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Preparation and Properties of Highly Purified Cytochrome P-450 and NADPH-Cytochrome P-450 Reductase from Pulmonary Microsomes of Untreated Rabbits

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SUMMARY

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Rabbit lung microsomal cytochrome P-450 has been purified to a specific content of 14.9 nmoles/mg of protein by *n*-octylamino-Sepharose 4B, hydroxylapatite, and DEAE-cellulose chromatography. NADPH-cytochrome P-450 reductase was purified from the same microsomal preparation to a specific activity of 47,700 nmoles of cytochrome *c* reduced per minute per milligram of protein, using *n*-octylamino-Sepharose 4B and 2',5'-ADP-agarose affinity chromatography. Both proteins were apparently homogeneous as judged from polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, and the respective yields of the two proteins were greater than 20% and 40%. A less highly purified and apparently distinct cytochrome P-450 fraction was also separated in lower yield in the course of purification, suggesting the presence of multiple forms of cytochrome P-450 in the lung. The major lung cytochrome P-450 fraction, when combined with lung NADPH-cytochrome P-450 reductase and phospholipid, was active in demethylating benzphetamine; activity was also demonstrated toward the substrates benzo[*a*]pyrene, 7-ethoxycoumarin, cyclohexane, dimethylnitrosamine, *N*-methyl-4-aminoazobenzene, *N*,*N*-dimethyl-4-aminoazobenzene, 4-ipomeanol, 2-(*N*-ethylcarbamoylhydroxymethyl)furan, and diethyl *p*-nitrophenylphosphorothionate (parathion). The NADPH-cytochrome P-450 reductases prepared from lung and liver microsomes appeared nearly identical as judged by their apparent subunit molecular weights, isoelectric focusing patterns, activities toward cytochrome *c*, and abilities to support benzphetamine demethylation with lung and liver cytochromes P-450. The major lung microsomal cytochrome P-450 has the same apparent subunit molecular weight (49,000) as the major form of liver microsomal cytochrome P-450 ($P\text{-}450_{LM-2}$) induced by phenobarbital (and differs from the other cytochromes P-450 of the liver in this regard) and reacts strongly with antibody prepared to this hepatic enzyme [but not antibody prepared to β-naphthoflavone-induced liver microsomal cytochrome P-450 ($P\text{-}450_{LM-4}$)] in Ouchterlony double-diffusion analyses. These hepatic and pulmonary cytochromes are, however, distinguished by their apparent isoelectric points and specificities toward certain substrates.

INTRODUCTION

The lung is a primary site through which airborne xenobiotics of toxic poten-

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tial gain access to the body; in addition, the lung is the target organ for a number of harmful materials ingested via other routes (1–5). Many of these toxic compounds require activation to elicit their activities, and the pulmonary mixed-function oxidase system appears to be capable of both activating and detoxifying a number of these materials (1, 5–9).

Both the hepatic (10–12) and the pulmonary (9, 13) mixed-function oxidase systems of the rabbit have been demonstrated to consist of cytochrome P-450 and the flavoprotein NADPH–cytochrome P-450 reductase;[1] in both cases, the proteins have been separated and systems active toward certain substrates have been reconstituted in the presence of added phospholipid (6, 9–13, 15–19). Evidence has been presented that multiple forms of cytochrome P-450 are present in hepatic microsomes of induced (17) or untreated rabbits (15, 17); these forms may be responsible for the substrate specificity observed *in vivo* under various conditions.

In the course of studies in this laboratory on the bioactivation of the lung toxin CMF[2] (5), several questions arose concerning the nature of the cytochrome P-450 and NADPH–cytochrome P-450 reductase of the lung, in particular regarding the presence of multiple forms of P-450 and its reductase in pulmonary microsomes and the similarity of these enzymes to those of the liver endoplasmic reticulum. To resolve these and other questions, these enzymes were highly purified and partially characterized. An NADPH–cytochrome P-450 reductase fraction and one cytochrome P-450 fraction are apparently homogeneous as judged by SDS–polyacrylamide gel electrophoresis; another, less highly purified but apparently distinct, P-450 fraction has also been obtained. Evidence is presented here that multiple forms of P-450 and perhaps NADPH–cytochrome P-450 reductase are present in the lung, and the similarities of the hepatic and pulmonary enzymes are discussed.

[1] An additional mixed-function oxidase system, consisting only of a flavoprotein oxidase (1, 14), is also present in the lung (and liver), but will not be considered here.

[2] The abbreviations used are: CMF, 2-(*N*-ethylcarbamoylhydroxymethyl) furan; SDS, sodium dodecyl sulfate; P-450, microsomal cytochrome P-450; $P\text{-}450_{LM}$, liver microsomal cytochrome P-450 [numbers designate the forms of hemoprotein as described by Haugen *et al.* (6, 17, 18)]; di-12 GPC, dilauroylglyceryl-3-phosphorylcholine.

MATERIALS AND METHODS

Purification of pulmonary cytochrome P-450. For each preparation, 15–20 male adult New Zealand rabbits (2–2.5 kg each, Vanderbilt Animal Care) were fasted overnight and killed with a blow to the base of the neck; the lungs were immediately excised, washed in 1.15% KCl, and trimmed (all purification steps were carried out at 0–4°). The microsomal fractions were prepared as previously described (6, 12), except that two washes with potassium pyrophosphate buffer were used and all buffers used in the preparation, storage, and solubilization included 0.1 mM fresh phenylmethylsulfonyl fluoride to protect against possible proteolysis. After storage overnight in 10 mM Tris-acetate (pH 7.4) buffer containing 1 mM EDTA and 20% (v/v) glycerol at −20° under N_2, the microsomes were diluted to a concentration of 2 mg/ml in 0.1 M potassium phosphate buffer (pH 7.25) containing 1 mM EDTA, 1 mM dithiothreitol, 20 μM butylated hydroxytoluene, and 30% glycerol; a 20% solution of sodium cholate was added dropwise to a final concentration of 0.6% (w/v) with stirring. After continued stirring for 90 min, the cholate extract was subjected to six 10-sec bursts of a Branson Sonifier (6A setting; with intermittent cooling for 1 min on ice) and centrifuged at 105,000 × *g* for 60 min.

The supernatant was applied to a 2.0 × 40 cm column of *n*-octylamino-Sepharose 4B that had been equilibrated with 0.1 M potassium phosphate (pH 7.25)–1 mM EDTA–1 mM dithiothreitol–20% glycerol–0.7% sodium cholate buffer (6, 19). The column was washed with 1000 ml of the equilibration buffer in which the cholate concentration had been lowered to 0.46%, and the P-450 was then eluted in a sharp peak by the addition of buffer (1000 ml) in which the cholate concentration had been lowered to 0.37% and Emulgen 913 (0.08%,

w/v) had been added (6, 19, 20). Further washing (1000 ml) with the same buffer eluted NADPH–cytochrome P-450 reductase in a separate peak. No additional P-450 or reductase was eluted from the column when further washing was done with the same buffer containing 0.4% Emulgen 913 and 0.4% sodium deoxycholate.[3]

The peak fractions containing P-450 were pooled, concentrated by ultrafiltration (Amicon PM-30 membrane), stirred with Amberlite XAD-2 beads for 30 min (6, 12, 18), filtered through glass wool, dialyzed twice for 8 hr against 30 volumes of 10 mM Tris-acetate (pH 7.4)–0.1 mM EDTA–0.1 mM dithiothreitol–20% glycerol buffer, and applied to a 1.0 × 10 cm column of hydroxylapatite equilibrated with the dialysis buffer. All of the cytochrome was bound and remained bound after washing with 100 ml each of 5 mM potassium phosphate (pH 7.25)–0.1 mM EDTA–0.1 mM dithiothreitol–20% glycerol buffer and the same buffer containing 0.2% Emulgen 913. The P-450 was then eluted with the same buffer containing 35 mM potassium phosphate (and 0.2% Emulgen 913); the column was washed until all of the cytochrome was eluted. Further washing with the same buffer containing 90 mM, 150 mM, and 300 mM phosphate did not remove more hemoprotein.

The first few P-450 fractions eluted and the tailing fractions were discarded, and the remainder of the peak tubes were pooled, concentrated, treated with XAD-2 beads as before, dialyzed overnight against 50 volumes of 5 mM potassium phosphate buffer (pH 7.7) containing 0.1 mM EDTA, 0.1 mM dithiothreitol, 20% glycerol, and 0.2% Emulgen 913, and applied to a 1.0 × 5 cm column of Whatman DE-52 cellulose equilibrated with the same buffer. Most of the P-450 was eluted in the void volume (fraction A). After washing with 100 ml of the equilibration buffer, the remainder of the P-450 was eluted with buffer in which the phosphate concentration had been raised to 35 mM (fraction B). Each P-450 fraction was concentrated, treated with XAD-2 beads, dialyzed for 8 hr against 30 volumes of 10 mM Tris-acetate (pH 7.4)–1 mM EDTA–20% glycerol buffer, and stored at −20°.

Purification of pulmonary NADPH–cytochrome P-450 reductase. The cytochrome *c* reductase-containing fractions eluted from the *n*-octylamino-Sepharose 4B column were pooled and applied to a 1.0 × 4 cm column of agarose–2′,5′-ADP (6, 21, 22). After the column was washed with 100 ml of 35 mM potassium phosphate (pH 7.7)–0.1 mM EDTA–0.1 mM dithiothreitol–0.2% Emulgen 913–20% glycerol buffer, the reductase was eluted in a sharp peak by the addition of the same buffer containing 5 mM 2′-AMP. The peak fractions were pooled and treated with calcium phosphate as previously described (6, 12), dialyzed for 8 hr against 30 volumes of 10 mM Tris-acetate (pH 7.4)–1 mM EDTA–20% glycerol, and stored at −20°.

Preparation of other enzymes. Rabbit liver P-450$_{LM-2}$ and P-450$_{LM-4}$ were purified as described (6, 17, 18) from the livers of rabbits treated with phenobarbital and β-naphthoflavone, respectively; the nominal specific contents of the preparations were 12.5 and 13.3 nmoles of P-450 per milligram of protein. Liver microsomal P-450$_{LM-4}$ as isolated by Haugen and Coon (17, 18) appears to be identical with P-450$_{LM-4b}$ isolated in this laboratory (6); to avoid confusion, the term P-450$_{LM-4}$ is used in this report. NADPH–cytochrome P-450 reductase was purified from the liver microsomes of untreated rabbits (22) as described (specific activity, 42,400 nmoles of cytochrome *c* reduced per minute per milligram of protein) and from phenobarbital-treated rats by essentially the same procedure as described for the rabbit lung reductase (specific activity, 39,800).

Assay procedures. Cytochrome P-450, cytochrome P-420, and hemoglobin were

[3] When the gel was prepared from cross-linked Sepharose (Sepharose CL-4B), approximately half the P-450 was not bound to the column in the cholate buffer. The *n*-octylamino-Sepharose column can be reused (without loss of capacity or resolution) after stepwise washing with 1500 ml each of (a) 0.4% Triton N-101–0.4% deoxycholate–0.1 M potassium phosphate (pH 7.25)–1 mM EDTA buffer, (b) distilled water, (c) 10% aqueous dioxane (v/v), (d) distilled water, (e) 0.5 M potassium phosphate (pH 7.25)–0.5 M KCl–1 mM EDTA buffer, and (f) distilled water.

assayed as previously described (6, 12). All P-450 assays were carried out in the presence of 0.2% Emulgen 913, as this detergent appears to prevent conversion of lung P-450 to cytochrome P-420 under the assay conditions (see below). Spectra were recorded with a Varian 635M instrument in the dual-beam mode. All protein assays were carried out according to Lowry *et al.* (23), with appropriate corrections to standard curves. NADPH–cytochrome P-450 reductase activity was assayed as described (6, 12) and is expressed in terms of nanomoles of cytochrome *c* reduced per minute per milligram of protein. Epoxide hydrase activity was assayed using [*G*-^{3}H]styrene oxide according to Oesch *et al.* (24), and NADH–ferricyanide reductase was assayed according to Takesue and Omura (25). Concentrations of nonionic detergent present in enzyme preparations were estimated using the method of van der Hoeven and Coon (12).

Assays measuring the oxidative demethylation of *d*-benzphetamine (6, 26, 27), dimethylnitrosamine (6), *N*-methyl-4-aminoazobenzene (6), and *N*,*N*-dimethyl-4-aminoazobenzene (6), the oxidative deethylation of 7-ethoxycoumarin (28), the hydroxylation of cyclohexane (29) and benzo[*a*]pyrene (6, 30), the conversion of 4-ipomeanol (6) and CMF (5, 6) to products covalently bound to protein, and the metabolism of parathion (6, 31) were carried out as described; results are the means of duplicate experiments.

Electrophoresis and isoelectric focusing. Polyacrylamide disc gel electrophoresis was carried out in the presence of 0.1% SDS as described by Laemmli (32); samples were heated at 100° for 3 min in 60 mM Tris-chloride (pH 6.8) buffer containing 1% SDS, 5% 2-mercaptoethanol, 0.001% bromphenol blue, and 10% glycerol prior to application to the discontinuous gel system. After electrophoresis at 20° at a constant current of 3 mamp/gel (1 mamp/gel while the samples entered the gel), the gels were stained with Coomassie brilliant blue R-250 according to Fairbanks *et al.* (33).

Apparent molecular weights were estimated using standard proteins as previously described (6, 18), except that β-galactosidase (mol wt 130,000) was included.

Isoelectric focusing was done at 5° using an LKB Multiphor 2117 system. The gels contained 5% acrylamide (3% cross-linking), 5% glycerol, 2% ampholyte (pH 3–10), 0.1% Emulgen 913, and 0.00012% riboflavin. Samples were dialyzed thoroughly against 5 mM glycine buffer (adjusted to pH 7.4 with Tris) containing 20% glycerol and applied to the slab gels (length, 23 cm) with 8 × 10 mm Whatman No. 3MM papers. The anode junction strip was soaked in 1 M H_3PO_4, and the cathode strip, in 1 M NaOH. The voltage was raised to 500 V over 3 hr (current ≦ 15 mamp) and maintained for an additional 16 hr. Pieces of gel were cut from the plate; each was mixed with 2 ml of degassed, distilled water for later pH measurement (at 5°). Staining was carried out as follows. (a) Protein staining was accomplished by fixing the gel in an aqueous solution of 30% (v/v) methanol, 3% (w/v) sulfosalicyclic acid, and 10% (w/v) trichloracetic acid with gentle shaking for 40 min, staining the gel for 40 min at 37° in the same mixture containing 0.1% Coomassie brilliant blue R-250, and destaining overnight in 500 ml of 25% ethanol–10% acetic acid (v/v) in the presence of lambs' wool (34) with gentle shaking. (b) Reductase activity was located by shaking the gel gently at 37° for 30 min (in the dark) in a solution of 20 μM nitro blue tetrazolium in 0.3 M potassium phosphate (pH 7.7), shaking for an additional 2 hr after adding 40 μM NADPH, and fixing in 10% acetic acid. (c) Staining for peroxidase activity was done essentially according to Welton and Aust (35), except that *o*-dianisidine (3,3′-dimethoxybenzidine) was used in place of benzidine, and 0.25 M sodium acetate (pH 5.0) buffer replaced the citrate buffer.

Materials. *n*-Octylamino-Sepharose 4B (6, 19), hydroxylapatite (36), calcium phosphate gel (37), sodium cholate (12), and [^{3}H]CMF (5, 6) were prepared as described. [4-^{14}C]4-Ipomeanol (2, 6) was synthesized by Dr. L. T. Burka, Vanderbilt University; *N*-methyl-4-aminoazobenzene and *N*,*N*-dimethyl-4-aminoazobenzene were gifts from Dr. J. A. Miller of the University of Wisconsin; and *d*-benzphetamine was donated by Dr. P. W. O'Connell of the Upjohn Company. 7-Ethoxycou-

marin was synthesized according to Ullrich and Weber (38); m.p. 88–89° [reported, 88–90° (uncorrected)]. Agarose-2′,5′-ADP and 2′-AMP were purchased from P-L Biochemicals; cholic acid and 3,3′-dimethoxybenzidine, from Eastman; Emulgen 913, from Kao-Atlas; Amberlite XAD-2, from Applied Science Laboratories; di-12 GPC, from Serdary Research Laboratories; and all reagents used for gel electrophoresis and isoelectric focusing, from Bio-Rad Laboratories. All other materials obtained from commercial sources were of reagent quality.

RESULTS

Purification of pulmonary cytochrome P-450 and NADPH-cytochrome P-450 reductase. A typical preparation is outlined in Table 1; both yields and purification factors were reproducible. Contaminating hemoglobin, methemoglobin, and cytochrome P-420 were completely removed from the octylamino-Sepharose 4B column in the 0.46% cholate wash fraction; prior steps to remove hemoglobin (9, 39) are unwarranted. Cytochrome b_5 remained tightly bound to the column and was eluted after the P-450 and its reductase. The P-450 recovered from the hydroxylapatite column was cleanly divided into two fractions (A and B) upon DEAE-cellulose chromatography, using 5 mM and 35 mM phosphate buffers in the presence of Emulgen 913 as described.

In a previously described partial purification of rabbit lung P-450, Arinc and Philpot (9) reported that epoxide hydrase activity was purified to the same extent (approximately 30-fold) as P-450. The P-450 fraction eluted from the octylamino-Sepharose 4B column here contained epoxide hydrase purified 4-fold with respect to the microsomes (which converted 1.2 nmoles of styrene oxide to its glycol per minute per milligram of protein). The P-450 fraction B (from the DEAE-cellulose column) contained hydrase purified 10-fold; however, in fraction A (in which the P-450 was purified 55-fold) the epoxide hydrase activity was only 2.1 nmoles/min/mg of protein, representing less than 2-fold purification.

Pulmonary NADPH-cytochrome P-450 reductase was separated from P-450 on the octylamino-Sepharose 4B column and was obtained in a highly purified state after subsequent affinity chromatography. When the reductase preparation was applied to a DEAE-cellulose column and (after washing) eluted with a KCl gradient (in the presence of 0.1% deoxycholate), only one peak of reductase activity was eluted (0.25 M KCl); the specific activity was not increased in the procedure. No NADH-ferricyanide reductase activity could be detected in the purified preparation.

Most of the Emulgen 913 (90–95%) could be removed from the enzymes by treatment with Amberlite XAD-2 beads. The level of detergent could be reduced another 10-fold by calcium phosphate treatment, but significant amounts (approximately

TABLE 1

Purification of pulmonary cytochrome P-450 and NADPH-cytochrome P-450 reductase

Fraction	Protein	P-450	Specific content	Purification	Yield	Reductase	Specific activity	Purification	Yield
	mg	*nmoles*	*nmoles/mg*	*-fold*	*%*	*units*	*units/mg*	*-fold*	*%*
Microsomes	312	75	0.27	(1)	(100)	35,700	114	(1)	(100)
Cholate extract	180	73	0.52	1.9	98	34,600	193	1.7	97
Octylamino-Sepharose	15	58	3.9	14	77				
Hydroxylapatite	3.2	23	7.0	26	38				
DEAE-cellulose									
5 mM phosphate	1.2	16.5	14.9	55	22				
35 mM phosphate	1.35	4.1	3.0	11	6				
Octylamino-Sepharose	17					19,700	1,140	10	55
Agarose-2′,5′-ADP	0.35					16,700	47,700	420	47

30%) of P-450 were lost in the process. No difference in benzphetamine demethylase activity (see below) was observed between preparations treated in these two manners. The enzymes were stable for at least several months when stored at −20°, as judged by the P-450 spectra and the activity observed toward benzphetamine in reconstituted systems.

Spectra of purified enzymes. The near-ultraviolet spectra of the purified P-450 (fraction A) are shown in Fig. 1A. The oxidized and dithionite-reduced preparations exhibited maxima at 417 and 414 nm, respectively; thus the oxidized hemoprotein would appear to exist largely in the low-spin form. In the presence of 0.2% Emulgen 913 or Triton N-101, the reduced-CO spectrum showed only a slight shoulder in the 420 nm region and a maximum was observed at 451.5 (±0.3)[4] nm. In the absence of detergent, the magnitude of the peak was somewhat greater and a maximum at 450.0 nm was observed, but a significant amount of cytochrome P-420 was formed. The same wavelength maxima were observed in the reduced-CO vs. reduced difference spectra.

The reason for the apparently greater amount of total CO-binding hemoprotein detected in the absence of detergent is presently unclear. Arinc and Philpot reported that the sum of detectable cytochromes P-450 plus P-420 is increased by the addition of phospholipid (9); however, the presence of concentrations of di-12 GPC up to 150 μM did not affect the spectra observed in these studies. The possibility should be considered that reduction was not complete in the presence of detergent, although the spectra did not change with time (up to 10 min) and were not enhanced by further additions of CO or $Na_2S_2O_4$. An alternative explanation is that the extinction coefficient of the reduced-CO complex of this particular hemoprotein may be altered by detergents (as the wavelength maximum appears to be).

Cytochrome P-450 fraction B contained about 40% of its total heme in the form of NADPH–cytochrome P-450 reductase preparation. Maxima were observed at 380 cytochrome P-420. The wavelength maximum of the reduced-CO complex was observed at 449.4 nm in buffer containing Emulgen 913. The maximum for the P-450 from the hydroxylapatite step, which contained both fractions A and B, was at 450.9 nm.

Figure 1B shows the near ultraviolet spectrum of the oxidized rabbit lung

[4] Mean ± standard deviation of two preparations (three spectra of each were recorded). The wavelength accuracy was better than ±0.5 nm.

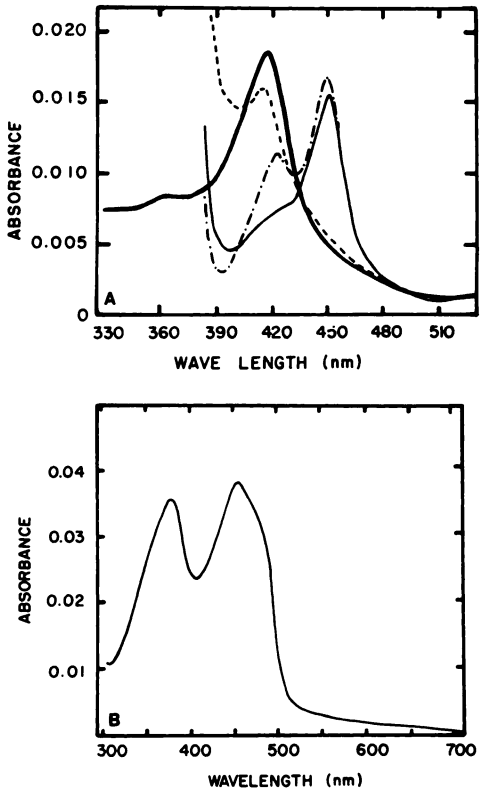


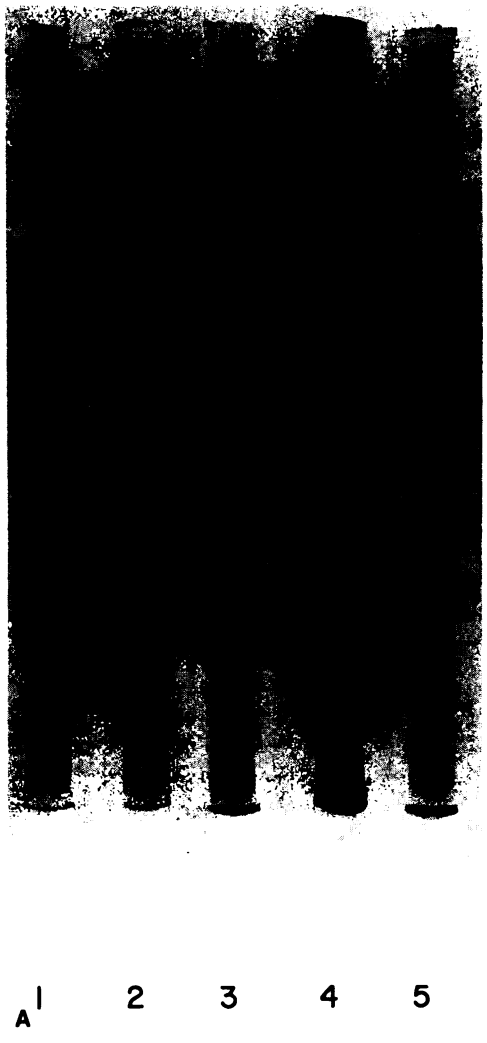
FIG. 1. *Absolute spectra of purified rabbit lung microsomal (A) and NADPH-cytochrome P-450 reductase (B)*

A. The sample cuvette contained 0.12 μM P-450 (8.1 μg/ml), 0.15 M potassium phosphate (pH 7.25), 1 mM EDTA, 20% glycerol, and 0.4% Emulgen 913. The oxidized (——), $Na_2S_2O_4$-reduced (- - -), and reduced-CO (——) spectra are shown. The reduced-CO spectrum (-·-·) was recorded in buffer which did not contain Emulgen 913. Reference cuvettes contained the appropriate buffer in the absence of protein.

B. The sample cuvette contained 0.19 mg/ml of the oxidized enzyme in 10 mM potassium phosphate (pH 7.7) buffer (with 1 mM EDTA and 20% glycerol present), and the reference cuvette contained only the buffer.

and 455 nm, as in the case of the flavoprotein isolated from rat liver microsomes (22, 40, 41). The preparation appeared to be devoid of contaminating heme as judged by the spectrum; the A_{380}:A_{455} ratio of 0.92 is within the range observed in this laboratory and reported by others (0.87–0.97) for the rat liver enzyme (22, 40, 41). The limited amount of purified material available did not allow determination of the nature of the flavin(s) present.

Electrophoresis and isoelectric focusing of purified enzymes. Cytochrome P-450 fraction A and the NADPH–cytochrome P-450 reductase appeared to be homogeneous as judged by SDS–polyacrylamide gel electrophoresis (Fig. 2). The apparent subunit molecular weight of the lung P-450 (fraction A) is 49,000, as compared with the values of 49,000 and 53,000 obtained with rabbit liver P-450$_{LM-2}$ and P-450$_{LM-4}$ (6, 17, 18) (gels not shown). Lung P-450 fraction B gave three major bands, the two more prominent of which had apparent molecular weights of 48,000 and 52,000. The NADPH–cytochrome P-450 reductases prepared from rabbit lung, rabbit liver, and rat liver microsomes all gave apparent



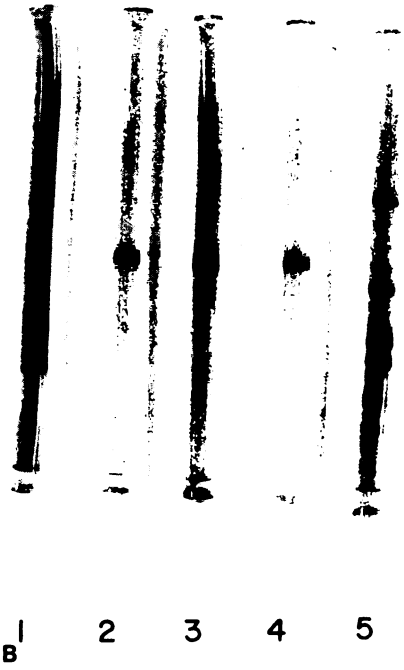


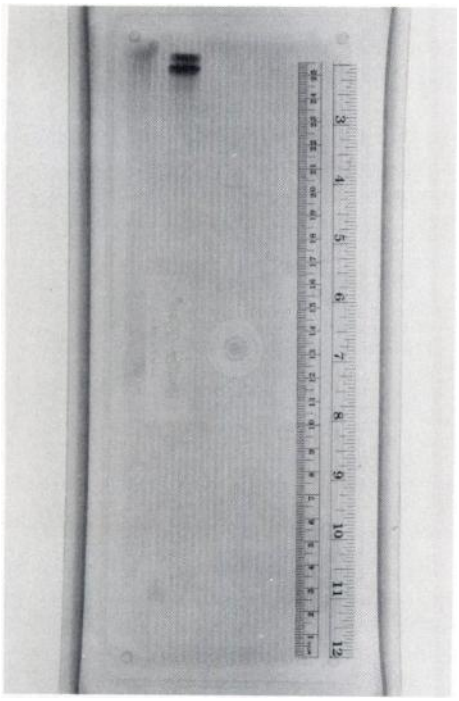
FIG. 2. *SDS-polyacrylamide gel electrophoresis of rabbit lung cytochrome P-450 (A) and NADPH-cytochrome P-450 reductase (B) fractions*

A. Gels were run from top to bottom (length, 10.5 cm) as described under MATERIALS AND METHODS; the concentration of acrylamide was 7.5%. Dye fronts were marked with wires. The indicated gels, from left to right, included: *1*, rabbit lung P-450 (fraction A), 0.55 μg; *2*, rabbit lung P-450 (fraction A), 2.9 μg; *3*, rabbit lung P-450 (fraction B), 4.0 μg; *4*, rabbit lung microsomes, 50 μg; *5*, β-galactosidase (2.0 μg), bovine serum albumin (1.0 μg), catalase (2.0 μg), glutamate dehydrogenase (2.0 μg), ovalbumin (2.0 μg), and aldolase (1.0 μg).

B. Conditions were the same as in Fig. 2A, except that the acrylamide concentration was 6.0%. Gels included: *1*, rabbit lung microsomes, 50 μg; *2*, rabbit lung reductase, 5.5 μg; *3*, rabbit liver reductase, 12.5 μg; *4*, rat liver reductase, 5.0 μg; *5*, standard proteins as in Fig. 2A.

subunit molecular weights of 79,000 (Fig. 2B).

Lung cytochrome P-450 fraction A migrated as a single band during isoelectric focusing on polyacrylamide gel, when gels were stained either for protein (Fig. 3) or for peroxidase activity (not shown). The apparent pI was 7.3 (at 20.5 cm in track 3 of Fig. 3), in comparison with 8.1 for liver P-450$_{LM-2}$ and 8.3 for liver P-450$_{LM-4}$. The pI values were taken from the peroxidase bands; both liver P-450 preparations contained what appeared to be minor protein impurities as judged by this technique. Lung P-450 fraction B exhibited a broad peroxidase band at an apparent pI range of 5.1–5.4 and Coomassie blue bands at pI values of 4.55, 4.8, 5.05, 5.2, and 5.35 (faintly visible at 7–11 cm in the photograph of Fig. 3, track *4*).



1 2 3 4

FIG. 3. *Isoelectric focusing of rabbit lung and liver microsomal P-450s*

The procedure was carried out as described under MATERIALS AND METHODS; the anode was at the bottom of the gel, and the gel was stained with Coomassie brilliant blue R-250. Samples included: *1*, liver P-450$_{LM-4}$, 39 μg; *2*, liver P-450$_{LM-2}$, 33 μg; *3*, lung P-450 (fraction A), 18 μg; *4*, lung P-450 (fraction B), 10 μg.

While the NADPH–cytochrome P-450 reductases from rabbit lung, rabbit liver, and rat liver all migrated as homogeneous species on SDS–polyacrylamide gel electrophoresis (Fig. 2B), all the reductase fractions gave several bands in these and repeated isoelectric focusing experiments (Fig. 4). Both the rabbit lung and liver reductase fractions gave two distinct major bands (pI 5.1 and 5.2) and at least two minor bands when stained either for protein with Coomassie blue or for NADPH–nitro blue tetrazolium reductase activity; the rat liver reductase fraction exhibited a greater number of bands (when stained by either method) in the range pI 4.7–5.4.

Immunological comparison of lung and liver cytochromes P-450. As shown in Fig. 5, rabbit lung P-450 (fraction A) reacted well with antibody prepared to rabbit liver P-450$_{LM-2}$, which, although virtually absent from untreated animals, is the major P-450 present in liver microsomes after phenobarbital treatment (17, 18). The rate of reaction, however, was considerably slower with the lung P-450 than with P-450$_{LM-2}$. The lung P-450 preparation was also examined under identical conditions for cross-reaction with liver P-450$_{LM-4}$, which is present in untreated animals but is induced by β-naphthoflavone and other polycyclic hydrocarbons (17, 18). In contrast to the single strong precipitin line observed between lung P-450 and anti-P-450$_{LM-2}$, no reaction between the lung enzyme and anti-P-450$_{LM-4}$ was visible when the indicated amounts of protein were used. No bands were observed when the P-450s were tested against preimmune serum. The results presented here are qualitative but suggest that the lung P-450 is considerably more immunologically similar to liver P-450$_{LM-2}$ than to P-450$_{LM-4}$. P-450$_{LM-2}$ and P-450$_{LM-4}$ have previously been shown by Coon *et al.* (42) not to share antigenic sites with each other.

Reconstitution of benzphetamine demethylase activity. A pulmonary system capable of metabolizing benzphetamine was reconstituted as described in Table 2.

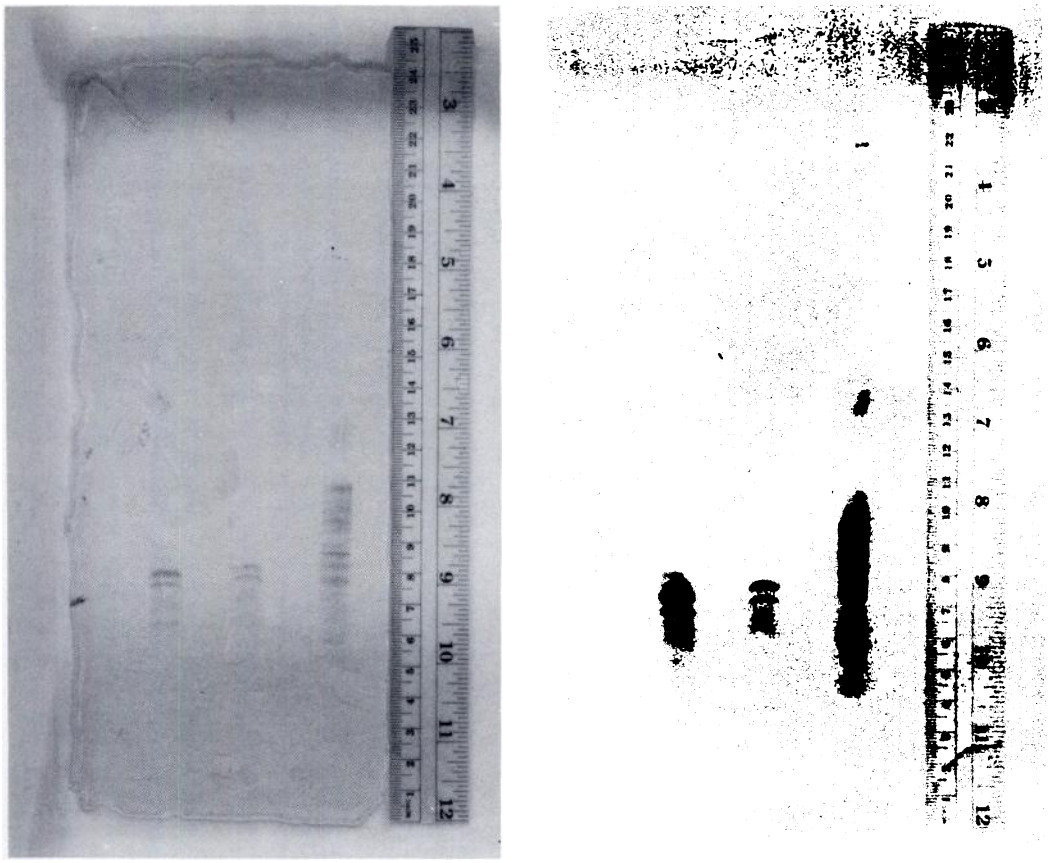


FIG. 4. *Isoelectric focusing of NADPH-cytochrome P-450 reductase fractions*

A. The procedure was carried out as described under MATERIALS AND METHODS, and the gel was stained for protein with Coomassie brilliant blue R-250. Samples included: *1*, rabbit liver reductase, 4.5 μg; *2*, rabbit lung reductase, 2.4 μg; *3*, rat liver reductase, 8.0 μg. The anode was at the bottom of the gel.

B. The procedure was carried out as described in Fig. 3A, and the gel was stained for nitro blue tetrazolium reductase activity. Samples are the same as in Fig. 4A; the anode was at the bottom of the gel.

Activity was completely dependent upon the presence of both P-450 and NADPH-cytochrome P-450 reductase and highly dependent upon the addition of di-12 GPC; in contrast to systems derived from rabbit and rat liver microsomes by similar procedures (6, 12), no enhancement of activity was observed upon the addition of deoxycholate. The di-12 GPC requirement and lack of deoxycholate requirement were observed with enzyme preparations from which detergent had been removed either with Amberlite XAD-2 beads or with calcium phosphate gel.

Both the lung P-450 (fraction A) and the major liver microsomal cytochrome P-450 ($P\text{-}450_{LM-2}$) isolated from phenobarbital-treated rabbits readily catalyzed the oxidative demethylation of benzphetamine in the presence of either lung or liver microsomal NADPH-cytochrome P-450 reductase (Fig. 6). The liver reductase was

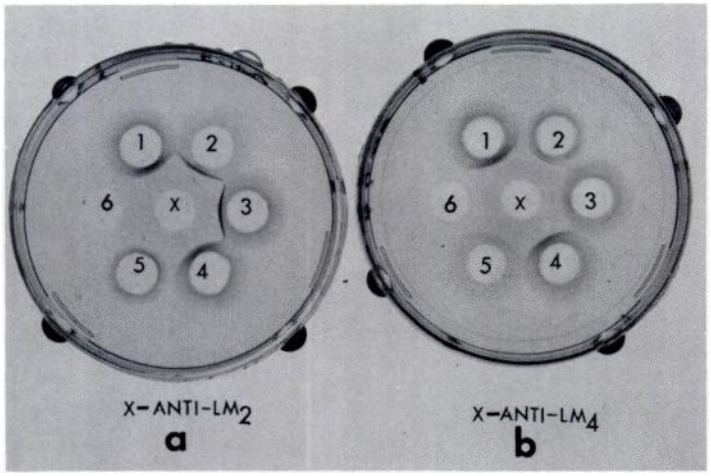


FIG. 5. *Ouchterlony diffusion analysis, showing reaction of hepatic and pulmonary P-450s with antibodies raised to hepatic P-450s*

The procedure was similar to that described by Coon *et al.* (42), except that antisera were derived from goats (43) instead of rabbits and deoxycholate was not used in the agar. The center well contained either 2.7 μM anti-P-450$_{LM-2}$ α-globulin (a) or 4.2 μM anti-P-450$_{LM-4}$ α-globulin (b). In experiment a, the wells contained: *1* and *4*, P-450$_{LM-2}$ plus P-450$_{LM-4}$ (1.7 μM total); *2*, P-450$_{LM-2}$ (1.5 μM); *3*, lung P-450 fraction A (1.7 μM); *5*, P-450$_{LM-4}$ (1.6 μM); *6*, buffer only. In experiment b, the wells contained: *1*, and *4*, P-450$_{LM-2}$ plus P-450$_{LM-4}$ (1.7 μM total); *2*, P-450$_{LM-4}$ (1.6 μM); *3*, lung P-450 fraction A (1.7 μM); *5*, P-450$_{LM-2}$ (1.5 μM); *6*, buffer only.

TABLE 2

Requirements for benzphetamine demethylation in reconstituted pulmonary system

The complete system contained 66 pmoles of rabbit lung P-450 (fraction A, 4.4 μg of protein), 140 units of rabbit lung NADPH-cytochrome P-450 reductase (2.9 μg of protein), 30 nmoles of di-12 GPC, 0.75 μmole of benzphetamine, 0.18 μmole of deoxycholate, 38 μmoles of potassium *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonate (pH 7.7), and 11 μmoles of $MgCl_2$ in a final volume of 0.75 ml. After incubation for 3 min, NADPH was added to 0.45 mM, incubation at 37° proceeded for 15 min, and formaldehyde production was measured as described under MATERIALS AND METHODS.

System	HCHO produced
	nmoles
Complete	31
−Reductase	0
−P-450	0
−Di-12 GPC	12
−Deoxycholate	31

slightly more active than the lung reductase in catalyzing the reaction with either P-450, and liver P-450$_{LM-2}$ was approximately twice as active as the lung cytochrome when coupled with either reductase.

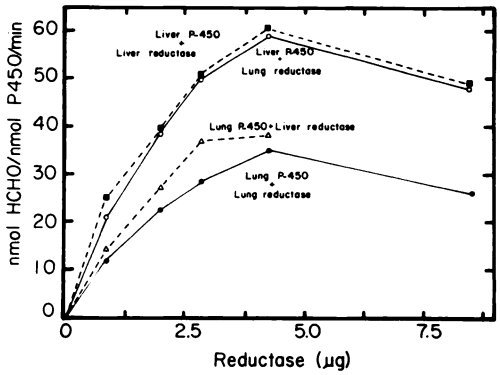


FIG. 6. *Demethylation of benzphetamine by rabbit lung P-450 (fraction A) and liver P-450$_{LM-2}$ as a function of varying concentrations of rabbit lung and liver NADPH-cytochrome P-450 reductase*

Conditions were the same as in Table 2, except that rabbit liver P-450$_{LM-2}$ (66 pmoles, 5.3 μg of protein) was substituted for the rabbit lung P-450 in the upper two curves and rabbit liver NADPH-cytochrome P-450 reductase was substituted as indicated.

Substrate specificity of pulmonary cytochrome P-450. The substrate specificity of the reconstituted pulmonary system toward a number of compounds of environmental concern was examined; the results

(expressed as P-450 turnover numbers, based on limiting P-450 in the presence of other components at optimal concentrations) are presented in Table 3. Cytochrome P-450 fraction A was relatively active in demethylating benzphetamine and the carcinogen dimethylnitrosamine [at both high and low substrate concentrations (44)], hydroxylating cyclohexane, and catalyzing the covalent binding of the lung toxins CMF and 4-ipomeanol to protein, as is the case with liver P-450_{LM-2} (6). The lung P-450 and liver P-450_{LM-2} (6, 17, 45) both exhibited little activity toward benzo[*a*]pyrene, in contrast to liver P-450_{LM-1} and P-450_{LM-7} isolated from untreated rabbits (6, 17, 45) and a partially purified P-450 fraction from lung microsomes of untreated rats (39). The lung P-450 resembles liver P-450_{LM-1} and P-450_{LM-7} and differs from liver P-450_{LM-2}, however, in readily catalyzing the demethylation of the carcinogens *N,N*-dimethyl-4-aminoazobenzene and *N*-methyl-4-aminoazobenzene (6).

Lung cytochrome P-450 fraction B was rather inactive toward most of the substrates with which it was tested; however, parathion was metabolized at significant rates (6, 20) by both fractions, although fraction A again was more active.

TABLE 3

Activity of reconstituted pulmonary cytochrome P-450/NADPH-cytochrome P-450 reductase systems toward various substrates

Assays were carried out as described under MATERIALS AND METHODS. In the cases of benzphetamine, cyclohexane, 7-ethoxycoumarin, benzo[a]pyrene, and parathion, the enzymes (reductase and P-450) and other components were added at the levels indicated in Table 2. With the other substrates, the amounts of both enzymes were doubled. Turnover numbers are based upon cytochrome P-450.

Substrate	Substrate concentration	Turnover number	
		Fraction A	Fraction B
	mM	*min*$^{-1}$	
Benzphetamine	1.0	37	2
Cyclohexane	10	28	2
7-Ethoxycoumarin	0.3	1.2	<0.1
Benzo[a]pyrene	0.08	0.10	0.03
Dimethylnitrosamine	200	5.2	
Dimethylnitrosamine	4.0	1.3	
N,N-Dimethyl-4-aminoazobenzene	0.10	3.8	
N-Methyl-4-aminoazobenzene	0.10	2.9	
CMF	1.0	3.0	0.04
4-Ipomeanol	1.0	3.3	0.04
Parathion conversion to:	0.05		
Paraoxon		1.2	0.6
Diethyl phosphorothionate		0.7	0.4
Diethyl phosphate		<0.1	0.2

DISCUSSION

Functionally active cytochrome P-450 and NADPH-cytochrome P-450 reductase have been purified from rabbit lung microsomes in greater than 20% and 40% yield, respectively. The SDS-polyacrylamide and isoelectric focusing gel patterns suggest that the P-450 A fraction, which apparently accounts for most of the P-450 of the lung microsomes under these conditions, is essentially homogeneous; the nominal specific content (14.9 nmoles/mg) is somewhat less than that expected on the basis of the apparent subunit molecular weight (i.e., 20.4 nmoles/mg), but possible overestimation of protein with the Lowry procedure (18, 46), the presence of apoenzyme (16, 18, 19), error in the estimation of the subunit molecular weight due to anomalous detergent binding, and the possibility that not all of the P-450 was reduced in the assays (see Fig. 1A) may explain the discrepancy. The trace amount of epoxide hydrase activity in the final P-450 preparation is apparently undetectable in electrophoresis or electrofocusing experiments; however, this activity may interfere in experiments with certain substrates.[5]

A second P-450 fraction (B) was also isolated, in lower yield and purity; on the basis of isoelectric focusing experiments, one or more P-450s (i.e., peroxidase-active proteins) distinct from fraction A are present. This P-450(s) is clearly distinguished

[5] The hydrase activity probably does not affect the covalent binding of CMF to tissue nucleophiles, as the microsomal reaction is unaffected by concentrations of styrene oxide, cyclohexene oxide, or 1,2-epoxy-3,3,3-trichloropropane as high as 3 mM (5).

from the P-450 of fraction A by SDS-gel electrophoresis and isoelectric focusing patterns, the wavelength maxima of the reduced-CO complexes (recorded in the presence of nonionic detergents), and the differences in activities toward the substrates assayed under fixed conditions.

The rabbit lung reductase preparation appears to be homogeneous as judged by SDS–polyacrylamide gel electrophoresis. The simplest explanation for the multiplicity of Coomassie blue and nitro blue tetrazolium bands observed upon isoelectric focusing is the presence of multiple forms of NADPH–cytochrome P-450 reductase, which appear to be present in both rabbit and rat liver microsomes (18). However, the multiple bands may be due to aggregative phenomena; this possibility cannot be ruled out at the present time and further studies are in progress.

The reductase fractions from the rabbit lung and liver could not be distinguished from each other by SDS–polyacrylamide gel electrophoresis or isoelectric focusing. The lung enzyme was slightly more active in reducing cytochrome *c*, but the fractions were nearly identical in supporting P-450-dependent benzphetamine demethylation, when activity was expressed on the basis of protein. The protease-derived fragments of rat liver and lung NADPH–cytochrome P-450 reductase were also shown by Buege and Aust (47) to be very similar as judged by immunological comparison, apparent subunit molecular weights, and salt dependence of cytochrome *c* reductase activity, although the liver enzyme was about twice as active as the lung protein in its activity toward cytochrome *c*.

The major lung cytochrome P-450 fraction resembles liver P-450_{LM-2}, a protein present in significant amounts only after phenobarbital induction (17, 18), with regard to apparent subunit molecular weight, immunological cross-reaction, and specificity toward some substrates (6), but is clearly distinguished by differences in behavior during purification (6, 12, 18), isoelectric focusing patterns, and activity toward certain substrates. The SDS-gel electrophoretic patterns clearly indicate that the major lung P-450 (fraction A) is distinct from any of the rabbit liver P-450s derived from untreated rabbits (6, 17, 18).

As a point of interest, it should be noted that Dus *et al.* (48, 49) have judged the pI of P-450_{LM-2} to be 4.2 and 5.4 in two separate experiments using electrofocusing in agarose gel. However, in this laboratory P-450_{LM-2} has consistently been observed to exhibit an apparent pI of about 8.0 in electrofocusing experiments using polyacrylamide gel, Bio-Gel P-200 (50), and Sephadex G-75 gel (50) in the presence of 0.05–0.5% nonionic detergent.

From the results of this work, it is concluded that multiple forms of cytochrome P-450 and possibly NADPH–cytochrome P-450 reductase are present in both the liver and the lung of the rabbit; the reductases of the two tissues are apparently identical, but the major lung P-450 is distinct from any of the liver P-450s. The procedures described here permit the purification of functionally active pulmonary cytochrome P-450 and its reductase in good yield and high purity for further characterization studies and for reconstitution of enzyme systems to study the metabolism of xenobiotics important to the lung.

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