Biphasic Decrease of Radioactive Hemoprotein from Liver Microsomal Carbon Monoxide-Binding Particles

Effect of Phenobarbital and Chlordane

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SUMMARY

The administration of δ-aminolevulinic acid-3,5-^{3}H to immature male rats leads to the incorporation of radioactivity into hemoprotein in the CO-binding particles. The incorporated radioactivity decreases in two distinct phases: a rapid phase with a half-life of 7–8 hr and a slow phase with a half-life of 46–48 hr. Treatment with phenobarbital or chlordane does not appreciably alter the ratio of the fast-phase component to the slow-phase component but does increase the half-life of the fast phase to 11–12 hr. The increase in the protoheme content of the CO-binding particles found after phenobarbital and chlordane treatment suggests that these compounds increase the amount of both the rapidly and slowly decaying components.

INTRODUCTION

The administration of δ-aminolevulinic acid-3,5-^{3}H to immature male rats leads to the incorporation of radioactivity into liver microsomal hemoprotein (1, 2). The hemoproteins of liver microsomes consist of cytochrome b_5 and a CO-binding hemoprotein (cytochrome P-450) (3–7). Selective solubilization of microsomal cytochrome b_5 with steapsin removes only a small portion of the radioactivity from the microsomes, leaving the labeled CO-binding hemoprotein attached to the undigested residue [termed CO-binding particles by Omura and Sato (3, 4)].

The existence of more than one CO-binding hemoprotein fraction has been suggested by studies which indicated a biphasic decrease in radioactive hemoproteins in the CO-binding particles (2) and by spectral studies of microsomal hemoprotein (8–14). Treatment of rats with 3-methylcholanthrene prior to labeling the hemoproteins in the CO-binding particles did not change the biological half-life of the rapidly disappearing hemoprotein fraction ($t_{1/2}$ = 7–8 hr) or the slowly disappearing fraction ($t_{1/2}$ = 46–48 hr), but 3-methylcholanthrene administration changed the proportion of the fast-phase component to the slow-phase component from 3.8:1 to 1:1 (2).

Since phenobarbital is a much more nonspecific inducer of the liver microsomal enzymes than is 3-methylcholanthrene (15), studies were initiated to determine the effect of phenobarbital treatment on the disappearance of labeled hemoprotein from rat liver microsomal CO-binding particles. The results presented here show that phenobarbital treatment does not appreciably alter the ratio of the fast-phase component to the slow-phase component, as did 3-methylcholanthrene, but does increase to 12 hr the half-life of the fast-phase component.

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METHODS

Immature male rats (60–70 g) of the Long-Evans strain were fed a commercial diet and water ad libitum. Sodium phenobarbital dissolved in 0.9% NaCl was administered intraperitoneally at a dose of 37 mg/kg twice daily for 10 days. Chlordane (technical grade; Velsicol Corporation) was dissolved in corn oil, and 25 mg/kg/day of the insecticide were administered intraperitoneally for 12 days. Control rats received the appropriate vehicle.

δ-Aminolevulinic acid-3,5-^{3}H (510 mCi/mmole; New England Nuclear Corporation) was injected intravenously (0.234 mg/kg) in 0.9% NaCl, and the animals were killed at various times after the injection. Liver microsomes were prepared as previously described (2) and stored as a pellet under a layer of 3 ml of 0.1 M KH_2PO_4-K_2PO_4, pH 7.4, frozen at −15° for 2–7 days before use. Previous studies from our laboratory (16) have shown that storage of liver microsomes as a pellet at −15° for as long as 14 days does not lead to any significant loss of microsomal enzyme activity responsible for the *N*-demethylation of ethylmorphine and the hydroxylation of pentobarbital and testosterone. Similar results were obtained for cytochrome P-450.[1] In contrast, when microsomes were stored frozen as a suspension rather than as a pellet, enzyme activity decreased with time (16). For determination of the protoheme content, the microsomes were stored frozen as a pellet for a maximum of 1 day. No studies on the stability of protoheme under these conditions are available, although, as mentioned above, the P-450 level does not change upon storage of such microsomal pellets.

Immediately before use, the microsomal pellets were thawed at room temperature, suspended in potassium phosphate buffer (microsomes equivalent to 250 mg of liver, wet weight, per milliliter), and incubated, as described previously (2), with 0.2% steapsin for 1 hr at 37° under nitrogen to solubilize cytochrome b_5 selectively. The pellet thus obtained on centrifugation for 2 hr at 105,000 × *g* contains 80–90% of the CO-binding pigment, mostly in the form of cytochrome P-420. The percentage of total CO-binding pigment recovered in this pellet was the same in control, PB-,[2] or MC-treated rats, indicating that no selective loss of CO-binding pigment had occurred with various drug treatments. In one experiment, the actual fraction of the CO-binding pigment recovered in the pellet was: control, 83%; PB-treated, 82%; and MC-treated, 81%. This preparation has been termed CO-binding particles by Omura and Sato (3, 4) because of its high content of CO-binding hemoprotein and because this cytochrome was the only spectrally observed hemoprotein in these particles.

The CO-binding particles were suspended in potassium phosphate buffer, and the radioactivity in 0.2 ml of the resuspended particles was measured in a liquid scintillation spectrometer, utilizing the scintillation mixture of Bray (17). The protein content was determined by the method of Sutherland *et al.* (18). Microsomal hemoproteins were determined as described by Omura and Sato (3, 4), using an Aminco-Chance dual-wavelength/split beam spectrophotometer. The protoheme content of the CO-binding particles was measured after converting the heme into pyridine-hemochromogen as described by Omura and Sato (3, 4). Cleavage of the heme-protein linkage was accomplished by addition of a mixture of HCl and acetone (19) or with methyl ethyl ketone (20).

RESULTS

Incorporation of radioactivity into the heme moiety of hemoprotein in CO-binding particles. PB (37 mg/kg twice daily) was administered to immature male rats daily for 4 days. On the 5th day, ALA-^{3}H was injected intravenously, and the rats were killed either 2 or 72 hr later. We have shown previously that the radioactivity from administered ALA-^{3}H is incorporated into the heme portion of microsomal hemo-

[1] Unpublished observations.

[2] The abbreviations used are: PB, phenobarbital; MC, 3-methylcholanthrene; ALA, δ-aminolevulinic acid.

protein in both control and MC-treated rats (2). Similar results have been obtained after treatment of rats with PB. Addition of cold trichloracetic acid solution (final concentration of 7%) to the CO-binding particles isolated 2 and 72 hr after the injection of ALA-^{3}H led to the precipitation of the protein and 99% of the radioactivity, indicating that the radioactivity was associated with protein. Treatment of the CO-binding particles with cold acid–acetone solubilized the heme and 99% of the radioactivity and precipitated the protein, indicating that the radioactivity was in the heme moiety. In contrast, treatment of the CO-binding particles with cold 90% acetone–10% water did not cleave the heme moiety, and the radioactivity was precipitated with the hemoprotein. Methyl ethyl ketone has also been shown to extract the heme from a hemoprotein (20), and extraction of the CO-binding particles with this solvent resulted in the extraction of over 95% of the radioactivity. Similar results were obtained with CO-binding particles derived from animals which were killed either 2 or 72 hr after ALA-^{3}H administration, indicating that at both times the radioactivity was contained principally in the heme moiety of a hemoprotein.

TABLE 1

Effect of phenobarbital treatment on incorporation of ALA-^{3}H into CO-binding particles

Immature male rats were given PB (37 mg/kg by injection twice daily) for 4 days. On the 5th day, ALA-^{3}H (0.234 mg/kg; specific activity, 510 mCi/mmole) was injected intravenously, and the animals were killed at various times after the injection. The values represent the means and standard errors from five or six animals.

Treatment	Time after ALA-^{3}H	CO-binding particles
	hr	*cpm/mg protein*
Control	1	15,473 ± 1340
PB	1	11,630 ± 304[a]
Control	2	15,053 ± 598
PB	2	12,448 ± 422[b]
Control	5	13,232 ± 1030
PB	5	11,285 ± 365[c]

[a] $p < 0.05$ difference from controls.
[b] $p < 0.01$ difference from controls.
[c] Not significantly different from controls.

Incorporation of radioactivity into CO-binding particles. Following the intravenous administration of ALA-^{3}H (0.234 mg/kg) to immature male rats, radioactivity appears in the CO-binding particles within 1 min and reaches a maximum within 30–60 min (2). Table 1 shows the effect of prior treatment with PB on the incorporation of ALA-^{3}H into the CO-binding particles. The results indicate a slightly decreased incorporation of ALA-^{3}H into hemoprotein in the CO-binding particles of PB-treated rats.

Disappearance of radioactive hemoprotein from CO-binding particles in rats receiving prior treatment with PB. Immature male rats were given PB (37 mg/kg twice daily) for 10 days. On the 5th day, ALA-^{3}H (0.234 mg/kg) was injected intravenously, and the animals were killed at various times thereafter. Figure 1 shows the disappearance of radioactivity with time from the CO-binding particles. As can be seen, the decrease in the radioactivity with time is biphasic, indicating the existence of at least two hemoprotein fractions. The ratio of the two hemoprotein fractions present in the CO-binding particles was calculated to be 3.3:1 as described in the legend to Fig. 1. The corrected $t_{1/2}$ of the fast phase was obtained by subtracting the counts per minute per milligram of protein contributed by the slow phase after extrapolating the slow-phase line back to zero time as described in Fig. 1. This calculation yielded a corrected $t_{1/2}$ of the fast phase of 12 hr and a $t_{1/2}$ of the slow phase of 49 hr. This is in contrast to results obtained with control and MC-treated rats, in which the $t_{1/2}$ of the fast-phase hemoprotein was 7–8 hr (2). A comparison between the disappearance of radioactive hemoprotein from control and PB-treated rats can be seen in Fig. 2. It is clear from the data presented that prior treatment with PB leads to an increase in the $t_{1/2}$ of the fast-phase hemoprotein fraction but does not affect the $t_{1/2}$ of the slow-phase fraction.

Disappearance of radioactive hemopro-

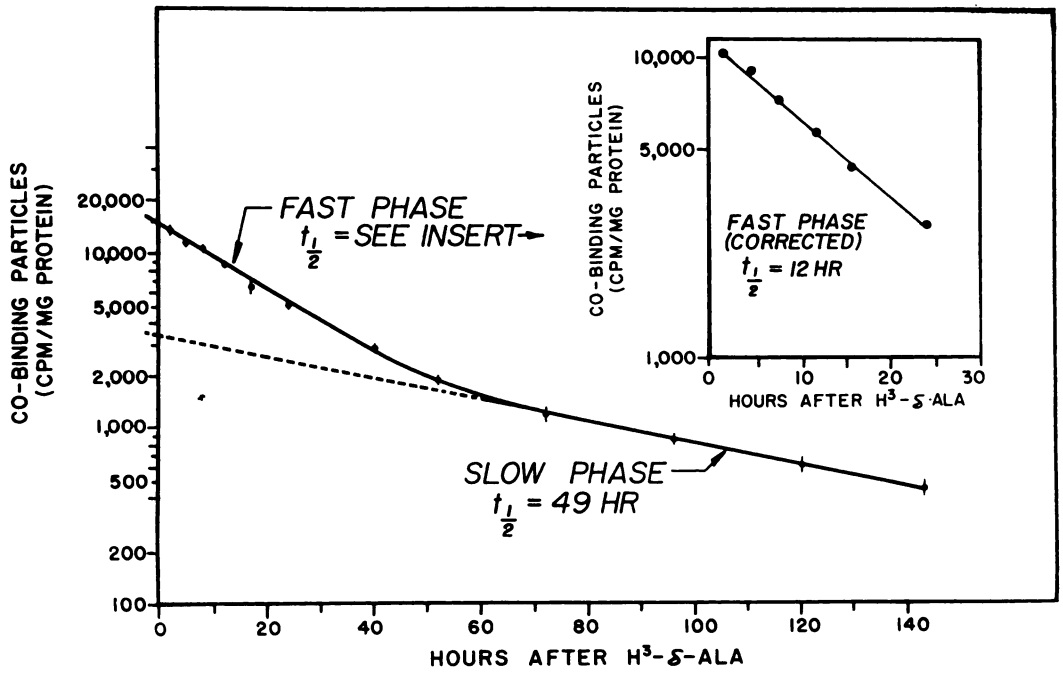

FIG. 1. *Disappearance of labeled hemoprotein from CO-binding particles obtained from PB-treated rats*

PB (37 mg/kg twice daily) was administered to rats for 4 days prior to the administration of δ-aminolevulinic acid-^{3}H (H^3-δ-ALA) (0.234 mg/kg). Animals were continued on PB throughout the study. Each value represents the mean ± standard error from four rats. Values used for the determination of the corrected half-life of the fast phase (inset) were obtained by first extrapolating the slow-phase line to zero time and then subtracting the values of the extrapolated slow phase from values of the uncorrected fast phase. The ratio of the fast-phase hemoprotein to the slow-phase hemoprotein was obtained by first extrapolating the lines for the two phases to zero time and then subtracting the zero-time intercept for the slow phase from the zero-time intercept of the uncorrected fast phase and dividing the result by the zero-time intercept for the slow phase.

tein from CO-binding particles in rats treated with chlordane. The possibility was considered that PB might be unique as an enzyme inducer in altering the half-life of the fast-phase hemoprotein fraction. Since the insecticide chlordane is an enzyme inducer with a specificity similar to that of PB for the liver microsomal enzymes (21, 22), chlordane treatment was used to study the disappearance of radioactive hemoprotein from rat liver CO-binding particles.

Immature male rats were given injections of chlordane (25 mg/kg/day) for 12 days. On the 7th day, ALA-^{3}H (0.234 mg/kg) was injected intravenously, and the animals were killed at various times thereafter. Figure 3 shows the effect of chlordane treatment on the biphasic disappearance of radioactivity from the CO-binding particles. The ratio of the two hemoprotein fractions was calculated to be 3.5:1, similar to that obtained for PB-treated rats. The corrected half-life for the fast-phase hemoprotein fraction was 11 hr, while the slow-phase fraction had a $t_{1/2}$ of 46 hr. These data indicate that chlordane treatment alters the $t_{1/2}$ of the fast-phase hemoprotein in a manner similar to PB.

Determination of protoheme content of CO-binding particles. The protoheme content of the CO-binding particles was determined so that the ratio of the fast-phase to slow-phase hemoprotein fractions and the protoheme content could be used to determine the relative amounts of the fast- and slow-phase fractions. Table 2 shows that MC treatment results in a 3–4-fold increase in the slow-phase hemoprotein fraction but

does not affect the concentration of the fast-phase component, as previously reported (2). In contrast, treatment of immature male rats with PB or chlordane led to an increase in both the fast- and slow-phase hemoprotein components, although the increase in the slow-phase component was greater after treatment with MC.

DISCUSSION

We have previously reported (1, 2) that the administration of ALA-^{3}H to immature male rats *in vivo* leads to the incorporation of radioactivity into the hemoprotein contained in rat liver CO-binding particles (steapsin-treated microsomes which do not contain cytochrome b_5). The use of ALA-^{3}H for the labeling of microsomal hemoprotein was considered the method of choice, because ALA is rapidly excreted (23) and heme is converted nearly quantitatively to bile pigment and excreted without reutilization (24, 25). A biphasic decrease in the radioactivity incorporated into the hemoprotein of the CO-binding particles obtained from both control and MC-treated rats was found (2). Half-life determinations of the fast-phase component ($t_{1/2}$ = 7–8 hr) and the slow-phase component ($t_{1/2}$ = 46–48 hr) revealed that MC treatment did not change the biological half-life of either hemoprotein fraction, but changed the ratio of the fast-phase fraction to the slow-phase fraction from 3.8:1 to 1:1. The protoheme content of the CO-binding particles indicated that treatment with MC increased by 3–4 times the amount of the slow-phase fraction but did not affect the amount of the fast-phase fraction (2).

The present study indicates that a bi-

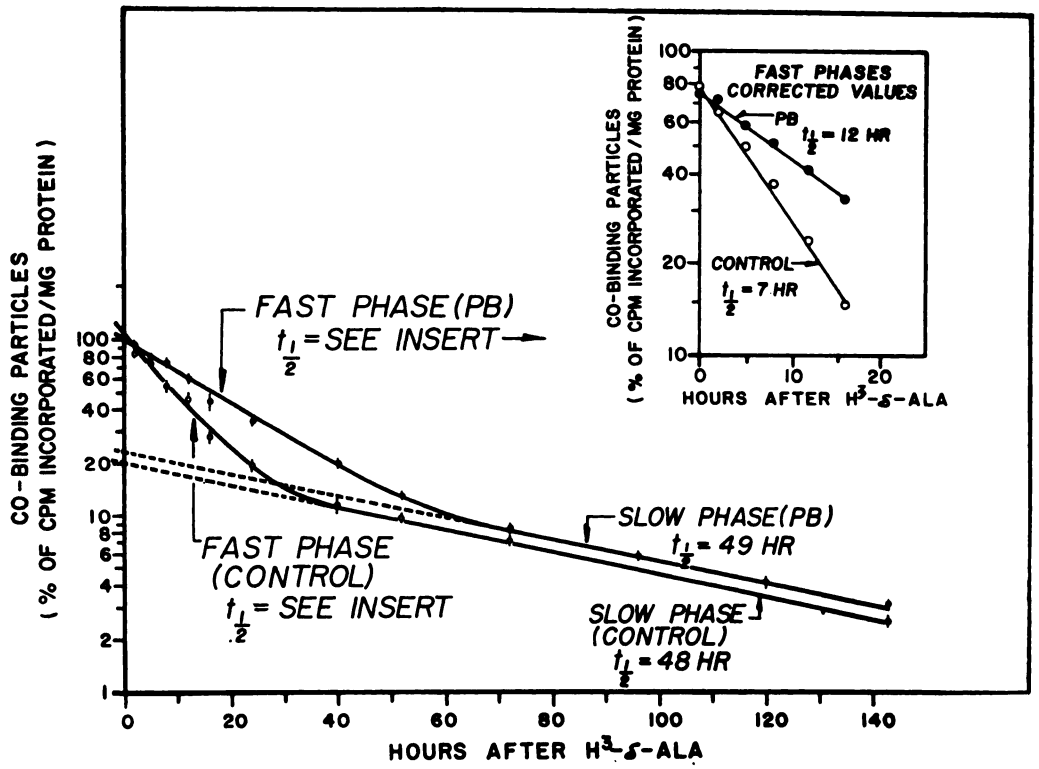

FIG. 2. *Disappearance of labeled hemoprotein from CO-binding particles obtained from control and PB-treated rats*

Rats were treated as described in Fig. 1. The zero-time intercept of the uncorrected fast phase was set equal to 100% for both control and PB-treated rats and graphed for comparative purposes. The half-lives of the various fractions were calculated as described in Fig. 1. H^3-δ-ALA is δ-aminolevulinic acid-^{3}H.

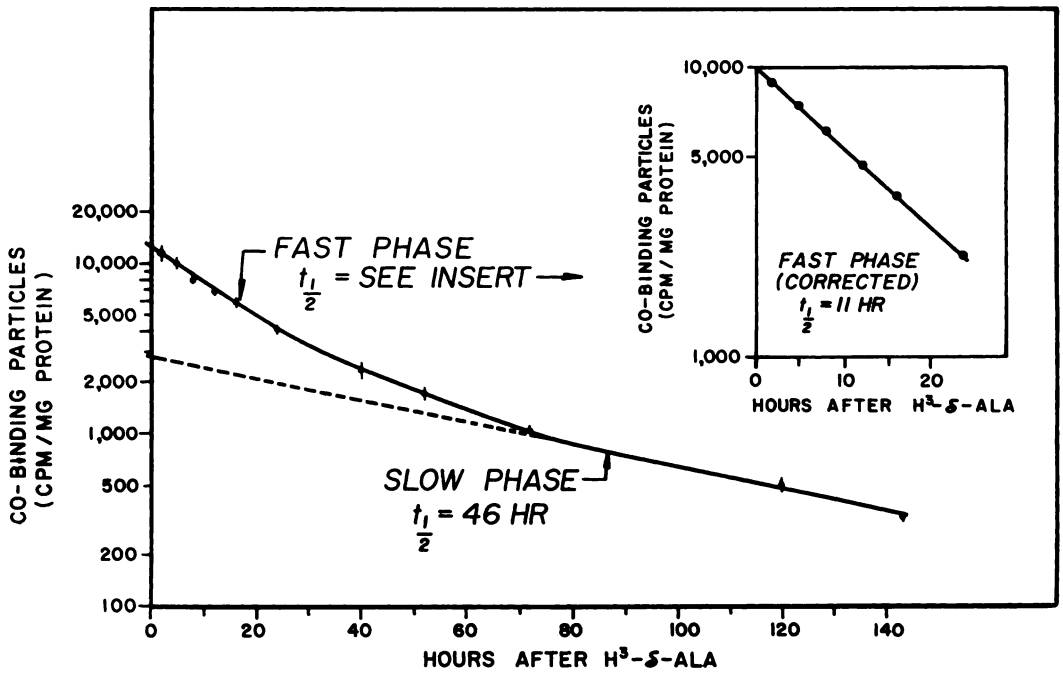

FIG. 3. *Disappearance of labeled hemoprotein from CO-binding particles obtained from chlordane-treated rats*

Rats were given chlordane (25 mg/kg/day) for 6 days prior to the administration of δ-aminolevulinic acid-^{3}H (H^3-δ-ALA) (0.234 mg/kg). Animals were continued on chlordane throughout the study. Each value represents the mean ± standard error for four rats. The half-lives of the various fractions and the ratio of the fast-phase hemoprotein to the slow-phase hemoprotein were calculated as described in Fig. 1.

phasic decrease in the radioactivity incorporated into the CO-binding particles also occurs in PB- or chlordane-treated animals. In contrast to the effect of MC, treatment with PB did not appreciably alter the ratio of the fast-phase fraction to the slow-phase fraction (3.3:1), but increased the half-life of the fast-phase component to 12 hr. The increase in the heme content of the CO-binding particles found after PB treatment has suggested that PB doubles the relative amount of both the fast- and slow-phase

TABLE 2

Determination of hemoprotein content of CO-binding particles

Immature male rats were given injections of MC (25 mg/kg/day for 3 days), PB (37 mg/kg twice daily for 4 days), or chlordane (25 mg/kg/day for 6 days), and the animals were killed 18–24 hr after the last injection. Ratios of the fast component to slow component were obtained as described in Fig. 1. The values represent the means of four to seven determinations; each determination was obtained from the pooled livers of two rats.

Treatment	Protoheme	Ratio of fast to slow fraction	Relative amounts	
			Fast component	Slow component
	mμmoles/mg protein			
Control	1.37 ± 0.07	3.8	1.1	0.3
MC	2.15 ± 0.09	1.0	1.1	1.1
PB	3.19 ± 0.09	3.3	2.5	0.7
Chlordane	2.19 ± 0.14	3.5	1.7	0.5

fractions. Similar results were obtained after chlordane treatment, although the increase in the amount of the fast- and slow-phase fractions after chlordane treatment (70%) was slightly less than that obtained after PB treatment.

As has been proposed previously (2), three possible explanations can be advanced to explain the biphasic decrease in the radioactivity incorporated into the CO-binding particles: (a) Two separate CO-binding hemoproteins are independently synthesized in the liver. Treatment of rats with inducers of liver microsomal enzymes would lead to an increase in the synthesis of one (induced by MC) or both of these hemoproteins (induced by PB and chlordane). (b) A single hemoprotein is synthesized in the liver, and this hemoprotein is catalytically converted to the second hemoprotein (slow-phase fraction) or metabolized. Treatment of rats with MC, PB, or chlordane could differentially affect this conversion. (c) A single hemoprotein exists in two physical forms, each with a different half-life. This might involve a change in the submicrosomal localization of the hemoprotein or a change in its lipid attachment, which could be differentially altered by treatment with MC, PB, or chlordane.

The present studies show that PB treatment significantly altered the biological half-life of the fast-phase component from 7 to 12 hr, but did not affect the half-life of the slow-phase component, while chlordane treatment increased the half-life of the fast-phase component from 7 to 11 hr. The possibility exists that PB and chlordane treatment stimulates the formation of a different fast-phase hemoprotein or that a third hemoprotein fraction is present in PB- or chlordane-treated rats. In addition, it is also possible that prior treatment with PB and chlordane leads to stabilization (decreased breakdown) of the fast-phase component. However, this possibility is not consistent with results which indicate that the specific activity of the labeled hemoprotein was not increased when PB was administered 1 hr after the hemoprotein was labeled.

A series of recent reports have indicated the existence of both low- and high-spin hemoproteins in submicrosomal particles that do not contain cytochrome b_5 (6, 7, 13, 14). Jefcoate *et al.* (13, 14) found that treatment of rabbits with MC led to an increase in the high-spin hemoprotein in these submicrosomal particles. It is interesting that Jefcoate *et al.* (13, 14) obtained ratios of the high- and low-spin hemoproteins present in control, PB-, and MC-treated rabbits that are in excellent agreement with the ratios and relative amounts of the two hemoprotein fractions described in this report (Table 2). It is therefore suggested that the two radioactive hemoprotein fractions observed in the CO-binding particles may represent the high- and low-spin hemoproteins.

Recent studies on the metabolism of drugs by liver microsomes have suggested a qualitative change in the enzyme system following MC treatment (26, 27). Alvares *et al.* (26) demonstrated a change in the K_m for benzpyrene hydroxylation after MC treatment, suggesting that a qualitative change in the enzyme system had occurred. Similarly, Sladek and Mannering (27) have proposed the appearance of a second enzyme system in rats treated with MC. However, the possibility of the existence of very small amounts of this second enzyme system in both control and PB-treated rats was not excluded (27).

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