A NEW DEVICE FOR THE DETERMINATION OF MICROSONAL CYTOCHROME P-450 IN RENAL TISSUE PREPARATIONS FROM VARIOUS SPECIES CONTAMINATED WITH MITOCHONDRIA AND HEMOGLOBIN

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Abstract—Using a spectrophotometer connected to a microcomputer, the carbon monoxide difference spectrum of renal microsomal dithionite-reduced cytochrome P-450 was measured avoiding the effects of the contaminating mitochondrial cytochromes and hemoglobin by subtracting the CO difference spectrum of a succinate-treated microsomal suspension from that of a dithionite-treated one. By this method, we quantitatively determined the microsomal cytochrome P-450 in kidneys from various species. The absorption peak of the renal microsomal cytochrome P-450 was about 452 nm in rats and about 450 nm in mice, hamsters, rabbits, guinea pigs, dogs, and pigs. Compared with the renal cytochrome P-450 contents in rats, the contents on a g tissue basis were greater in pigs, dogs, and mice and were about the same in hamsters and rabbits. The renal cytochrome P-450 contents in guinea pigs were less than those in rats.

Cytochrome P-450 plays a major role in the metabolism of xenobiotics as a component of the hepatic microsomal mixed-function oxidase system (1). Cytochrome P-450 is also reported to be present in other organs such as kidney (2-4), lung (5), adrenals (6), and intestine (7). Among these organs, kidney is one of the most frequently reported for its cytochrome P-450 content and xenobiotic-metabolizing activities (3, 4). But renal microsomal fractions prepared by the usual method for the liver are contaminated by a large amount of hemoglobin and mitochondria (8). When these microsomes are treated with carbon monoxide (CO) under reduced conditions, hemoglobin and mitochondrial cytochromes exhibit peculiar changes in the absorbance around 450 nm and obscure the peak of the cytochrome P-450 spectrum. Moreover, the content of renal cytochrome P-450 is lower than that of the liver. For these reasons, a correct CO difference spectrum of microsomal cytochrome P-450 in the kidney is not obtainable by the method of Omura and Sato (9).

In this study, using a spectrophotometer connected to a microcomputer, we were able to obtain a CO difference spectrum of reduced renal microsomal cytochrome P-450 in which most of the influences of contaminating hemoglobin and mitochondria were excluded; and we determined the renal microsomal cytochrome P-450 contents of various species.
MATERIALS AND METHODS

Animals: Male rats (Wistar strain, 9–10 weeks of age), mice (ddy strain, 9 weeks), Syrian golden hamsters (9 weeks), guinea pigs (Hartley strain, 400–500 g), rabbits (Beagle strain, ca 4 kg), and male and female pigs (Göttingen miniature pig strain, 7 to 16 months) were used.

Preparation of microsomes: Rabbits were anaesthetized with sodium pentobarbital (80 mg/kg, i.v.). Pigs and dogs were asphyxiated with carbon dioxide and the other animals were killed by decapitation. Kidneys were then quickly removed, minced in ice-cold isotonic KCl solution (i-KCl), and were homogenized in a Potter-type Teflon-glass homogenizer in 3 volumes of ice-cold i-KCl (Figs. 1 and 5 and Table 2), 0.25M sucrose (Figs. 2, 3, and 6 and Table 1) or 20% glycerol solution in 0.1 M potassium phosphate buffer (pH 7.4) containing 10^-4 M EDTA and 10^-4 M dithiothreitol (Figs. 7, 8, and 9 and Table 3). The homogenate was centrifuged at 8,000 x g for 20 min, and the supernatant obtained was centrifuged further at 105,000 x g for 60 min to sediment the microsomes. The microsomal pellet was resuspended in the i-KCl (unwashed microsomes). For the purpose of removing the contaminating hemoglobin, the microsomal suspension was recentrifuged at the same force and suspended again in the i-KCl (washed microsomes).

To prepare the mitochondrial suspension, the kidney homogenate was centrifuged at 600 x g for 10 min, and the supernatant obtained was centrifuged at 8,000 x g for 20 min. To decrease the contaminating microsomes in the mitochondrial fraction, the precipitate obtained was resuspended in the i-KCl or 0.25M sucrose solution and recentrifuged at 2,000 x g for 20 min. The resultant precipitate was resuspended in the same solution and centrifuged again at the same centrifugal force. The pellet was suspended in the i-KCl, and this was used as the mitochondrial suspension.

Assay of cytochrome P-450: A "high sensitivity spectrophotometer SM401" connected up to a 16K bite microcomputer system 77-02 (Union Giken, Osaka, Japan) was used. This microcomputer has the ability to store 5 independent spectra in its memory, each of them consisting of 500 points in the scanning span. The accuracy of the wavelength is within 0.2 nm. Quartz cuvettes (1 cm light path, 4 ml) were used. The microsomal suspension (1.0 ml) obtained from 0.5 g of the wet kidney and 0.1 M sodium phosphate buffer (pH 7.4, 2.0 ml) was placed in three cuvettes. Succinate solution (150 mM, pH 7.4, 0.2 ml) was also placed in two cuvettes to reduce the contaminating mitochondrial cytochromes under anaerobic conditions. Using the succinate-free cuvette as a reference, the succinate-containing cuvettes were scanned between 400 nm and 500 nm repeatedly until reduction became maximum. Each of these two cuvettes containing a fully reduced microsomal suspension was used as a reference and a sample, and it was confirmed that there was no absorbance difference between them. Carbon monoxide (CO) gas was then bubbled through the sample cuvette, and the CO difference spectrum of the reduced mitochondrial cytochromes and contaminating hemoglobin (Spectrum A) was recorded. A few grains of dithionite were then added into the two cuvettes, and the CO difference spectra of the reduced mitochondrial cytochromes and contaminating hemoglobin (Spectrum B) were measured. From the difference between these two spectra, the CO difference spectrum of the reduced cytochrome P-450 was obtained (Spectrum B-A).

The content of cytochrome P-450 was...
calculated from the difference of optical density at 490 nm and the absorption peak around 450 nm using the molar extinction coefficient reported for hepatic microsomal cytochrome P-450 (91 mM⁻¹ cm⁻¹) (9).

Assays of protein and other enzymes: Protein was determined by the method of Lowry et al. (10). NADPH cytochrome c reductase, glucose 6-phosphatase, and cytochrome oxidase activities were determined by the method of Masters et al. (11), Schwanson (12), and Orii and Okunuki (13), respectively.

Chemicals: All chemicals were of commercially available analytical grade.

RESULTS

The renal microsomal fraction prepared by the method indicated in "Materials and Methods" was contaminated by a large amount of hemoglobin. When CO gas was bubbled through these microsomal suspensions after the addition of a few grains of dithionite, the contaminating hemoglobin showed an absorption peak at about 420 nm and a trough at about 434 nm, apart from the absorption peak of the cytochrome P-450 at about 456 nm (Fig. 1A). Therefore, when cytochrome P-450 was assayed by the method of Omura and Sato (9), the contaminating hemoglobin caused the shift in the peak of the cytochrome P-450 to the longer wavelength. The peak at 420 nm could be greatly reduced by the washing procedure, but the peak at about 456 nm was not influenced to as great an extent (Fig. 1A). Thus, the shift of the absorption peak of cytochrome P-450 seemed not to be caused mainly by the contaminating hemoglobin.

When the cytochrome P-450 was assayed by the method of Johannesen and DePierre (5) using ascorbate and phenazine ethosulfate to eliminate the influences of the contaminating hemoglobin and methemoglobin, two peaks were found close together, one at 428 nm and the other at 450 nm and they partly overlapped (Fig. 1B). Therefore it was difficult to make a quantitative analysis of the renal microsomal cytochrome P-450 by the method of Johannesen and DePierre (5).

It has been shown that the renal microsomal fraction was contaminated abundantly by mitochondria (8). When the reduced mitochondrial suspension was bubbled by CO gas, it exhibited a peculiar absorbance peak at 428 nm and a trough at 445 nm (Fig. 2). Thus there was the probability that the contaminating mitochondria obscured the peak of the CO difference spectrum of cytochrome P-450. This mitochondrial contamination was not completely eliminated by strengthening the centrifugal force (Fig. 3).

We then tried to obtain the CO difference spectrum of the reduced renal microsomal cytochrome P-450 without the influence of the contaminating hemoglobin and mitochondria. At first the renal microsomal suspension was treated with succinate under anaerobic conditions and the change of absorbance between 400 and 500 nm was followed until it reached a maximum (Figs. 4a and 5a). Because the addition of dithionite to the succinate-treated microsomal suspension did not influence the absorbance between 440 and 500 nm (the data for the microsomal fraction is not indicated in the figures), it was suggested that the contaminating mitochondrial cytochromes were completely reduced. When we used the mitochondrial suspension, the addition of a few grains of dithionite to the succinate-treated mitochondrial suspension had no effect on the absorbance between 440 and 450 nm (Fig. 6). The difference spectrum of the succinate-treated microsomal suspension which was heavily contaminated by hemoglobin has a peak at about 434 nm (Fig. 4a), and the CO difference spectrum of this microsomal suspension has a peak at about 418 nm and a trough at about 433 nm (Fig.
Fig. 1. Measurement of rat renal cytochrome P-450 by the method of (A) Omura and Sato (9), (B) Johannesen and Depierre (5), and (C) Jakobsson and Cinti (8). (A): Microsomal suspension (3.0 ml) obtained from 0.5 g of wet tissue was placed in each sample and reference cuvette. A few grains of dithionite were added to both cuvettes and the difference spectrum was recorded twice before and after bubbling CO through the sample cuvette. The CO difference spectrum of dithionite-reduced cytochrome P-450 was obtained from the difference between these spectra. a) Microsomes were prepared by the centrifugation (105,000×g for 60 min) of the postmitochondrial supernatant (unwashed microsomes). b) Microsomes were obtained by the recentrifugation (105,000×g for 60 min) of the microsomal suspension used in a) (washed microsomes). (B): The microsomal suspension was adjusted to 250 μM in ascorbate and 2.5 μM in phenazine ethosulfate, and CO was bubbled through this suspension for 1 min. These suspensions were divided between two cuvettes, and the cytochrome P-450 was determined as described in (B). (C): Washed microsomal suspension (6.0 ml) obtained from 1.0 g of wet tissue was adjusted to 5 mM in succinate, then CO was bubbled through for 1 min. This suspension was divided between two cuvettes and the cytochrome P-450 was determined as described in (B).
4b). The characteristics of these spectra were about the same as those of deoxyhemoglobin (data not indicated in the figure). These data suggested that the contaminating hemoglobin was also changed into deoxyhemoglobin by the succinate treatment. When there was little contamination of hemoglobin, a trough at about 445 nm which is peculiar to the

**Fig. 2.** Measurement of CO difference spectrum of rat renal mitochondrial suspension. Mitochondrial suspension (3.0 ml) obtained from 0.125 g of wet tissue was placed in each cuvette. a) The mitochondrial suspension was reduced by the addition of succinate in both sample and reference cuvettes and the spectrum was obtained by the difference between the absorbances before and after bubbling CO through the sample cuvette. b) The spectrum was obtained from the difference between the absorbance before and after the addition of a few grains of dithionite to both the sample and reference cuvettes of a).

**Fig. 3.** Sedimentation of rat renal microsomes and mitochondria estimated by the activities of the marker enzymes. Glucose 6-phosphatase (G6Pase) and cytochrome oxidase were used as microsomal and mitochondrial marker enzymes, respectively. As a homogenizing solution, 0.26 M sucrose was used in this experiment. Almost the same results were obtained with 20% glycerol solution in 0.1 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 0.1 mM dithiothreitol.

**Fig. 4.** Measurement of rat renal microsomal CO difference spectrum of reduced cytochrome P-450 contaminated by hemoglobin (ca. 15 µg/ml). Microsomal suspension (3.0 ml) obtained from 0.5 g of wet tissue was placed in each cuvette. a) Succinate-induced changes of difference spectrum. Times after the addition of succinate (10 mM) were indicated in the figure. b) CO-induced difference spectrum of succinate-treated renal microsomes. c) CO-induced difference spectrum of dithionite-treated renal microsomes. d) CO-induced difference spectrum of microsomal cytochrome P-450 was calculated by the subtraction of b) from c).
mitochondrial cytochrome oxidase appeared in the CO difference spectrum (Fig. 5b). Accordingly, using these succinate-treated microsomal suspensions, we obtained the CO difference spectrum of the contaminating reduced mitochondria and deoxyhemoglobin in the microsomal fraction (Figs. 4b and 5b). The spectrum was stored in the microcomputer memory, a few grains of dithionite were added to both the reference and sample cuvettes, and the CO difference spectrum of the reduced microsomes and mitochondria and deoxyhemoglobin was obtained (Figs. 4c and 5c). From the difference between these spectra we obtained the CO difference spectrum of the dithionite-reduced microsomal cytochrome P-450 (Figs. 4d and 5d). In these spectra of the rat, there was a peak at about 452 nm and a small peak at about 420 nm. The wavelength of the latter peak is about the same as that of the peak of the CO difference spectrum of the deoxyhemoglobin, and the height of the peak was increased by storing the microsomal suspension under an aerobic condition at 4°C and decreased by washing. Thus this peak at about 420 nm seemed to be caused mostly by the me-

Fig. 5. Measurement of rat renal microsomal CO difference spectrum of reduced cytochrome P-450 in which contamination by hemoglobin was much less than that used in Fig. 4 (ca. 3 μg/ml). See the legend of Fig. 4 for other details.

Fig. 6. Reduction of washed mitochondria by succinate and dithionite. Mitochondrial suspension (3.0 ml) obtained from 0.125 g of rat renal wet tissue was placed in each cuvette. a) Reduction by succinate (10 mM). Times after the addition of succinate (10 mM) were indicated in the figure. b) Reduction by the further addition of a few grains of dithionite.

<table>
<thead>
<tr>
<th>Microsome (ml)</th>
<th>Mitochondria (ml)</th>
<th>Content of cytochrome P-450 (nmol/cuvette)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.0</td>
<td>0</td>
<td>exp. 1</td>
</tr>
<tr>
<td>1.0</td>
<td>0.1</td>
<td>1.06</td>
</tr>
<tr>
<td>1.0</td>
<td>0.2</td>
<td>1.13 (1.12)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.3</td>
<td>1.18 (1.19)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.4</td>
<td>1.22 (1.25)</td>
</tr>
<tr>
<td>1.0</td>
<td>0.5</td>
<td>1.32 (1.32)</td>
</tr>
<tr>
<td>0</td>
<td>0.5</td>
<td>0.31</td>
</tr>
</tbody>
</table>

Washed microsomes and mitochondria from 0.25 g of wet tissue in 1.0 ml of each suspension were used. In exp. 1, i-KCI was used to resuspend the 8,000×g precipitated fraction, and in exp. 2, 0.25 M sucrose was used. Figures in parentheses are expected values calculated from the data obtained for the microsomes or mitochondria only. n.d.: not-detected.
hemoglobin which could be reduced to the deoxyhemoglobin by dithionite, but not by succinate (data was not shown). The probability that cytochrome P-420 plays some role in forming this peak at about 420 nm seems to be small. Because this peak was small and did not increase on storing the microsomal suspension under anaerobic conditions, we can ignore the influence of the contaminating methemoglobin on this cytochrome P-450 assay. When cytochrome P-450 was assayed by the method described here using succinate, the determination was also not influenced by the addition of the renal mitochondria to the microsomal suspensions (Table 1).

When this method was applied to the renal homogenate, a peak at about 420 nm caused by the contaminating hemoglobin interfered with the cytochrome P-450 determination, and a clear spectrum was not obtained. However, when the contaminating hemoglobin was reduced by precipitating the fraction by centrifugation and resuspending, the cytochrome P-450 determination became possible by this method (Fig. 7).

When we compared this method with the method of Omura and Sato (9), the cytochrome P-450 content was 23–25% greater in our method (Table 2).

When we measured rat renal microsomal cytochrome P-450 by the method of Jakobsson et al. (8), the spectrum obtained

![Graph](image)

**Table 2. Determination of rat renal microsomal cytochrome P-450 obtained from various fractions**

<table>
<thead>
<tr>
<th>Microsomal fraction*</th>
<th>Content of cytochrome P-450 (nmole/g)</th>
<th>Cytochrome oxidase activity###</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Succinate## (A)</td>
<td>Omura's### (B)</td>
</tr>
<tr>
<td>5,000 x g</td>
<td>3.95</td>
<td>3.02</td>
</tr>
<tr>
<td>7,000 x g</td>
<td>3.68</td>
<td>2.73</td>
</tr>
<tr>
<td>9,000 x g</td>
<td>3.17</td>
<td>2.42</td>
</tr>
<tr>
<td>10,000 x g</td>
<td>3.12</td>
<td>2.38</td>
</tr>
</tbody>
</table>

*: Microsomal fractions were prepared by centrifugation (105,000 x g for 60 min) of the supernatant fraction obtained by centrifugation of the supernatant fraction (600 x g for 10 min) at the force indicated in the table. These were washed once as indicated in the legend of Fig. 1A. ##: indicates the data obtained by the method described in "Materials and Methods". ###: Indicates the data obtained by the method of Omura and Sato (9). ####: Indicates the percent activity of the 600 x g 10 min supernatant fraction.
had a horizontal part between 480 nm and 500 nm, but there were two peaks overlapping each other, one at 426 nm and the other at 451-452 nm (Fig. 1C). When we compared our method with that of Jakobsson and Cinti (8), there was a good correlation in the quantitative data for renal microsomal cytochrome P-450 between them (Fig. 8). But when we use 105 mM$^{-1}$ cm$^{-1}$ (5) as a molar extinction coefficient for the dithionite difference spectrum of the CO-treated microsomal cytochrome P-450 obtained by the method of Jakobsson and Cinti (8), the content was about 7% lower than that obtained by the

![Graph of Fig. 8](image_url)

Fig. 8. Correlation between cytochrome P-450 determinations of rat kidney obtained by the method of Jakobsson and Cinti (8) (ordinate) and the method described in "Materials and Methods" (abscissa).

![Graph of Fig. 9](image_url)

Fig. 9. Species difference of renal microsomal cytochrome P-450. Washed microsomes (3.0 ml) obtained from 0.5 g of wet tissue were used.

<table>
<thead>
<tr>
<th>Species</th>
<th>Peak of cytochrome P-450 (nm)</th>
<th>Content of cytochrome P-450 (n mole/g tissue)</th>
<th>Content of cytochrome P-450 (n mole/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mouse</td>
<td>449.7±0.1 (7)</td>
<td>4.31±0.36 (7)</td>
<td>0.240±0.010 (7)</td>
</tr>
<tr>
<td>Rat</td>
<td>451.9±0.1 (27)</td>
<td>2.35±0.17 (19)</td>
<td>0.161±0.007 (19)</td>
</tr>
<tr>
<td>Hamster</td>
<td>450.3±0.2 (5)</td>
<td>2.36±0.07 (7)</td>
<td>0.219±0.021 (7)</td>
</tr>
<tr>
<td>Guinea pig</td>
<td>450.7±0.2 (6)</td>
<td>1.65±0.14 (7)</td>
<td>0.147±0.017 (7)</td>
</tr>
<tr>
<td>Rabbit</td>
<td>450.0±0.0 (5)</td>
<td>2.72±0.32 (7)</td>
<td>0.156±0.018 (7)</td>
</tr>
<tr>
<td>Dog</td>
<td>450.8±0.0 (3)</td>
<td>5.08±1.04 (3)</td>
<td>0.223±0.040 (3)</td>
</tr>
<tr>
<td>Pig*</td>
<td>450.4±0.1 (4)</td>
<td>15.28±3.49 (3)</td>
<td>0.732±0.145 (3)</td>
</tr>
<tr>
<td>female</td>
<td>450.3±0.1 (5)</td>
<td>6.35±0.52 (5)</td>
<td>0.287±0.019 (5)</td>
</tr>
</tbody>
</table>

The values in the table represent the means±S.E.M., and the numbers of determinations are given in parentheses. *: In these animals, the renal cortex was used to prepare the microsomal fraction, and in the other animals, whole kidney except for papilla was used. **: Cytochrome P-450 content was calculated from the molar extinction coefficient (91 M$^{-1}$ cm$^{-1}$) determined for hepatic cytochrome P-450 (9).
method reported in this paper.

The peak of the CO difference spectrum of the dithionite-reduced renal microsomal cytochrome P-450 was about 452 nm in rats as previously indicated in this paper. In other species such as mice, hamsters, guinea pigs, rabbits, dogs, and pigs, the peak was at about 450 nm (Fig. 9, Table 3). Compared with the results from rats, the cytochrome P-450 contents of pigs, dogs, and mice were greater and those of guinea pigs lower. The cytochrome P-450 contents of hamsters and rabbits were about same as in rats.

**DISCUSSION**

For the determination of cytochrome P-450, Johannesen and DePierre (5) pretreated a lung microsomal suspension with phenazine ethosulfate and ascorbate to decrease the influence of contaminating methemoglobin. However, this method was disadvantageous for the renal microsomes since in addition to the absorbance peak at about 450 nm, there was another peak at about 428 nm caused by microsomal cytochrome b5, and these peaks overlapped each other. Moreover, the influence of the contaminating mitochondrial cytochromes could not be ignored for the renal microsomal cytochrome P-450 determination.

Jakobsson and Cinti (8) pretreated the renal microsomal suspension with succinate to eliminate the influence of the contaminating mitochondrial cytochromes and obtained the dithionite-difference spectrum of cytochrome P-450. In this method, there was also an absorbance peak at about 428 nm which affects the determination of cytochrome P-450 as mentioned by Johannesen and DePierre (5).

In this communication, we obtained the CO difference spectrum of the dithionite-reduced renal microsomal cytochrome P-450 of various species in which most of the influences of the contaminating hemoglobin and mitochondrial cytochromes were excluded and found that the peak of the CO difference spectrum of the reduced renal microsomal cytochrome P-450 in rats was about 452 nm. This was the same wavelength as reported by Ellin and Orrenius (14) for rats and by Ichihara et al. (15) for a partially purified porcine renal microsomal cytochrome P-450. On the other hand, the peak of renal cytochrome P-450 was about 450 nm in mice, hamsters, guinea pigs, rabbits, dogs, and pigs. The difference between our results and those of Ichihara et al. (15) in pigs cannot be clearly explained at present. However, in a recent paper, Masters et al. (16) reported that the absorption peak of purified renal microsomal cytochrome P-450 obtained from the miniature pig was at 450 nm, so strain difference seems to be one of the causes.

We also indicate that there is a difference, by about 25%, between the results obtained by the method of Omura and Sato (9) and by the method described here with respect to the quantitative determination of the renal microsomal cytochrome P-450 contaminated with hemoglobin and mitochondria.

There was a cytochrome P-450-like peak in the CO difference spectra of the reduced mitochondrial fraction (Experiment 1 in Table 1). The presence of cytochrome P-450 in the renal mitochondria has been reported (17–19), so there was a probability that the mitochondria cytochrome P-450 was also measured by this method. However, as indicated in experiment 2, in Table 1, and in Fig. 2, we could not find the peak at 450 nm, and in Fig. 3, about 20% of the cytochrome P-450 was sedimented by using a lower centrifugation force (2,000×g for 20 min), in good correlation with the loss in the activity of glucose-6-phosphatase, a marker enzyme of microsomes. From these results, it was suggested that the peak at about 450 nm from the renal mitochondrial fraction resulted from the contaminating microsomal cytochrome.
P-450. On the other hand, succinate was the most effective electron donor for the cytochrome P-450 involving the renal mitochondrial 25-hydroxy-vitamin D₃-1-hydroxylase (19). Thus, even if cytochrome P-450 existed in the rat mitochondria, it would be reduced by the addition of succinate together with other cytochromes existing in the mitochondria and would not interfere with the cytochrome P-450 determination in the microsomal fraction.

As a conclusion, by the method using a microcomputer-equipped spectrophotometer, both qualitative and quantitative analyses of the renal microsomal cytochrome P-450 in tissue preparations contaminated with mitochondria and hemoglobin may be done relatively quickly.

REFERENCES