Characterization of a Rat Lung Microsomal Fraction Obtained by Sepharose 2B Ultrafiltration

Jorge Capdevila, Sten W. Jakobsson, Bengt Jernström, Otto Helia, and Sten Orrenius

Department of Forensic Medicine, Karolinska Institute, S-104 01 Stockholm 60, Sweden

SUMMARY

A new procedure for obtaining rat lung microsomes essentially free of interfering hemoproteins has been developed. The method includes Sepharose 2B column chromatography of the 12,000 × g supernatant of lung homogenates, followed by ultracentrifugation of the material eluted in the void volume. Microsomes isolated in this manner contain specific levels of cytochromes b₅ and P-450 and of NADPH-cytochrome c reductase that are among the highest ever reported for a rat lung microsomal fraction.

After treatment of rats with 3-methylcholanthrene, the specific content of cytochrome P-450 in lung microsomes is doubled and that of cytochrome b₅ increases 1.5 times. Several spectral differences between hepatic and lung microsomal cytochrome P-450 are apparent. In lung microsomes, the maximum of the reduced CO-bound cytochrome complex in a difference spectrum is at 453 nm for the noninduced hemoprotein and shifts to 451 nm after 3-methylcholanthrene induction. In contrast, no significant change in the ethylisocyanide difference spectra of reduced microsomes is obtained after induction; moreover, the spectra obtained with induced and noninduced cytochrome P-450 are similar to the one shown by hepatic microsomes from polycyclic hydrocarbon-treated rats. Furthermore, spectrophotometric studies on n-octylamine binding to control and induced lung cytochrome P-450 yielded results different from those previously obtained with rabbit liver microsomes.

It is concluded that the cytochrome P-450 present in rat lung microsomes before and after 3-methylcholanthrene treatment of the animals is distinctly different from the liver hemoprotein.

INTRODUCTION

The hepatic microsomal aryl hydrocarbon monooxygenase system has been extensively studied. The system is known to be inducible by polycyclic hydrocarbons, and it is now accepted that it metabolizes carcinogetic compounds to the corresponding arene epoxides by means of a cytochrome P-450-dependent mixed-function oxidase (5, 18, 19, 26). Evidence that these epoxides are the active intermediates in carcinogenesis is also accumulating (6, 12, 18).

The lung is the organ immediately exposed to carcinogenic compounds present in urban air and tobacco smoke; moreover, polycyclic hydrocarbons induce tumors in the respiratory tract of the rat (3), in other species (13, 30), and most probably in man. However, only a few reports concerning the properties of the lung microsomal electron transport components of different species have appeared in the literature (9, 11, 14, 28, 35) and, with the exception of a recent publication (23), little is known about the properties of the terminal oxidases of this cytochrome P-450 system.

Three main circumstances are probably responsible for this lack of knowledge with regard to lung microsomes. First, the lung contains large amounts of connective tissue, which make it difficult to homogenize. Second, the microsomal fractions obtained by differential centrifugation methods are almost always contaminated by hemoglobin. Third, the amounts of monooxygenase components in lung microsomes are very low compared with hepatic microsomes.

The aim of this study was to obtain a rat lung microsomal fraction suitable for spectral and catalytic studies. By applying a novel chromatographic procedure originally used by Tangen et al. (34) to isolate liver microsomes, a rat lung microsomal fraction, essentially free of interfering hemoproteins and with the highest specific levels of the electron transport components ever reported, was obtained. The effects of inducers on the rat lung mixed-function oxidase components, as well as some spectral properties of the induced and noninduced cytochrome P-450, are described.

MATERIALS AND METHODS

General. Male Sprague-Dawley rats (200 to 250 g) were used. The animals were kept in stainless steel cages in a room maintained at 25°C and 50% humidity and were given pelleted food (Anticimex Avelsfoder 213, supplied by Astra-Ewos AB, Södertälje, Sweden) and tap water ad libitum.

Microsomal fractions were prepared from both noninduced and induced rats. The inducer used was either PB or 3-MC.
in 0.9% NaCl (40 mg/ml) or 3-MC in corn oil (5 mg/ml), each administered by i.p. injection. The animals received 20 mg 3-MC per kg body weight daily for 3 days or 80 mg PB per kg body weight daily for 4 days. All animals were starved overnight before being killed by decapitation.

Preparation of Microsomes. To remove blood and bronchial fluids, the lungs were perfused in situ with 50 ml of ice-cold 0.15 M KCl solution through the right heart chamber via the truncus pulmonalis. The lungs were removed, dissected free of larger bronchi, and placed in ice-cold 0.15 M KCl. The tissue fragments were then washed 2 or 3 times with the same solution, placed between filter paper for drying, and subsequently weighed. Thereafter, the lungs were transferred to a 150 mM phosphate buffer, pH 7.5, containing 10% glycerol (v/v). The material was minced, washed twice with buffer, and homogenized in a glass-Teflon Potter-Elvehjem homogenizer with 25 strokes at 500 rpm. The resulting homogenate was diluted to a 20% suspension (w/v based on fresh tissue weight) and was spun twice at 12,000 g for 15 min each time. The supernatant was then spun at 105,000 g for 90 min. The pellets thus obtained were filtered through 2 layers of gauze and chromatographed on Sepharose 2B, essentially as described by Tangen et al. (34). In some cases the perfused and minced lung tissue was homogenized in 0.25 M sucrose as described above. The homogenate was diluted to a 20% suspension and spun twice at 12,000 × g. The resulting supernatant was then spun at 105,000 × g. The pelleted microsomal fraction was subsequently washed twice by ultracentrifugation with a 0.15 M KCl solution.

In the chromatographic step, a Sepharose 2B column (height, 40 cm; width, 5 cm) equipped with flow adapters was used (a generous gift of Pharmacia, Uppsala, Sweden). Elution was carried out by upward flow at a constant flow rate of 1.3 ml/min. The gel had been equilibrated with the homogenization buffer, which was also used for elution. Elution was continuously monitored with a LKB 4700 Uvicord at 259 nm, and the fractions corresponding to the void volume (V0) of the gel (cf. Chart 2) were pooled and spun down at 105,000 × g for 90 min. The pellets thus obtained were regarded as the microsomal fraction. Absorbance of the 2nd absorption peak shown in Chart 2 was measured at 420 nm and the fractions giving the highest absorption values were pooled and called the “hemoglobin fraction”. A summary of the procedure is shown in Chart 1.

Analytical Methods. Protein was determined according to the method of Lowry et al. (20), using bovine serum albumin as a standard. The reduced CO-bound cytochrome P-450 difference spectra were measured according to the method of Omura and Sato (27). The carboxyhemoglobin-balanced CO-difference spectra of cytochrome P-450 were obtained as follows. The rectangular tandem cells (Helma Cells, Inc., Jamaica, N. Y.), each containing 2 chambers one behind the other and having identical light paths (4.7 mm), were used. In the compartments facing the photomultiplier, a suspension of microsomes (1 mg protein per ml) reduced with dithionite was added, while to the other compartments an oxidized microsomal suspension was added and the base line was then recorded. Carbon monoxide was subsequently bubbled through the reduced suspension in the sample beam and through the oxidized suspension in the reference beam, and the difference spectrum was recorded. Cytochrome P-450 was determined by calculating the absorption difference between 450 and 490 nm in the resulting spectrum, assuming an extinction coefficient of 91 mM⁻¹·cm⁻¹, and making the necessary corrections for the difference in the light paths. This technique was devised in order to eliminate any spectral interference due to contaminating hemoglobin. Cytochrome b₅ was determined according to the method of Omura and Sato (27). NADPH-cytochrome c reductase activity was determined according to the method of Dalnlner (7), and succinate-cytochrome c reductase activity was determined by the method of Sottocasa et al. (33). The ethyl isocyanide difference spectra and the n-octylamine difference spectra were determined by the methods of Imai and Sato (15) and Jefcoate et al. (17), respectively.

All spectral studies were carried out in a 0.15 M phosphate buffer, pH 7.5, 10% v/v glycerol suspension using an Aminco DW-2 double-beam spectrophotometer set in the split beam mode. When conventionally prepared microsomes were used, glycerol was deleted.

Chemicals. 3-MC, benzo(a)pyrene, NADH, NADPH, cytochrome c and n-octylamine were purchased from Sigma Chemical Co., St. Louis, Mo. Carbon monoxide was obtained from Aga, Stockholm, Sweden. All other chemicals were of analytical grade and were obtained from a local commercial source (Kebo, Stockholm, Sweden) and used without further purification.

RESULTS

Isolation of Microsomes. As described in “Materials and Methods,” the lungs were perfused with a cold 0.9% NaCl...
solution before homogenization. This decreased the amount of hemoglobin in the homogenate, and most of the bronchial fluids present in the organs were also washed out. In experiments in which the perfusion was omitted, a 30 to 50% decrease in microsomal NADPH-cytochrome c reductase activity was obtained, possibly due to the presence of surfactants in the saliva and in the mucus which might release the enzyme from microsomes or inactivate it. Twenty % (w/v) homogenates gave the highest final yields of microsomal NADPH-cytochrome c reductase and cytochrome P-450, whereas lower or higher tissue concentrations resulted in losses of both components. Moreover, the use of a 20% homogenate yielded a microsomal fraction less contaminated by succinate-cytochrome c reductase activity. To avoid cytochrome P-450 conversion into cytochrome P-420, 10% glycerol was always included in the buffer system used during the Sepharose 2B filtration. When this was not done, a small shoulder at around 420 nm was consistently seen in the reduced, CO-bound cytochrome P-450 minus carboxyl hemoglobin difference spectrum recorded by double-chambered cuvets, as described in "Materials and Methods." The hemoglobin contamination in the final microsomal preparation was also highly dependent on the ionic strength of the buffer system used. Concentrations of phosphate buffer lower than 150 mM resulted in an increase of microsomal-bound hemoglobin.

In Chart 2, the elution profile obtained after Sepharose 2B column chromatography of the 12,000 x g supernatant is shown. The microsomal fraction was eluted in the V₀ of the column, far separated from the 2nd absorption peak representing the cytosol and hemoglobin. The Kᵥ values for the microsomal fraction and the hemoglobin were 0.002 and 0.842, respectively.

Table 1 shows the recoveries and the relative specific activities of NADPH-cytochrome c reductase (used as a marker for endoplasmic reticulum) and succinate-cytochrome c reductase (used as a mitochondrial marker) measured in 6 different fractions obtained during the isolation of the microsomal fraction. With regard to the protein distribution, it is important to point out that the values shown are related to the weight of the perfused material that contained substantial amounts of the perfusion medium. Forty-eight % of the protein present in the filtered homogenate was lost in the 12,000 x g pellet. Almost 100% of the protein loaded into the column could be recovered in the 2 fractions. However, for the sake of the purity of the microsomal fraction, the material was pooled, as shown in Chart 2, and does not represent all the eluted protein in the absorption peak. Of the total protein present in the 12,000 x g supernatant and loaded into the Sepharose 2B column, 7.5% was collected at the V₀ of the gel and 59% was recovered in the so-called hemoglobin fraction which, in this case, represents a mixture of cytosol and soluble proteins. Only 34% of the NADPH-cytochrome c reductase activity present in the homogenate was recovered in the 12,000 x g supernatant and, moreover, its specific activity was decreased, indicating a loss of activity probably due to an incomplete homogenization procedure. As shown in the table, about 84% of the enzyme activity loaded onto the column was recovered in the pooled fractions at the V₀; its relative specific activity was increased 7.8 times above the activity of the homogenate. The total recovery of microsomal...
real-bound enzyme activity was much lower than that reported for rat liver preparations (8) and somewhat lower than the values given by Hook et al. (14) for rabbit lung. The microsomal fraction was contaminated to a small extent by succinate-cytochrome c reductase activity but, on the other hand, there was no spectral manifestation of cytochrome oxidase in the reduced versus oxidized spectra when either dithionite or succinate was used as reducing agent.

Fig. 1 shows an electron micrograph of the microsomal fraction isolated by the Sepharose 2B column chromatography method. Closed vesicles of different sizes surrounded by a double-layer membrane are abundant. No mitochondrial structures can be identified. However, some contamination

Table 1

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Protein mg/g lung wet wt</th>
<th>% recovery</th>
<th>NADPH cytochrome c reductase</th>
<th>% recovery</th>
<th>Relative specific activity*</th>
<th>% recovery</th>
<th>Relative specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>27.7 ± 4.5</td>
<td>100</td>
<td>100</td>
<td>1.0</td>
<td>100</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>12,000 × g pellet</td>
<td>13.3 ± 2.6</td>
<td>48.0</td>
<td>60.1 ± 4.1</td>
<td>1.2</td>
<td>89.0 ± 7.0</td>
<td>1.86</td>
<td></td>
</tr>
<tr>
<td>12,000 × g supernatant</td>
<td>13.6 ± 2.4</td>
<td>49.0</td>
<td>34.3 ± 3.0</td>
<td>0.70</td>
<td>5.3 ± 0.5</td>
<td>0.11</td>
<td></td>
</tr>
<tr>
<td>V0 Sepharose</td>
<td>1.03 ± 0.08</td>
<td>3.7</td>
<td>28.8 ± 3.2</td>
<td>7.8</td>
<td>1.8 ± 0.4</td>
<td>0.48</td>
<td></td>
</tr>
<tr>
<td>Microsomes</td>
<td>1.02 ± 0.09</td>
<td>3.6</td>
<td>25.2 ± 3.5</td>
<td>7.1</td>
<td>1.2 ± 0.3</td>
<td>0.33</td>
<td></td>
</tr>
<tr>
<td>Hb fraction</td>
<td>8.1 ± 2.0</td>
<td>29.0</td>
<td>4.7 ± 0.9</td>
<td>0.16</td>
<td>0</td>
<td>0</td>
<td></td>
</tr>
</tbody>
</table>

*The relative specific activity (purification factor) is the specific activity of the respective fraction divided by the specific activity of the homogenate. The specific activities in the homogenate were for NADPH cytochrome c reductase and succinate cytochrome c reductase 11.0 ± 1.5 and 9.85 ± 1.0 nmoles cytochrome c reduced per min per mg protein, respectively.

Hb, hemoglobin.
by Golgi profiles cannot be excluded. The vast majority of the vesicles resemble the smooth ER isolated from rat liver and no definite rough ER structures are seen. However, the presence of rough ER, as well as of free ribosomes, has been reported for rabbit lung microsomal preparations (14). The apparent lack of these structures in our samples could be due to a washing effect during the chromatography step.

When microsomes from the same pool of lungs were isolated either by the Sepharose 2B method or by the conventional ultracentrifugation procedure, described in "Materials and Methods," the results given in Table 2 were obtained. The specific levels of NADPH-cytochrome c reductase and cytochromes P-450 and b5 in the Sepharose 2B-isolated microsomes were about twice those obtained by the conventional procedure. Moreover, the total yield of NADPH-cytochrome c reductase was higher, and the contamination by succinate-cytochrome c reductase was considerably lower, in the case of the Sepharose preparation. Most important, however, was the much lower hemoglobin contamination obtained in the case of the Sepharose microsomes. Thus, the ratio between nmoles of cytochrome P-450 per mg of protein and nmoles hemoglobin per mg of protein was 33.4 in the case of the Sepharose microsomes, and 0.29 in the case of the conventionally isolated microsomes. It should be pointed out that the microsomal fraction isolated by the ultracentrifugation method was washed twice and that repeated washing did not result in any marked decrease in the hemoglobin contamination.

Spectral Studies. The possibility of isolating lung microsomes with a fairly low hemoglobin contamination provided by the Sepharose 2B column chromatography procedure permitted spectral studies of the microsomal hemoproteins before and after pretreatment with inducers, such as PB and 3-MC.

In contrast to rat liver microsomes, PB treatment did not increase the specific content of cytochrome P-450 of b4, the microsomal protein content, or the specific activity of NADPH-cytochrome c reductase. On the other hand, as shown in Table 3, 3-MC treatment did result in an induction of cytochrome P-450 (217% of control), cytochrome b4 (144% of control) and NADPH-cytochrome c reductase (133% of control). There was no obvious increase in the content of microsomal protein after 3-MC treatment. The values given in Table 3 are the maximum that could be obtained after 3 days of treatment. Longer periods of pretreatment did not result in any further increase in the specific activities or concentrations of the enzymes studied.

The CO difference spectra of microsomes from control and 3-MC treated rats are shown in Chart 3, A and B. In both cases, Curve 1 shows the difference spectrum obtained by scanning a CO-bubbled sample cuvet against a reference cuvet containing microsomes. The symmetric absorption peak with a maximum at 423 nm represents the carboxy hemoglobin still present in the microsomes. Although, in the absolute spectrum, the Soret band of carboxy hemoglobin is at 420 nm, in our CO difference spectra of the microsomal suspension the peak is at 423 nm, probably due to some unbalanced oxy- and deoxyhemoglobin. Curves 2 in Chart 3, A and B, show CO difference spectra of dithionite-reduced microsomes obtained according to the method of Omura and Sato (27). Control microsomes showed one absorption peak at about 421 nm, mainly representing contaminating carboxy hemoglobin, and another at 453 nm, corresponding to the CO-bound reduced lung cytochrome P-450. The spectra obtained with microsomes from 3-MC-treated rats were similar, but the 2nd absorption peak was now at 451 nm. To obtain a CO difference spectrum of reduced cytochrome P-450 free of spectral interference by hemoglobin, the device shown in Chart 3, inset, was used. Curve 3 of Chart 3, A and B, represent such carboxy hemoglobin-balanced CO difference spectra of the control and 3-MC-induced hemoproteins. With control microsomes, the absorption maximum was again at 453 nm and shifted 2 nm to the blue after 3-MC induction.

Lung microsomes from either control or 3-MC-treated rats showed no type I binding spectrum with hexobarbital or aminopyrine. This lack of binding was observed when either Sepharose 2B or conventionally prepared microsomes were used. Furthermore, SKF 525-A at high concentrations (around 20 mM) produced only a very weak spectral

---

### Table 2

Comparison of 2 different preparations of rat lung microsomes

The values given are the means of at least 4 experiments ± S.D. The microsomes were prepared as described in "Materials and Methods," using control rats.

<table>
<thead>
<tr>
<th></th>
<th>Protein (mg/g tissue)</th>
<th>NADPH cytochrome c reductase (units/mg protein/min)</th>
<th>Succinate cytochrome c reductase (units/mg protein/min)</th>
<th>Cytochrome P-450 (nmoles/mg protein)</th>
<th>Cytochrome b4 (nmoles/mg protein)</th>
<th>Hemoglobin A (423-408/mg protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sepharose microsomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific activity or content% recovery</td>
<td>1.02 ± 0.09</td>
<td>78.1 ± 4.6</td>
<td>3.25 ± 0.6</td>
<td>0.084 ± 0.014</td>
<td>0.101 ± 0.009</td>
<td>0.0025 ± 0.0002</td>
</tr>
<tr>
<td>% recovery</td>
<td>3.6 ± 0.8</td>
<td>25.2 ± 3.5</td>
<td>1.2 ± 0.3</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ordinary microsomes</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Specific activity or content % recovery</td>
<td>1.75 ± 0.25</td>
<td>33.4 ± 6.0</td>
<td>15.5 ± 1.8</td>
<td>0.043 ± 0.009</td>
<td>0.071 ± 0.011</td>
<td>0.164 ± 0.02</td>
</tr>
<tr>
<td>% recovery</td>
<td>6.0 ± 1.8</td>
<td>11.5 ± 2.1</td>
<td>10.4 ± 1.25</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*1 unit = 1 n mole cytochrome c reduced.

* Refer to Table 1.
Table 3
Effect of 3-MC treatment on Sepharose 2B-isolated rat lung microsomes
The values are given as the mean of 10 experiments ± S.D. Isolation of microsomes, treatment of animals, and enzyme measurements were done as described in “Materials and Methods.”

<table>
<thead>
<tr>
<th></th>
<th>Protein (mg/g tissue)</th>
<th>Cytochrome $b_5$ (nmole/mg protein)</th>
<th>Cytochrome P-450 (nmole/mg protein)</th>
<th>NADPH cytochrome c reductase (units/mg protein)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>1.016 ± 0.085</td>
<td>0.101 ± 0.009</td>
<td>0.084 ± 0.014</td>
<td>78.1 ± 4.6</td>
</tr>
<tr>
<td>3-MC induced</td>
<td>1.15 ± 0.17</td>
<td>0.145 ± 0.0010</td>
<td>0.182 ± 0.024</td>
<td>104 ± 14</td>
</tr>
<tr>
<td>% of controls</td>
<td>113.2</td>
<td>144.0</td>
<td>217.0</td>
<td>133.3</td>
</tr>
</tbody>
</table>

$^a$ 1 unit = 1 nmole cytochrome $c$ reduced in 1 min.

Chart 3. CO difference spectra of lung microsomes from control (A) and 3-MC-treated (B) rats. Curves 1, CO difference spectra of oxidized microsomes: Curves 2, CO difference spectra of dithionite-reduced microsomes. (Curves 1A and 2A, 0.86 mg protein per ml; Curves 1B and 2B, 0.30 mg protein per ml). Spectra 1 and 2 were recorded using single-chambered cuvets. Curves 3, carboxyhemoglobin balanced, CO difference spectra of dithionite-reduced microsomes. Spectra 3 were recorded using the system shown in the inset. The graphs show the CO difference spectra of dithionite-reduced cytochrome P-450 for a light path of 4.7 mm. (Curve 1A, 1.72 mg protein per ml; Curve 1B, 0.60 mg protein per ml). – –, base lines. Base line corrections did not change the apparent peak positions. See “Materials and Methods” for further details.

manifestation of a type I binding when added to either control or 3-MC-induced microsomes. Finally, 3,4-benzpyrene produced a weak type I spectral change (peak at about 390 nm and trough at about 420 nm) when added to lung microsomes from 3-MC-pretreated rats.

The type II spectral change obtained upon addition of $n$-octylamine to induced and noninduced lung microsomes is shown in Chart 4. In both cases, a typical $n$-octylamine type II spectral change was obtained. Control microsomes showed a single trough at 411 nm and a peak at 434 nm, whereas microsomes from 3-MC-treated rats produced a double trough with minima at 394 and 411 nm and a peak at 434 nm. When the reciprocal of the ligand concentration was plotted versus the reciprocal of the spectral change, the $K_s$ values given in Chart 4, inset, were obtained. The $K_s$ value for control microsomes was about 36 µM, while the corresponding value for microsomes from 3-MC-treated rats was about 380 µM. The trough at 394 nm found with 3-MC-induced cytochrome P-450 appeared to be saturated even at very low concentrations of $n$-octylamine, and when
the concentration of the ligand was gradually increased, the intensity of the trough remained constant, indicating a high affinity for the ligand.

The difference spectra obtained by addition of ethylisocyanide to oxidized, NADPH, and dithionite-reduced microsomes, isolated from either control or 3-MC-induced animals, are shown in Chart 5, A and B. Curves 1 of both A and B show the difference spectra obtained by adding the ligand to an oxidized suspension of microsomes from control (Chart 5A) and induced rats (Chart 5B). The asymmetric peak obtained in both cases had a maximum at 434 nm, similar to the one reported for a rat liver (25). Although our microsomes were still contaminated by very small amounts of hemoglobin, this could not account for the difference spectra obtained (25). Moreover, hemoglobin isolated from the hemoglobin fraction of the Sepharose 2B column and diluted to concentrations similar to those contaminating our microsomal preparations did not give any spectral change in either the oxidized or dithionite-reduced microsomes from control treated rats (0.55 mg protein per ml; 0.11 nmole cytochrome P-450 per ml; n-octylamine added to a final concentration of 1.5 mM). The apparent $K_s$ values for n-octylamine (5 to 1500 $\mu$M) with microsomes from control rats or 13 concentrations of n-octylamine (15 to 1500 $\mu$M) with microsomes from treated rats. The values given in the inset were calculated from the regression lines obtained from the corresponding double-reciprocal plots and are the average of 3 experiments for each case ($r$ for control microsomes = 1.008 and for microsomes from 3-MC-treated rats = 0.9973).

Chart 4. n-Octylamine type II spectral change of lung microsomes from control and 3-MC-treated rats, recorded by using single-chambered cuvets. Curve 1, Control microsomes (1.87 mg protein per ml; 0.18 nmole cytochrome P-450 per ml; n-octylamine added to a final concentration of 0.100 mM); Curve 2, Microsomes from 3-MC-treated rats (0.55 mg protein per ml; 0.11 nmole cytochrome P-450 per ml; n-octylamine added to a final concentration of 0.100 mM). The apparent $K_s$ values for n-octylamine (5 to 300 $\mu$M) with microsomes from control rats or 13 concentrations of n-octylamine (15 to 1500 $\mu$M) with microsomes from treated rats. The values given in the inset were calculated from the regression lines obtained from the corresponding double-reciprocal plots and are the average of 3 experiments for each case ($r$ for control microsomes = 1.008 and for microsomes from 3-MC-treated rats = 0.9973).

Chart 5. Ethylisocyanide difference spectra of lung microsomes from control (A) and 3-MC- (B) treated rats recorded by using single chambered cuvets. Control microsomes, 0.94 mg protein per ml; 0.09 nmole cytochrome P-450 per ml. Microsomes from 3-MC-treated rats, 0.55 mg protein per ml; 0.11 nmole cytochrome P-450 per ml. Curves 1, oxidized microsomes; Curves 2, NADPH-reduced microsomes; Curves 3, dithionite-reduced microsomes. The microsomes were in each case suspended in 0.15 M phosphate buffer, pH 7.5, 10% glycerol, v/v. Ethylisocyanide was added to a final concentration of 10 mM. Base line corrections did not change the apparent peak positions.
reduced ethylisocyanide difference spectra. When the oxidized suspension of microsomes was first reduced with NADPH and then the ethylisocyanide difference spectrum was recorded, Curves 2 of Chart 5, A and B, were seen. With the noninduced material, the spectra showed a small shoulder at 430 nm and a peak at 458 nm, whereas the microsomes from 3-MC-treated rats revealed a shoulder at 430 nm and a peak at 456 nm. As is the case of oxidized hepatic microsomes, NADPH mainly reduced the abnormal disiocyanide form of cytochrome P-450 (15). The dithionite-reduced ethylisocyanide-bound difference spectra of the induced and non-induced hemoproteins are given in Curves 3 of Chart 5, A and B. With microsomes from control and induced rats, the curves show a normal Soret band at 430 nm, whereas the anomalous Soret band was at 458 nm with control microsomes, compared with 456 nm with those from induced rats. No change in the peak position of the normal Soret band of reduced ethylisocyanide-bound cytochrome P-450 or of the oxidized ethylisocyanide-bound cytochrome P-450 was observed after induction. When the ratios $\Delta A$ 430 nm - 490 nm/$\Delta A$ 458 nm - 490 nm, and $\Delta A$ 430 nm - 490 nm/$\Delta A$ 456 nm - 490 nm at pH 7.5 were calculated for microsomes from control and 3-MC-treated rats, values of 0.64 and 0.70, respectively, were obtained.

**DISCUSSION**

The Sepharose 2B column chromatography procedure introduced for the isolation of rat lung microsomes is based on the ability of the gel to separate particulate and high-molecular-weight material (exclusion limit around 20 x 10^6 MW) from dissolved and lower size particles. The only limitation is that the particle size should be small enough to allow free distribution in the outer volume of the Sepharose (34). The size range of our microsomes is from 0.05 to 0.3 $\mu$m, as calculated from the electron micrographs, and they pass readily between the gel beads. (These spaces ideally range from 9 to 38 $\mu$m in diameter.)

The source of tissue used throughout this study was the whole rat lung. Although no attempts were made to characterize from which particular group of cells our microsomes were derived, it seems important to point out that they probably came from the type II and Clara cells of the bronchial epithelium, since these cell types are known to contain the major portion of the lung ER (4).

The specific levels of NADPH-cytochrome c reductase and of cytochromes $b_5$ and P-450 in the Sepharose-isolated microsomes are the highest reported in the literature for rat lung microsomes (cf. Refs 23, 28), but they are lower than those found in rabbit lung microsomes (9, 11, 14, 35). However, some of the published values for the concentration of cytochromes P-450 and $b_5$ should be considered carefully, since the preparations used were contaminated by hemoglobin to a significant extent. In our case, the molar ratio between cytochromes P-450 and $b_5$ and hemoglobin is very low and no spectral interference, by either hemoglobin or cytochrome oxidase, could be detected with the technique used to obtain the reduced CO-bound cytochrome P-450 difference spectrum.

Upon 3-MC treatment, there is an induction of cytochromes $b_5$ and P-450 and a small increase in the activity of the NADPH-cytochrome c reductase. The increase in lung cytochrome $b_5$ following 3-MC pretreatment is in contrast to what happens in liver and kidney microsomes, where no induction effect has been found (1, 2, 21, 23, 24, 26). An increased specific content of rat lung cytochrome $b_5$ after 3-MC induction has recently also been reported by Matsubara et al. (23). Whether this indicates a different regulatory mechanism for the synthesis or the degradation of this particular pigment in lungs as compared with the other 2 organs is not yet clear, but it seems an interesting possibility.

The peak position of our induced and noninduced reduced CO-bound hemoprotein deserves some comment. Control microsomes exhibit a maximum at 453 nm in the dithionite-reduced CO-bound difference spectrum, as well as in the dithionite-reduced CO-bound minus carboxy hemoglobin difference spectrum. On the other hand, with microsomes from 3-MC-treated rats, the peak is at 451 nm in both types of difference spectra. These maxima are different from those found in liver microsomes from control and 3-MC-treated rats (2, 22). It is interesting to note that in the reduced CO-bound difference spectrum, carboxy hemoglobin is unbalanced and contributes to the absorption band appearing in the crowded region at about 420 to 424 nm. (A small contribution to the absorption in this region by reduced CO-bound cytochrome P-420 cannot be completely excluded.) On the other hand, in the reduced CO-bound minus carboxy hemoglobin difference spectrum, carboxy hemoglobin and methemoglobin are balanced and oxyhemoglobin is the only component that could disturb the spectrum. As seen in the graphs, no absorption band is apparent in the region from 415 to 425 nm, indicating no visible interference by either unbalanced oxyhemoglobin or reduced CO-bound cytochrome P-420. Matsubara et al. (23) have also reported a 2-nm shift to the blue for rat lungs cytochrome P-450 after 3-MC treatment but, in their case, the shift was from 450 to 448 nm, similar to what is obtained with liver microsomes (21, 22). The reason for these apparent differences in peak position is not yet clear, but they could be due to differences in either the source of animals, the microsomal isolation procedure, or the spectral technique used. Furthermore, rat kidney microsomal cytochrome P-450 (29), as well as the highly purified and apparently homogeneous rabbit liver cytochrome P-450 (36), show absorption maxima at 452 nm in the reduced CO-bound difference spectrum and in the reduced CO-bound absolute spectrum, respectively. The possible role of different membrane environments for the absorption of the reduced CO-bound cytochrome is not clearly understood, and such differences could explain the observed differences in peak position which, alternatively, may also represent differences in the apoprotein molecule itself.

The lack of a type 1 spectral change upon addition of hexobarbital or aminopyrine to our rat lung microsomes is in contrast to what happens with liver microsomes (10, 22, 31), and indicates that rat lung microsomes do not contain a type I binding site at all or, alternatively, that although the
type I binding site is present and the drugs are binding to it, the spectral manifestation of the phenomenon is absent due perhaps, to a low extinction coefficient for the ΔA produced and/or a low concentration of the hemoprotein. The former possibility appears to be supported by the very weak type I signal obtained at high concentrations of SKF 525-A.

As in the case of the reduced CO-bound difference spectra of microsomes from control and 3-MC-treated rats, the abnormal Soret peak of reduced ethylisocyanide-bound cytochrome P-450 shifted 2 nm to the blue after induction. This is similar to what occurs with liver microsomes after pretreatment of the animals with polycyclic hydrocarbons, but in this case, the positions of the maxima are different from those reported here (2, 16). Furthermore, the ratio between the “430 nm form” and the “455 nm form” obtained at pH 7.5 with lung microsomal preparation was not significantly changed after 3-MC induction, and the ratios calculated are similar to those obtained (at pH 7.5) with liver microsomes from 3-MC-treated rats (1, 2, 16, 21, 22, 24).

The binding spectra obtained upon addition of n-octylamine to lung microsomes from control and 3-MC-treated rats did not correlate with the ethylisocyanide-produced difference spectra, nor did they agree with those previously obtained with rabbit liver microsomes (17). The reason for these discrepancies is still unclear, although it should be pointed out that a similar lack of correlation between n-octylamine and ethylisocyanide-induced microsomal difference spectra has been reported (22, 24). Since the low concentration of cytochrome P-450 in the lung microsomes precludes electron paramagnetic resonance studies at the present time, a characterization of the spin state of lung cytochrome P-450 and a possible effect thereon by 3-MC treatment of the animals will not be obtained until a soluble and purified lung cytochrome P-450 preparation can be obtained for such studies.

Finally, it can be concluded that both 3-MC-induced and noninduced rat lung cytochrome P-450 are clearly different from the cytochrome P-450 present in rat liver microsomes. However, the lung hemoprotein obtained after 3-MC treatment does not appear as conclusively different from the preexisting pigment as judged by the spectral techniques used in this report.

REFERENCES


Characterization of a Rat Lung Microsomal Fraction Obtained by Sepharose 2B Ultrafiltration

Jorge Capdevila, Sten W. Jakobsson, Bengt Jernström, et al.

*Cancer Res* 1975;35:2820-2829.

**Updated version**
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/35/10/2820

**E-mail alerts**
Sign up to receive free email-alerts related to this article or journal.

**Reprints and Subscriptions**
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

**Permissions**
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.