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# Chromatography of Radioactive Microsomal Hemoproteins on Diethylaminoethyl Cellulose

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SUMMARY

Administration of $^{14}C$- or $^{3}H$-δ-aminolevulinic acid to immature male rats resulted in the incorporation of radioactivity into liver microsomal hemoprotein. Column chromatography of "solubilized" microsomes or CO-binding particles[4] obtained from rats treated with phenobarbital or 3-methylcholanthrene revealed that essentially all the radioactivity was associated with fractions containing cytochromes $b_5$, P-450, P-448, and P-420. The results indicated that radioactivity incorporated into microsomes and CO-binding particles after administration of $^{14}C$- or $^{3}H$-δ-aminolevulinic acid is associated almost exclusively with cytochromes, and that very little, if any, nonspecific binding of heme occurs.

## INTRODUCTION

Administration of 3-methylcholanthrene to rats causes the appearance in liver microsomes of a CO-binding pigment with spectral characteristics different from the cytochrome found in untreated or phenobarbital-treated rats (2–6). This cytochrome has been termed cytochrome $P_1$-450 (2), cytochrome P-448 (3–5), and cytochrome P-446 (6). Jefcoate, Gaylor, and Calabrese (7, 8), using electron spin resonance, have recently shown the presence of both high- and low-spin forms of cytochrome P-450 in rabbits. Studies from our laboratory (9–11), utilizing radioactive δ-aminolevulinic acid, have shown a biphasic decrease in the radioactivity incorporated into the hemoprotein of CO-binding particles (microsomes devoid of cytochrome $b_5$) obtained from immature male rats, also indicating more than one hemoprotein fraction.[5] Although several suggestions were made to explain the nature of the two radioactive hemoprotein fractions (9, 10), one of these—that the fraction with the half-life of 7 hr may represent cytochrome P-450 while the fraction with the half-life of 48 hr may represent cytochrome P-448—is unlikely, since recent evidence suggest that little, if any, cytochrome P-448 is present in microsomes obtained from control and phenobarbital-treated rats (12, 13). Whether both fractions possess different

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[4] Microsomes and CO-binding particles were solubilized according to the method of Lu and Coon (1). The term solubilized is used to denote the supernatant fraction obtained after centrifugation at 165,000 × *g* for 90 min.

[5] As suggested previously (9, 10), the hemoprotein "fractions" can be either individual CO-binding hemoproteins or a hemoprotein(s) bound in more than one way to the membrane.

catalytic activities remains to be demonstrated. In contrast to our studies, which showed a biphasic decrease in specific activity of labeled CO-binding hemoprotein in CO-binding particles, Greim and his associates (14, 15) recently concluded that there was a monophasic decline, and they suggested that the biphasic decline in radioactivity observed in our laboratory may have been caused by nonspecific binding of labeled heme to microsomal protein. Since the methodology and the strain and age of the rats used by these investigators differed from those used for our earlier work, we have repreated the studies of Greim and his associates. In addition, liver microsomes and CO-binding particles have been solubilized and chromatographed on DEAE-cellulose according to the method of Lu and Coon (1) to characterize further the radioactivity incorporated into the CO-binding particles following the administration of $^{14}C$- or $^{3}H$-δ-aminolevulinic acid. The results presented below indicate that the radioactivity incorporated into CO-binding particles is associated almost exclusively with cytochromes and that very little, if any, nonspecific binding of heme occurs. Our findings further strengthen the concept that at least two CO-binding hemoprotein fractions occur in liver microsomes.

## METHODS

### *Studies on Turnover of Microsomal CO-Binding Hemoprotein*

Adult male Wistar rats (Royal Hart Farms, New Hampton, N. Y.) weighing 140–150 g were used for the studies on the turnover of cytochrome P-450. The method of Greim (15) was used for removal of cytochrome $b_5$ from microsomes and the subsequent extraction of cytochrome P-450 heme. δ-Aminolevulinic acid-3,5-$^{3}H$ with a specific activity of 500 mCi/mmole in 0.3 ml of 0.9% NaCl was injected into the tail vein (0.125 mg/kg). Liver microsomes were prepared using 1.15% KCl in 0.05 M Tris-HCl (pH 7.5) as previously described (15). The microsomal pellets were resuspended and washed twice with Tris-KCl to remove hemoglobin, and cytochrome $b_5$ was removed with pig pancreatic lipase (Serva, Heidelberg) as described by Greim (15). The pellet, termed CO-binding particles, obtained after centrifugation at 165,000 × *g* for 2 hr was suspended in Tris-KCl and sonicated for 1 min as described by Greim (15). The suspension was subsequently extracted with 4 volumes each of ether, chloroform, and acetone for 10 min. After the chloroform extraction, both the organic and aqueous phases were removed, leaving only the intermediate protein layer for acetone extraction. The sediment from the acetone extraction, containing the CO-binding pigment, was resuspended in 5 ml of 1.15% KCl and extracted with 6 volumes of 5 N HCl in acetone. The heme in the acetone-HCl supernatant solution was then extracted into 30 ml of hexane. After evaporation of the organic phase, the heme was dissolved in 1 ml of acetone, and its hemochromogen content was determined by the method of Omura and Sato (16, 17). Radioactivity was determined in a liquid scintillation spectrometer utilizing the scintillation mixture of Bray (18). The recovery of heme and radioactivity from CO-binding particles was 50–55%.

### *Column Chromatography of Microsomal Hemoprotein*

For studies involving the use of column chromatography, immature male rats (60–70 g) of the Long-Evans strain (Blue Spruce Farms, Altamont, N. Y.) were fed a commercial diet and water ad libitum. 3-Methylcholanthrene was dissolved in corn oil, and 25 mg/kg of the polycyclic hydrocarbon were administered intraperitoneally once daily for 3 days. Sodium phenobarbital dissolved in 0.9% NaCl was administered intraperitoneally at a dose of 37 mg/kg twice daily for 4 days. δ-Aminolevulinic acid-3,5-$^{3}H$ (588 mCi/mmole; New England Nuclear Corporation) or δ-aminolevulinic acid-4-$^{14}C$ (12.7–33.8 mCi/mmole; Tracerlab) was injected intravenously (0.5 mg/kg) in 0.9% NaCl, and the animals were killed either 2 or 72 hr thereafter.

Liver microsomes were prepared from 33% homogenates (15–30 pooled rat livers) in 0.25 M sucrose as previously described (9). The microsomal pellets were washed and

resuspended in 1.15% KCl twice to ensure complete removal of the soluble fraction. The washed microsomal pellets were used immediately or layered with 3 ml of 0.1 M $KH_2PO_4$–$K_2HPO_4$ (pH 7.4), and stored at −15° for 1–7 days before use. Previous studies from our laboratory (19) 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 cytochrome P-450 or enzyme activity responsible for the *N*-demethylation of ethylmorphine or the hydroxylation of pentobarbital and testosterone.

To prepare CO-binding particles, the microsomal pellets were thawed at room temperature, suspended in 0.1 M potassium phosphate buffer, pH 7.4 (microsomes equivalent to 250 mg of liver, wet weight, per milliliter), and incubated as previously described (9, 10) with 0.2% steapsin for 1 hr at 37° under nitrogen to solubilize cytochrome $b_5$. The pellet obtained on centrifugation for 2 hr at 165,000 × *g* contains 40–50% of the total microsomal protein, 80–90% of the CO-binding pigment, mostly in the form of cytochrome P-420, and none of the cytochrome $b_5$. This preparation has been termed "CO-binding particles" by Omura and Sato (16, 17) because of its high content of CO-binding hemoprotein (a 2-fold purification of CO-binding pigment heme) and because this cytochrome was the only spectrally observed hemoprotein in these particles.

Microsomes or CO-binding particles were solubilized according to the method of Lu and Coon (1). A suspension of CO-binding particles or microsomes containing 800–1000 mg of protein in 35 ml of 0.25 M sucrose was stirred for 20 min at 4° with 24 ml of glycerol, 8 ml of 1.0 M potassium citrate (pH 7.6), 8 ml of 1.0 M KCl, 0.8 ml of 0.1 M dithiothreitol, and 4.1 ml of 10% sodium deoxycholate, and the clarified solution which resulted was centrifuged at 165,000 × *g* for 90 min. Over 90% of the CO-binding pigment was recovered in the supernatant fraction. This supernatant fraction was filtered through glass wool and diluted with 3 volumes of cold distilled water to a final protein concentration of 2–3 mg/ml, and 60 ml were applied to a column of DEAE-cellulose (2.5 × 30 cm) equilibrated with 0.1 M Tris-HCl buffer, pH 7.8, containing $10^{-4}$ M dithiothreitol and 0.05% sodium deoxycholate. The column was eluted with a linear concentration gradient of 0–0.5 M KCl in the Tris buffer solution described by Lu and Coon (1). The flow rate of the column was adjusted to 42–48 ml/hr, and the eluate was collected in 11–13-ml fractions.

Cytochrome $b_5$ was determined by the method of Omura and Sato (16, 17). Estimates of cytochromes P-420, P-450, and P-448 were made by bubbling CO through the contents of the sample cuvette, then reducing the contents of both cuvettes with $Na_2S_2O_3$, and measuring the difference spectra in an Aminco-Chance split-beam, dual-wavelength spectrophotometer in the split-beam mode.

Radioactivity in the column effluent was determined by taking 0.2 ml from each fraction and measuring the radioactivity in a liquid scintillation spectrometer utilizing the scintillation mixture of Bray (18). In double isotope experiments, the radioactivity was determined by the channels ratio method as described by Okita *et al.* (20).

## RESULTS

### *Turnover of Microsomal Hemoprotein*

We have repeated the studies by Greim and his associates and found that the specific activity of heme in CO-binding pigment in the adult rat does not decrease as a single exponential after administration of radioactive δ-ALA,[6] as they reported (14, 15); on the contrary, the half-life of radioactive heme from CO-binding particles increases with time (Fig. 1), as we previously reported in the immature rat (9–11). In addition, Table 1 shows that the ratioactivity in CO-binding particles (expressed as counts per minute per millimicromole of heme) is the same as the specific activity of heme from the CO-binding particles purified by the extraction procedure of Greim (15). These results indicate that little or no nonspecifically

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

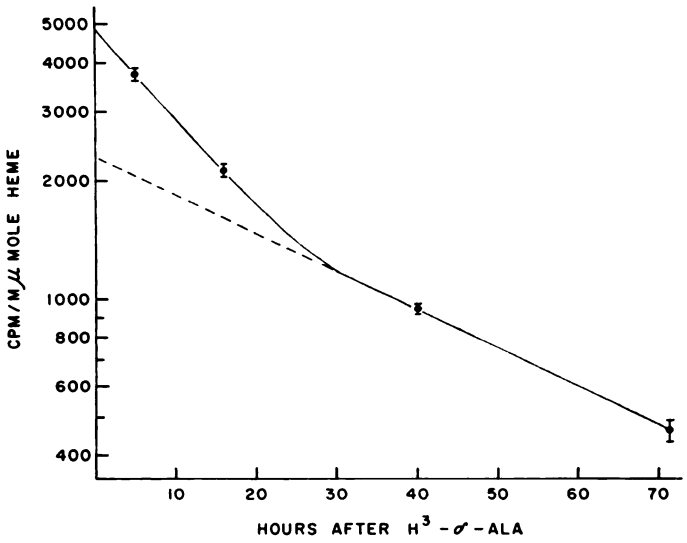


FIG. 1. *Rate of decline of radioactive heme from CO-binding particles*

$^3$H-δ-ALA (0.125 mg/kg; 500 mCi/mmole) was administered intravenously to adult male rats, which were killed at various times thereafter. CO-binding particles were prepared and the heme extracted as described in METHODS. Each value represents the mean ± standard error of eight rats.

bound heme is present in CO-binding particles. Interestingly, two differences were observed between the results obtained with adult Wistar rats and immature Long-Evans rats. The ratio of fast-phase hemoprotein to slow-phase hemoprotein in the adult male Wistar rat studied here is considerably lower than that previously found with the immature male Long-Evans rat, and the slow-phase hemoprotein in the adult Wistar rat has a $t_{1/2}$ of 32 hr while the $t_{1/2}$ in the immature Long-Evans rat is 46 hr.

*Column Chromatography of Solubilized Microsomes*

The CO difference spectra of the solubilized CO-binding pigment from microsomes of PB- and 3-MC-treated rats are shown in Fig. 2. Of the total amount of CO-binding pigment solubilized from microsomes from PB-treated rats, about 70% was recovered as cytochrome P-450 and 30% as cytochrome P-420, while over 90% of the CO-binding pigment from 3-MC-treated rats was recovered as cytochrome P-448. These findings suggest that cytochrome P-448 is more stable during the solubilization procedure than is cytochrome P-450.

*PB-treated rats.* Chromatography of the solubilized microsomal preparation from PB-treated rats that had received $^{14}$C-δ-ALA revealed that radioactivity was closely associated with the CO-binding hemoproteins and cytochrome $b_5$ rather than with absorbance at 280 mμ (Fig. 3). Studies using the $A_{280}$:$A_{260}$ ratio method (21) for protein determination revealed that in the first 55 fractions more than 90% of the absorbance at 280 mμ was due to protein, while large amounts of nucleic acid contributed to the 280 mμ absorbance in the remaining fractions.

A large amount of protein was eluted in the first 15 fractions, but little or no cytochrome P-420, P-450, or radioactivity was present. Spectral evidence indicated the presence of a small amount of hemoglobin in tubes 5–10; however, hemoglobin would not be labeled to any appreciable extent because of its extremely long half-life and because of the rapid elimination of δ-ALA from the body (22). In addition, δ-ALA, as opposed to glycine, is not a good precursor for hemoglobin heme (23).

Although 70% of the solubilized CO-binding pigment in liver microsomes from PB-treated rats was present as cytochrome P-450 prior to column chromatography, most

TABLE 1

*Specific activity of heme in CO-binding particles and purified heme obtained from CO-binding particles*

Adult male Wistar rats (140–150 g) received intravenous injections of $^3$H-δ-ALA (500 mCi/mmole; 0.125 mg/kg) and killed at various times thereafter. CO-binding particles and isolation of heme were measured by the method of Greim (15), as described in METHODS. The specific activity of heme in CO-binding particles was determined by measuring the radioactivity and heme in the CO-binding particles as described in the text. Values represent the means and standard errors for the livers of four individual animals, or the means of four pooled livers.

| Expt | Time after $^3$H-δ-ALA | CO-binding particles | Purified heme |
|---|---|---|---|
| | *hr* | *cpm/mμmole heme* | |
| 1 | 5 | 3462 ± 110 | 3514 ± 57 |
| | 16 | 2236 ± 23 | 2165 ± 43 |
| | 40 | 966 ± 43 | 966 ± 25 |
| | 72 | 525 ± 41 | 461 ± 33 |
| 2 | 5 | 3607 | 3979 ± 132 |
| | 16 | 2017 | 2117 ± 154 |
| | 40 | 917 | 928 ± 25 |
| | 72 | 483 | 475 ± 51 |

of the CO-binding pigment eluted from the column was in the form of cytochrome P-420, indicating that cytochrome P-450 is converted to cytochrome P-420 on the column. This is consistent with the finding that in addition to a sharp peak of cytochrome P-420 in tube 48 (0.24 M KCl), cytochrome P-420 was also found in high amounts in the fractions that contained cytochrome P-450. The recovery of radioactivity, cytochrome $b_5$, and material absorbing at 280 mμ from the column was approximately 70%, 85–90%, and 80%, respectively. The total recovery of CO-binding pigment was not determined precisely, since approximately 15 min were required for maximum absorption at 420 or 450 mμ after the addition of CO and subsequent reduction of the hemoprotein with dithionite. In the present study the absorption at 2 min after the addition of CO was used for the estimation of the relative amounts of cytochromes P-420 and P-450 in the various fractions.

*3-MC-treated rats.* Chromatography of the solubilized microsomal preparation from 3-MC-treated rats that had received $^3$H-δ-ALA 2 hr before sacrifice revealed that the elution of radioactivity paralleled the elution of CO-binding hemoprotein and cytochrome $b_5$ rather than absorbance at 280 mμ (Fig. 4). Since the radioactivity incorporated into microsomal CO-binding pigment at 2 hr after δ-ALA administration represents a combination of both fast- and slow-phase hemoprotein fractions (9–11), it was important to investigate the chromatographic profile of radioactivity at 72 hr after the administration of δ-ALA, which represents only the slow-phase hemoprotein fraction (9–11). Figure 5 shows the results obtained when solubilized microsomes obtained from 3-MC-treated rats killed 72 hr after $^3$H-δ-ALA were chromatographed. The elution profile of radioactivity and CO-binding pigment is very similar to that shown in Fig. 4. Since the radioactivity incorporated into cytochrome $b_5$ decreased more slowly than that incorporated into the CO-binding pigment, the major radioactive peak found when animals were killed 72 hr after the administration of $^3$H-δ-ALA was associated with cytochrome $b_5$ rather than with CO-binding hemoprotein (Figs. 4 and 5). This is to be expected, since the $t_{1/2}$ of cytochrome $b_5$ is approximately 2–2.5 days (14, 15, 24, 25) while the $t_{1/2}$ values of hemoproteins in CO-binding particles are 7 and 48 hr. In addition, maximum labeling of the CO-binding pigment heme after injection of radioactive δ-ALA has been shown to occur within 1 hr (9, 15), while maximum incorporation into cytochrome $b_5$ occurs between 12 and 20 hr after injection of the isotope (15, 25). Recoveries of radioactivity, cytochrome $b_5$, and material absorbing at 280 mμ from the column were essentially the same for solubilized microsomes prepared 2 or 72 hr after the administration of $^3$H-δ-ALA. It is interesting that the specific activity of the CO-binding pigment decreased by 80% over the 70-hr time interval studied (Figs. 4 and 5), while previous studies from our laboratory (9) have shown that the radioactivity in CO-binding particles obtained from 3-MC-treated rats decreased by 79% over the same 70-hr period.

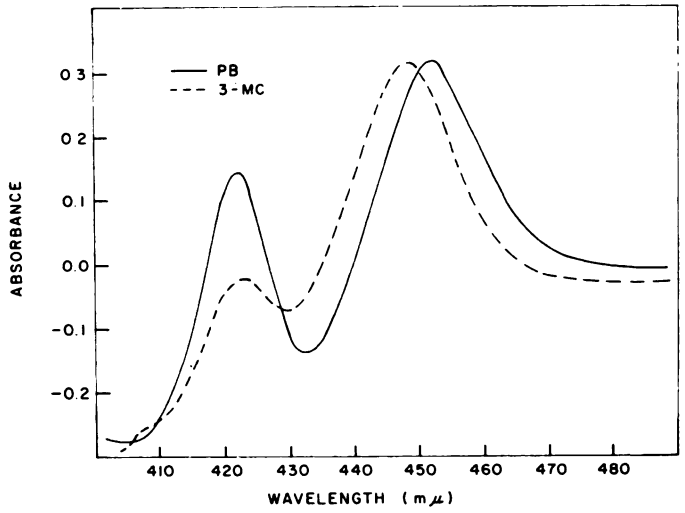


FIG. 2. *Effect of PB or 3-MC on CO-binding pigments in solubilized liver microsomes*
Immature male rats were treated with PB (37 mg/kg twice daily) or 3-MC (25 mg/kg once daily) for 4 and 3 days, respectively. Liver microsomes were prepared and solubilized as described in METHODS. The final protein concentration was 3 mg/ml. CO difference spectra were determined by the method of Omura and Sato (16, 17).

These results further strengthen the concept that the radioactivity incorporated into the CO-binding particles after an injection of radioactive δ-ALA is present as CO-binding hemoprotein and is not bound nonspecifically to microsomes. Since the elution profile of radioactivity was the same at 2 and 72 hr after the administration of labeled δ-ALA, these results also show that the CO-binding pigment which decreases with a $t_{1/2}$ of 7–8 hr has chromatographic characteristics similar to the pigment which decreases with a $t_{1/2}$ of 46–48 hr.

### *Column Chromatography of Solubilized CO-Binding Particles*

Figure 6 shows the CO difference spectrum of the solubilized hemoprotein obtained from CO-binding particles. The cytochrome P-450 from PB-treated rats was converted to cytochrome P-420 by steapsin treatment, while about 10–20% of the cytochrome P-448 from 3-MC-treated rats appears to have resisted conversion to cytochrome P-420. Attempts to convert cytochrome P-448 quantitatively to cytochrome P-420 were not successful. This suggests that in 3-MC-treated rats the hemoprotein or the environment surrounding it is more resistant to steapsin action than the hemoprotein from PB-treated animals, and thus complete conversion to cytochrome P-420 is prevented. As previously reported, cytochrome $b_5$ was not found in the CO-binding particles (9, 10, 16, 17).

*PB-treated rats.* Figure 7 shows that the radioactivity from solubilized CO-binding particles was eluted primarily in conjunction with the spectra of cytochrome P-420, the fraction containing the maximum content of radioactivity also containing the highest concentration of hemoprotein (tube 59; KCl concentration, 0.29 M). A measurable amount of radioactivity (10% of the total radioactivity eluted from the column) was associated with the large protein peaks eluted between tubes 5 and 15. In contrast to the results obtained with solubilized microsomal preparations, the absorbance at 280 mμ of all the samples obtained from chromatography of solubilized CO-binding particles was due primarily to protein, and little or no nucleic acid was present in the various fractions. Tube 6 contained a trace amount of a substance with the spectral characteristics of hemoglobin, while tube 11 contained a hemoprotein with maximum absorption at 420 mμ. Treatment of microsomes with

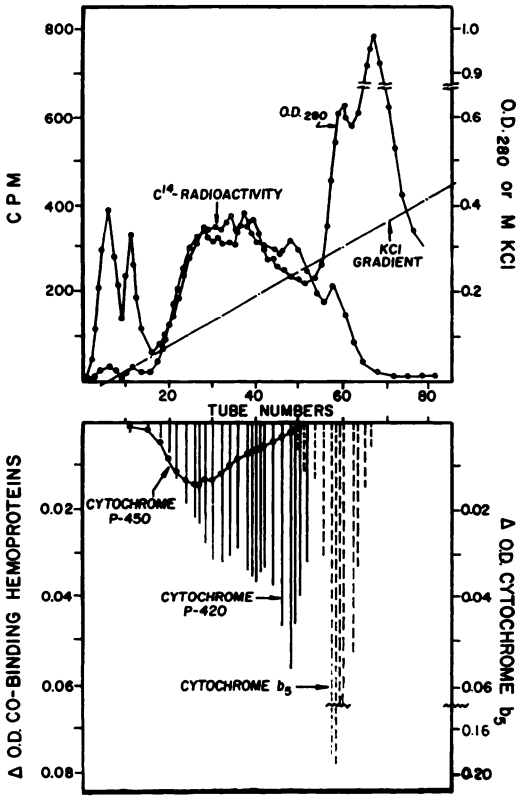


FIG. 3. *Chromatographic profile of solubilized microsomes from PB-treated rats 2 hr after administration of $^{14}$C-δ-ALA*

PB (37 mg/kg twice daily) was administered intraperitoneally to immature male rats for 4 days. On the fifth day, $^{14}$C-δ-ALA (0.5 mg/kg) was injected intravenously, and the rats were killed 2 hr later. Microsomes were prepared, solubilized, and chromatographed on a DEAE-cellulose column as described in METHODS.

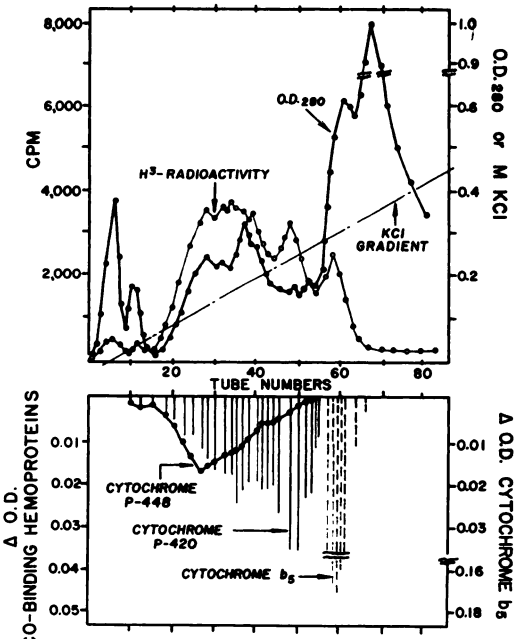


FIG. 4. *Chromatographic profile of solubilized microsomes from 3-MC-treated rats 2 hr after administration of $^3$H-δ-ALA*

3-MC (25 mg/kg once daily) was administered intraperitoneally to immature male rats for 3 days. On the fourth day, $^3$H-δ-ALA (0.5 mg/kg) was injected intravenously, and the rats were killed 2 hr later. Microsomes were prepared, solubilized, and chromatographed on a DEAE-cellulose column as described in METHODS.

steapsin during the preparation of CO-binding particles resulted in the complete solubilization of cytochrome $b_5$, and no evidence of this cytochrome was found in the column eluates obtained from CO-binding particles.

The elution profiles of microsomal hemoproteins at both 2 and 72 hr after radioactive δ-ALA administration was studied, since radioactivity in CO-binding particles at the earlier time represents label incorporated into both the fast- and slow-phase hemoprotein fractions, while radioactivity at the later time (72 hr) is associated with the slow-phase hemoprotein fraction (9, 10). To co-chromatograph solubilized CO-binding particles obtained from rats 2 and 72 hr after δ-ALA administration, either $^{14}$C- or $^3$H-δ-ALA was administered to rats which were killed 2 or 72 hr later, respectively. CO-binding particles were prepared, solubilized, and mixed so that 18% of the total radioactivity was $^{14}$C and 82% was $^3$H. The results of column chromatography of this mixed sample can be seen in Fig. 8. The recovery of $^{14}$C and $^3$H from the column was 60% and 63%, respectively. The $^{14}$C and $^3$H were eluted in an almost identical manner, and most of the radioactivity was associated with cytochrome P-420. The fraction containing the highest amounts of $^{14}$C and $^3$H, tube 59, also contained the highest concentration of cytochrome P-420. The major

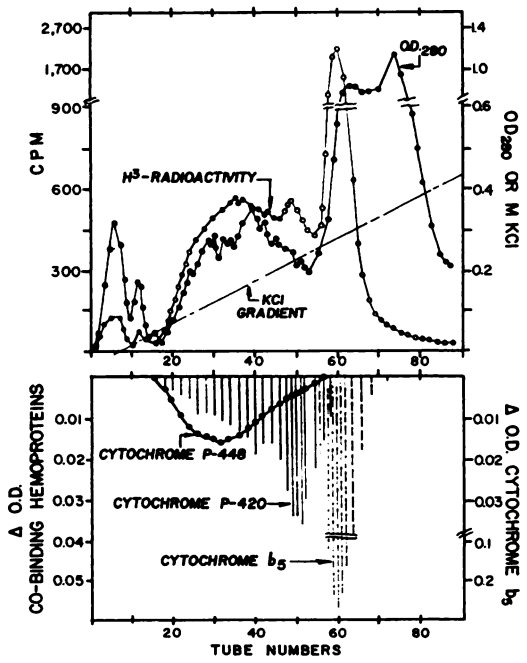


FIG. 5. *Chromatographic profile of solubilized microsomes from 3-MC-treated rats 72 hr after administration of ³H-δ-ALA*

3-MC (25 mg/kg once daily) was administered intraperitoneally to immature male rats for 6 days. On the fourth day, ³H-δ-ALA (0.5 mg/kg) was injected intravenously, and the rats were killed 72 hr later. Microsomes were prepared, solubilized, and chromatographed on a DEAE-cellulose column as described in METHODS.

difference in the elution of $^{14}C$ and $^{3}H$ can be seen in tubes 3–10. Since this difference between the 2- and 72-hr preparations was not seen when solubilized microsomes were chromatographed (Figs. 4 and 5), and for other reasons stated earlier, the steapsin treatment probably altered the slow-phase hemoprotein, which would be expected to predominate in the 72-hr sample.

*3-MC-treated rats.* Column chromatography of solubilized CO-binding particles from 3-MC-treated rats gave results different from those obtained with PB-treated rats (Fig. 9). Although the elution of material absorbing at 280 mμ was similar to that observed with PB-treated rats, a greater number of fractions contained large amounts of both cytochrome P-420 and radioactivity (compare Figs. 7 and 9). In addition, a large amount of cytochrome P-448 was eluted along with cytochrome P-420. This is in contrast to the results obtained with microsomes, in which cytochrome P-448 was eluted before the major portion of the cytochrome P-420 (Fig. 4). Trypsin activity present in the crude steapsin (16, 17) used to prepare CO-binding particles may account for the differences in elution properties of the hemoprotein present in microsomes and CO-binding particles; alternatively, under the conditions used, the CO-binding particles may not have been solubilized to the same extent as whole microsomes.

Rats treated with 3-MC received either $^{3}H$-δ-ALA (72 hr prior to sacrifice) or $^{14}C$-δ-ALA (2 hr prior to sacrifice), and the solubilized CO-binding particles were mixed and

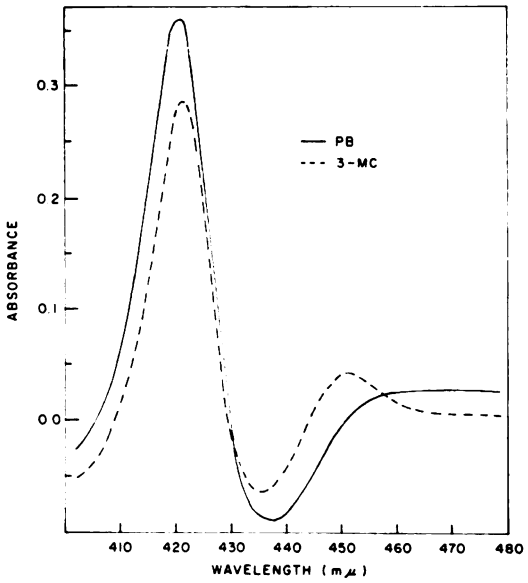


FIG. 6. *Effect of PB and 3-MC on CO-binding pigments in solubilized CO-binding particles (microsomes devoid of cytochrome $b_5$)*

Immature male rats were treated with PB (37 mg/kg twice daily) or 3-MC (25 mg/kg once daily) for 4 and 3 days, respectively. CO-binding particles were prepared and solubilized as described in METHODS. The final protein concentrations were 2.2 and 2.6 mg/ml, respectively. CO difference spectra were determined by the method of Omura and Sato (16, 17).

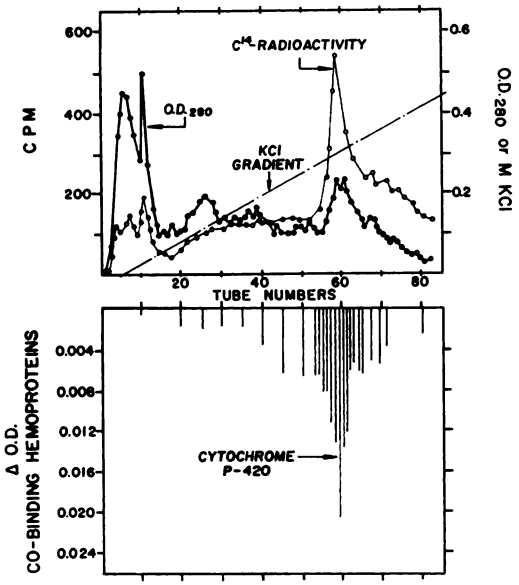


FIG. 7. *Chromatographic profile of solubilized CO-binding particles (microsomes devoid of cytochrome $b_5$) from PB-treated rats 2 hr after administration of $^{14}C$-δ-ALA*

PB (37 mg/kg twice daily) was administered intraperitoneally to immature male rats for 4 days. On the fifth day, $^{14}C$-δ-ALA (0.5 mg/kg) was injected intravenously, and the rats were killed 2 hr later. CO-binding particles were prepared, solubilized, and chromatographed on a DEAE-cellulose column as described in METHODS.

co-chromatographed. As was found with CO-binding particles obtained from PB-treated rats (Fig. 8), no large differences between the elution of $^{14}C$ (2 hr) or $^{3}H$ (72 hr) were observed (Fig. 10), except for the presence of more radioactivity in the early fractions derived from the 72-hr sample.

## DISCUSSION

The recent utilization of radioactive δ-aminolevulinic acid to label microsomal hemoprotein (9–11, 14, 15, 24–27) has made it possible to study the synthesis, degradation, and distribution of the cytochromes present in liver microsomes. The existence of more than one CO-binding hemoprotein fraction in liver microsomes of immature Long-Evans rats has been indicated by studies which demonstrated a biphasic decrease in radioactive hemoproteins in the CO-binding particles (microsomes devoid of cytochrome $b_5$) as reported by Levin, Alvares, and Kuntzman (9–11). However, Greim and his associates (14, 15) have reported a monophasic decrease in radioactive hemoprotein from CO-binding particles obtained from adult male Wistar rats treated with $^{14}C$-δ-ALA. They suggested that the discrepancy between the two laboratories could be due to the presence in our preparation of nonspecifically bound heme, and that extraction of the CO-binding particles with organic solvents removes this nonspecifically bound material. We have repeated the studies of Greim and his associates and found that the disappearance of radioactive hemoprotein from the CO-binding particles of the adult Wistar rat is not monophasic but is in fact biphasic, and that the specific

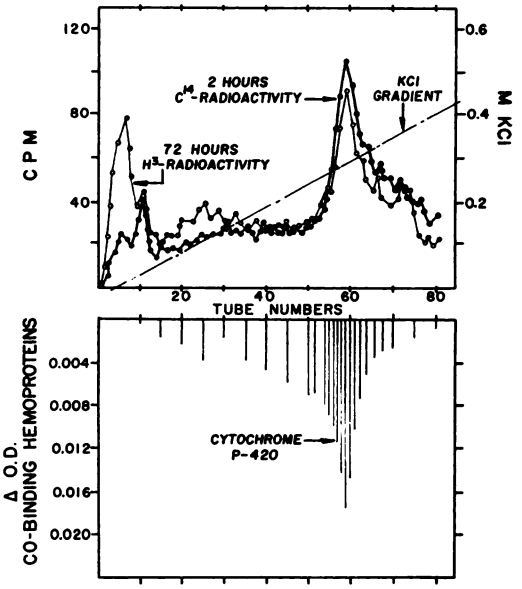


FIG. 8. *Chromatographic profile of solubilized CO-binding particles (microsomes devoid of cytochrome $b_5$) from PB-treated rats*

PB (37 mg/kg twice daily) was administered intraperitoneally to immature male rats for 4–7 days. On the fifth day, $^{14}C$- or $^{3}H$-δ-ALA (0.5 mg/kg) was injected intravenously, and the rats were killed 2 or 72 hr later, respectively. CO-binding particles were prepared, solubilized, and chromatographed on a DEAE-cellulose column after the two preparations had been mixed so that 18% of the total radioactivity was $^{14}C$ (2-hr sample) and 82% was $^{3}H$ (72-hr sample).

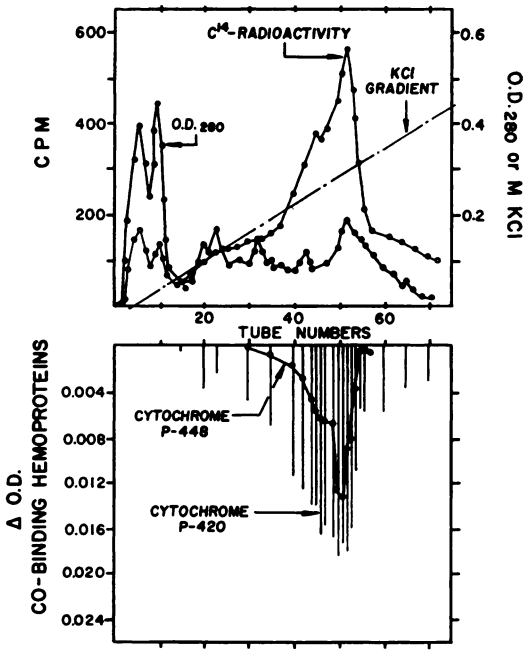


FIG. 9. *Chromatographic profile of solubilized CO-binding particles (microsomes devoid of cytochrome $b_5$) from 3-MC-treated rats 2 hr after administration of $^{14}C$-δ-ALA.*

3-MC (25 mg/kg once daily) was administered intraperitoneally to immature male rats for 3 days. On the fourth day, $^{14}C$-δ-ALA (0.5 mg/kg) was injected intravenously, and the rats were killed 2 hr later. CO-binding particles were prepared, solubilized, and chromatographed on a DEAE-cellulose column as described in METHODS.

activity of the CO-binding particles (expressed as counts per minute per millimicromole of heme) was identical with that of the extracted heme, indicating that little or no nonspecifically bound heme was present. The results presented above indicate that the ratio of the fast-phase to slow-phase hemoprotein in the adult Wistar rat is considerably lower than that previously reported for the immature Long-Evans rat, and that the $t_{1/2}$ of the slow-phase hemoprotein in the adult Wistar rat is somewhat shorter than that for the immature Long-Evans rat (9, 10). Similar results have recently been obtained by Meyer and Marver (28), who demonstrated a biphasic decrease of labeled microsomal hemoprotein in the adult male Sprague-Dawley rat. These investigators also indicated the absence of nonspecifically bound radioactivity and a lower ratio of fast- to slow-phase hemoproteins than that previously reported for the immature Long-Evans rat (9–11).

The results obtained here confirm that a biphasic disappearance of radioactive hemoprotein from rat liver CO-binding particles occurs, and our observations also suggest an age and/or strain difference in the hemoprotein content of CO-binding particles. Since age and strain differences in the metabolism of drugs and steroids are well recognized, similar variations in the turnover and ratio of microsomal hemoproteins are not surprising.

The present studies indicate that the administration of radioactive δ-ALA to rats leads to the incorporation of radioactivity into the CO-binding pigments cytochromes P-450 and P-448, as well as into cytochrome $b_5$. Column chromatography of microsomes

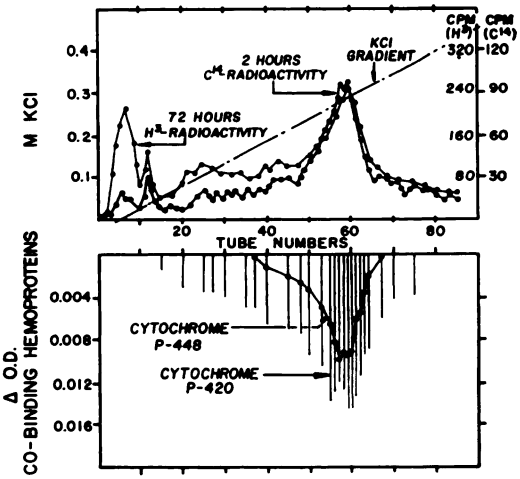


FIG. 10. *Chromatographic profile of solubilized CO-binding particles (microsomes devoid of cytochrome $b_5$) from 3-MC-treated rats*

3-MC (25 mg/kg once daily) was administered intraperitoneally to immature male rats for 3–6 days. On the fourth day, $^{14}C$- or $^{3}H$-δ-ALA (0.5 mg/kg) was injected intravenously, and the rats were killed 2 or 72 hr later, respectively. CO-binding particles were prepared, solubilized, and chromatographed on a DEAE-cellulose column after the two preparations had been mixed so that 18% of the total radioactivity was $^{14}C$ (2-hr sample) and 82% was $^{3}H$ (72-hr sample).

or CO-binding particles that had been solubilized according to the method of Lu and Coon (1) revealed that essentially all the radioactive label was associated with the fractions containing cytochromes $b_5$, P-450, and P-448, as well as with cytochrome P-420, a breakdown product of cytochromes P-450 and P-448. In contrast, using an identical column procedure, Alvares *et al.* (29) have shown that when $^3$H-3-MC was administered to rats the radioactivity remaining in microsomes 24 hr later was more closely associated with the protein absorbance at 280 mμ than with hemoprotein. These results indicate that the radioactivity in solubilized microsomes or CO-binding particles, which decreases biphasically after the administration of radioactive δ-ALA, is present in microsomal cytochromes rather than nonspecifically bound to other constituents of microsomes.

Column chromatography of solubilized microsomes from 3-MC-treated, immature Long-Evans rats 2 or 72 hr after the administration of radioactive δ-ALA revealed similar elution patterns of labeled CO-binding pigment and cytochrome $b_5$ at both time intervals. The radioactivity which chromatographed along with the CO-binding pigment decreased during the 70-hr period by the amount predicted from our previous studies, in which a biphasic decrease of radioactivity in CO-binding particles was measured (9–11). These observations provide additional evidence that little or no nonspecifically bound heme is present in CO-binding particles.

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