 to 60 min. Essentially no difference in the incorporation of \(^3H\) \(\delta\)-ALA into the CO-binding particles could be shown between control and 3-MC-treated rats.

**Disappearance of Radioactive Hemoprotein from CO-binding Particles**—Fig. 5 shows the disappearance of radioactivity with time from the CO-binding particles of control and 3-MC-treated rats injected with \(^3H\) \(\delta\)-ALA as described above. Since essentially the same amount of \(^3H\) \(\delta\)-ALA is incorporated into the CO-binding particles from control and 3-MC-treated rats (Fig. 4), the zero time value for both groups was set equal to 100% so that the results could be compared easily. The data indicate a biphasic disappearance of radioactivity from the CO-binding particles obtained from both control and 3-MC-treated rats, indicating the existence of at least two hemoprotein fractions. The ratio of the two hemoprotein fractions present in the CO-binding particles from control rats was calculated to be 3.8:1 as described under Fig. 5. However, in 3-MC-treated rats the ratio of the two hemoprotein fractions was 1:1. The results of Fig. 5 confirm our earlier finding that, 72 hours after the administration of \(^3H\) \(\delta\)-ALA, 2 to 3 times more radioactivity remains in the CO-binding particles of 3-MC-treated rats when compared to control rats (30). The corrected \(t_1\) of the fast phase was obtained by subtracting the counts per min contributed by the slow phase from extrapolating back to zero time. This calculation yielded a corrected \(t_1\) of the fast phase of 7 to 8 hours in control and 3-MC-treated rats (Fig. 5). Since the \(t_1\) of the slow phase hemoprotein was the same in control and 3-MC-treated rats, i.e., 46 to 48 hours, the difference in the radioactivity remaining in the CO-binding particles after 72 hours can be explained by an increased amount of the slow fraction in the 3-MC treated rats.

**Determination of Protopheme Content of CO-binding Particles**—The protopheme content of the CO-binding particles was determined so that the relative amounts of the two hemoprotein fractions present in control and 3-MC-treated rats could be calculated. Immature male rats were injected with 3-MC (25 mg per kg per day) for 3 days, and the animals were killed 24 hours after the last injection. 3-MC treatment increased the protopheme content of the CO-binding particles from 1.35 mmoles per mg of protein to 2.15 mmoles per mg of protein. The ratios of the two hemoprotein fractions (3.8:1 in control and 1:1 in 3-MC-treated rats, Fig. 5) present in the CO-binding particles and the protopheme content were used to calculate the relative amounts of the fast and slow phase hemoprotein. This calculation indicated that 3-MC treatment did not change the content of the fast phase hemoprotein, but caused a 3- to 4-fold increase in the content of the slow phase hemoprotein; i.e., the relative amount of fast component hemoprotein to slow component hemoprotein was 1.1:0.3 in control rats and was 1.1:1.1 in 3-MC-treated animals.

**DISCUSSION**

The present studies show that administration of \(^3H\) \(\delta\)-ALA to rats leads to the incorporation of radioactivity into the microsomal fraction of liver and that, following the selective removal of cytochrome \(b_1\) with steapsin treatment, the radioactivity remaining is in the heme portion of microsomal hemoprotein or hemoproteins. Addition of cold trichloroacetic acid to the CO-binding particles led to the precipitation of the protein and radioactivity, indicating that the radioactivity was associated with protein. Treatment of the CO-binding particles with acid-acetone or methyl ethyl ketone led to the cleavage of the heme moiety from the protein, and the radioactivity was associated with the heme fraction. In addition, Barou and Teply (31) have shown that treatment of animals with an inhibitor of heme synthesis (aminotriazole) decreases the incorporation of \(^14C\) \(\delta\)-ALA into total microsomal heme. Since the only spectrally observable hemoproteins in liver microsomes are cytochrome \(b_1\) and the CO-binding pigment (21, 22, 32-34), it is clear that the
labeled component in the CO-binding particles after removal of cytochrome \( b_2 \) is the CO-binding pigment.

The results presented here show a biphasic decrease in the radioactivity incorporated into the CO-binding particles in both control and 3-MC-treated rats. Half-life determinations of the fast phase component \((\tau = 7 \text{ to } 8 \text{ hours})\) and the slow phase component \((\tau = 46 \text{ to } 48 \text{ hours})\) indicated that 3-MC treatment did not change the biological half-life of either hemoprotein fraction. However, treatment of rats with 3-MC changed the ratio of the fast phase component to the slow phase component from 3.8:1 to 1:1. Determination of the total protoheme content of the CO-binding particles indicated that treatment of rats with 3-MC leads to a 3- to 4-fold increase in the concentration of the slow phase component while not affecting the concentration of the fast phase component in liver microsomes.

Three possible explanations can be advanced to explain the biphasic decrease in radioactive hemoproteins. (a) Two separate CO-binding hemoproteins are independently synthesized in the liver. Treatment with 3-MC would lead to an increase in the synthesis of only one of these hemoproteins. (b) A single hemoprotein is synthesized in the liver, and this hemoprotein is catalytically converted to a second hemoprotein or broken down. 3-MC would then accelerate the conversion of the first hemoprotein to the second. (c) A single hemoprotein exists in two physical forms, and 3-MC treatment facilitates the change to the more stable form. This might involve a change in the submicrosomal localization of the hemoprotein or a change in its lipid attachment.

Although all three possibilities have to be considered, the data presented in this paper demonstrate two hemoprotein fractions in liver CO-binding particles. It is possible that the two hemoprotein fractions described in this report, whether they be different hemoproteins or different physical forms of the same hemoprotein, represent separate catalytic entities for the hydroxylation of drugs and steroids. This would be consistent with previous observations indicating that one microsomal hemoprotein fraction could not alone impart specificity for testosterone hydroxylation in the 7β, 16α, and 6β positions on the steroid nucleus and that a minimum of two factors was necessary (4–10). However, it has not been established that the specificity for microsomal oxidation is imparted by the hemoprotein. It is possible that the hemoprotein is attended by several specific substrate-binding proteins that put the substrate in the proper position for interaction with the oxygenated cytochrome. The two hemoprotein fractions, as discussed above, could be attended by different specific substrate-binding proteins.

Nishibayashi and Sato (33) and Miyake, Gaylor, and Mason (34) have recently obtained submicrosomal particles containing cytochrome \( P_{450} \) with little or no contamination by cytochrome \( \text{P}_{60} \) or cytochrome \( b_2 \). Their studies have shown that the structure of oxidized cytochrome \( P_{450} \) in the vicinity of the heme is similar to that of ordinary \( b \)-type cytochromes and that the heme iron is in the low spin state. The spectrum of oxidized cytochrome \( P_{450} \) in their preparations was unusual in that it showed a small absorption band in the 600 to 650 nm region. Nishibayashi and Sato (33) pointed out that the appearance of peaks in the 600 to 650 nm and 450 to 500 nm regions is believed to be a spectral expression of high spin ferrihemoprotein. They suggested, therefore, that it is possible that a small amount of a ferrihemoprotein of the high spin type is present in the oxidized cytochrome \( P_{450} \) preparation. Nishibayashi and Sato (33) concluded that it is likely that the peak at 650 nm is inherent to oxidized cytochrome \( P_{450} \), which exists as a mixture of a large amount of a low spin form and a small amount of a high spin form. Jefcoate and Gaylor (30), using electron spin resonance, have recently shown that the presence of the high spin form in rabbit liver submicrosomal particles is increased by prior treatment of the animals with 3-methylcholanthrene. Similarly, with a crude microsomal preparation, it has also been found that 3-MC treatment leads to the appearance of an absorption peak at 647 nm in the oxidized hemoprotein spectrum (18). The selective action of 3-MC on one of the microsomal hemoproteins is consistent with the data presented in this paper, and it is therefore possible that cytochrome \( P_{450} \), \( P_{60} \), and the high spin form all represent the hemoprotein with a \( \tau = 48 \) hours described here and that cytochrome \( P_{450} \) and the low spin form represent the microsomal hemoprotein with a \( \tau = 7 \) hours. Proof for the suggestion that each hemoprotein has catalytic activity will have to await the solubilization and purification of the microsomal hemoproteins in an active form.

Previous studies on the half-life of microsomal protein have yielded conflicting results. The original studies of Shuster and Jick (38) with \( ^{3} \)H-leucine indicated that the \( \tau \) of total microsomal protein was 2 to 3 days. Arias, Doyle, and Schimke (37) obtained similar results with \( ^{14} \)C-guanidino-L-arginine. However, in both studies, the determination of the protein half-life was started at 24 hours after the administration of the isotope. The studies presented here would also show a \( \tau \) of about 2 days if the determination were started at 24 hours after the injection of \( ^{3} \)H-5-ALA instead of at 2 to 5 hours. Studies by Schmid, Marver, and Hammaker (18), utilizing a model of protein synthesis, suggested that cytochrome \( P_{450} \) had a half-life of less than 12 hours. Holtzman and Gillette (38) recently estimated the half-life of aniline hydroxylase to be about 5 to 7 hours. Indeed, the latter studies are in agreement with the results presented here for the fast phase hemoprotein component (\( \tau = 7 \) to 8 hours). It seems, therefore, that the methodology used for estimating the half-life of microsomal protein has possibly led to the differential results.

REFERENCES

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