

BIOSYNTHESIS OF CYTOCHROME P-450  
ON MEMBRANE-BOUND RIBOSOMES  
AND ITS SUBSEQUENT INCORPORATION INTO  
ROUGH AND SMOOTH MICROSOMES IN RAT HEPATOCYTES

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ABSTRACT

Intracellular sites of synthesis of cytochrome P-450 and the subsequent incorporation of it into membrane structures of the endoplasmic reticulum (ER) in rat hepatocytes have been studied using an antibody monospecific for phenobarbital-inducible cytochrome P-450. The cytochrome is synthesized mainly on the "tightly bound" type of membrane-bound ribosomes whose release from the membrane requires treatment with puromycin in a high salt buffer (500 mM KCl, 5 mM MgCl<sub>2</sub>, and 50 mM Tris-HCl [pH 7.5]). Subsequently the cytochrome is incorporated directly into the rough ER membranes with its major part exposed to the outer surface of the membrane and accessible to proteolytic enzymes added externally. The newly synthesized molecules, which appeared first in the rough membrane, are translocated to the smooth membrane, and are then distributed evenly between the two types of microsomal membranes in ~1 h. Administration of cycloheximide, an inhibitor of protein biosynthesis, did not significantly inhibit the transfer of the enzyme from the rough to the smooth ER. It is suggested, therefore, that the translocation of the newly synthesized cytochrome P-450 between the rough and smooth microsomes is mainly due to the lateral movement of the molecules in the plane of the membranes rather than to the attachment and detachment of the ribosomes on the microsomal membranes after the ribosomal cycle for protein synthesis.

**KEY WORDS** biosynthesis · cytochrome P-450 · cycloheximide · membrane-bound ribosomes · rough microsomes · smooth microsomes

It is generally agreed that the basic structure of the biomembrane is a bimolecular layer of phos-

pholipid in which amphipathic protein components are deposited and sometimes span (38, 43, 44). Since the lipid layer may hamper transmembrane movement of the charged macromolecules (38), it is highly prerequisite to an understanding of the asymmetric assembly of endoplasmic reticulum (ER) membrane to elucidate the intracellu-

lar site of synthesis of individual components and their subsequent distribution process in the membrane (38, 40).

In most eukaryotic cells, there are two populations of cytoplasmic ribosomes; one is free in the cytoplasm and the other is bound to the ER membranes. It is known that these two types of ribosomes, in general, synthesize different kinds of protein; soluble proteins for the cytoplasm such as ferritin (16, 37), arginase (46) etc., are synthesized on free ribosomes, whereas membrane-bound ribosomes are engaged in the synthesis of secretory proteins, such as serum albumin (37, 45), immunoglobulin (50), chymotrypsinogen (42), casein (11), etc.

However, the situations are somewhat complicated with regard to the site of synthesis of membrane proteins. Some membrane proteins are synthesized on the membrane-bound ribosomes (13, 25, 31, 36, 41), whereas others are synthesized on the free ribosomes (23-25, 36).

Cytochrome P-450 is one of the major intrinsic proteins of the ER membrane of hepatocytes and has recently been reported from our laboratory to be synthesized mostly on membrane-bound ribosomes (29). In confirmation and extension of our previous results, the present report describes how the nascent peptides of cytochrome P-450 are inserted into the ER membrane after their release from the ribosomes and how the newly synthesized molecules subsequently spread over the surface of rough and smooth microsomal membranes.

## MATERIALS AND METHODS

### *Treatment of Animals*

Male Sprague-Dawley rats weighing 150-200 g were used. They were fed ad libitum on laboratory chow and fasted overnight before sacrifice.

### *Purification of Cytochrome P-450 and Preparation of Anti-Cytochrome P-450 Antibody*

Cytochrome P-450 was purified from rats treated with a daily injection of phenobarbital (10 mg/100 g body weight) for 7 d according to the procedures of Imai and Sato (19) with slight modifications, details of which will be reported elsewhere.<sup>1</sup> The specific content of the purified sample was 17 nmol/mg of protein with an

<sup>1</sup> Mikawa, R., Y. Fujii-Kuriyama, M. Negishi, and Y. Tashiro. Manuscript in preparation.

overall yield of ~10% of total content in the microsomes. SDS-acrylamide gel electrophoresis of the purified preparation gave a single band of protein, when stained with Coomassie Brilliant Blue.

Antisera against the purified cytochrome P-450 were prepared as described previously (21). The immunoglobulin (IgG) fractions of the rabbit antiserum were obtained by repeated fractionations with ammonium sulfate.

The purity of the anti-cytochrome P-450 IgG was tested by the Ouchterlony agar diffusion technique (data not shown) and SDS-gel electrophoresis of the immunoprecipitates.

The antibody effectively inhibited the demethylation activity of benzphetamine by normal and phenobarbital-induced microsomes, whereas the corresponding activity by methylcholanthrene-induced microsomes was only partially inhibited. In the case of hydroxylating activity of benzo (a) pyrene, inhibitory effects of the antibody were observed with phenobarbital-induced microsomes but not with methylcholanthrene-induced ones. The antibody, therefore, seems to react specifically with cytochrome P-450 of phenobarbital-inducible type. The details of these results will be reported elsewhere.<sup>1</sup>

### *Preparation of Free, Loosely Bound, and Tightly Bound Ribosomes*

Free ribosomes and rough microsomes were first prepared from the postmitochondrial supernate of liver homogenates essentially as described by Blobel and Potter (4), and the ribosomes of the rough microsomes were further separated into loosely membrane-bound and tightly membrane-bound ribosomes by the high salt treatment of Adelman et al. (1) as follows:

Rat livers (20 g) were homogenized in 2.5 vol of 0.25 M sucrose in 50 mM Tris-HCl (pH 7.5), 25 mM KCl, 5 mM MgCl<sub>2</sub> (TKM buffer) in a Potter Teflon homogenizer and centrifuged at 12,500 rpm for 10 min in a Hitachi RP 65 T rotor (equivalent to Beckman 65 rotor; Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The sucrose concentration of the postmitochondrial supernate was adjusted to 0.88 M by the addition of 2.5 M sucrose in TKM buffer, and 5.8 ml of the resultant supernate was layered over a discontinuous sucrose gradient of 2.25 M (2 ml), 2.0 M (1 ml), and 1.35 M sucrose (3 ml) in TKM buffer. After centrifugation at 40,000 rpm for 24 h in a Hitachi RPS-40 T rotor (equivalent to Beckman SW 40 T rotor), free ribosomes were obtained as transparent precipitates and the rough and the smooth microsomes were banded at the interfaces of 2.0- to 1.35-M and 1.35- to 0.88-M sucrose layers, respectively.

The rough microsomes, diluted with 2 vol of 0.25 M sucrose in TKM, were collected as pellets by centrifugation at 40,000 rpm for 30 min in a Hitachi RP 40 rotor (equivalent to Beckman 40 rotor) and then resuspended in 0.25 M sucrose containing 50 mM Tris-HCl (pH 7.5),

500 mM KCl, 5 mM MgCl<sub>2</sub> (TK<sub>500</sub>M). After incubation at 30°C for 15 min, the sucrose concentration of the reaction mixture was adjusted to 1.0 M in TK<sub>500</sub>M and the mixture was layered over the top of three layers of sucrose solutions: 0.5 ml of 1.35 M sucrose in TK<sub>500</sub>M, 1 ml of 2.0 M sucrose in TK<sub>500</sub>M, and 2 ml of 2.25 M sucrose in TKM. 2 ml of 0.25 M sucrose in TK<sub>500</sub>M was placed on the top of the sample layer, and the gradient was centrifuged at 40,000 rpm for 24 h in a Hitachi RPS-40T rotor. Loosely bound ribosomes were obtained as a pellet, and several bands of microsomes in the 1.35-M sucrose layer were pooled for the preparation of tightly bound ribosomes. The microsomes collected by centrifugation were dissolved in 0.25 M sucrose in TKM containing 1% sodium deoxycholate (DOC), pH 8.0, and 4% Triton X-100 and centrifuged in the discontinuous sucrose gradient of 1.35 M (0.5 ml), 2.0 M (1 ml), and 2.25 M (2 ml) sucrose in TKM at 40,000 rpm for 20 h. The tightly bound ribosomes were obtained as a pellet. The yields of ribosomes were 42, 10.8, and 110.8 in optical density units at 260 nm for free, loosely bound, and tightly bound ribosomes, respectively.

#### Preparation of Rough and Smooth Microsomes

Rough and smooth microsomes were prepared essentially as described previously (31). Homogenates of rat liver (~10 g) in 4 vol of 0.88 M sucrose containing 0.5 mM MgCl<sub>2</sub> were centrifuged at 15,000 rpm for 20 min in a Hitachi RP 40 rotor. The supernate was layered on the discontinuous sucrose density gradient (2.5 ml of 1.35 M and 2.5 ml of 2.1 M sucrose in TKM) and centrifuged at 35,000 rpm for 20 h in an RPS-40T rotor. The 2.1-M sucrose layer was introduced to avoid contamination of microsomes with free ribosomes as used by Cardelli et al. (5). The rough and the smooth microsomal fractions were washed with 1.15% KCl containing 10 mM EDTA (pH 7.5) and then with 0.1 M potassium phosphate buffer (pH 7.5) successively. Protein, RNA, and cytochrome P-450 content of the rough and the smooth microsomal fractions are shown in Table I.

#### Labeling of Nascent Peptides In Vivo and In Vitro

For labeling in vivo of the cytochrome, 20  $\mu$ Ci/100 g body weight of radioactive leucine (1-L-[<sup>14</sup>C]leucine, 57 mCi/mmol, Commissariat a L'Energie Atomique, France) or 100  $\mu$ Ci/100 g body weight of a <sup>3</sup>H-amino acid mixture (1 mCi/ml, The Radiochemical Center, Amersham, England) was injected into rats in isotonic saline through a caudal vein.

Labeling in vitro of the nascent peptides on ribosomes with [<sup>3</sup>H]puromycin (1 mCi/0.65 mg/ml, [methoxy-<sup>3</sup>H]puromycin 2 HCl, New England Nuclear, Boston, Mass., or 3.5 Ci/mmol, [8(n)-<sup>3</sup>H]puromycin 2 HCl, The

TABLE I  
Recovery and Contents of RNA and Cytochrome P-450 in the Rough and the Smooth Microsomes

	Protein	RNA	P-450
	mg/g liver	$\mu$ g/mg protein	nmol/mg protein
Rough	4.2 $\pm$ 0.8	207 $\pm$ 20	0.71 $\pm$ 0.1
Smooth	9.0 $\pm$ 1.0	33.0 $\pm$ 5.0	1.1 $\pm$ 0.2

The values listed are the mean values of eight preparations of the rough and the smooth microsomes. The microsome preparations were washed with 0.1 M potassium phosphate buffer (pH 7.5).

Radiochemical Center) was carried out as described in the previous papers (1, 29). The ribosomal fractions (free, loosely bound, or tightly bound ribosomes, 1–4 mg/ml) and the rough microsomal fraction containing an equivalent amount of ribosomes were incubated with [<sup>3</sup>H]puromycin (~10 M excess over ribosomes content) in 50 mM Tris-HCl, pH 7.5, containing 750 mM KCl and 5 mM MgCl<sub>2</sub> (TK<sub>750</sub>M buffer) at 25°C for 15 min and dialyzed overnight against 0.1 M Tris-HCl (pH 8.0).

#### Immunoprecipitation of Nascent Cytochrome P-450 with the Antibody

Detection of nascent cytochrome P-450 peptides in ribosomal fractions was carried out as described previously (29). The dialyzed ribosome solution (~1 mg of ribosomes) which had been supplemented with purified cytochrome P-450 (0.39 nmol) as a carrier was incubated with 30  $\mu$ l of anti-cytochrome P-450 IgG (45 mg/ml) in 0.5 ml of 0.1 M Tris-HCl (pH 8.0) at 20°C for 1 h and then kept overnight at 4°C. This amount of the antibody was shown to be sufficient to attain maximum immunoprecipitation under the present experimental conditions.

For immunoprecipitation of cytochrome P-450 in microsomes, the washed microsomal fractions (10 mg/ml of cold water) were solubilized by mixing with an equal volume of detergent-glycerol solution (2% sodium cholate, 0.8% Emulgen 913 and 32% glycerol in 0.1 M potassium phosphate buffer [pH 7.25]). The mixtures were kept on ice for 15 min and then centrifuged at 40,000 rpm for 2 h in a Hitachi RP 40 rotor. To the resultant supernate (0.5 ml) containing 1.5–2.0 nmol of cytochrome P-450, the anti-cytochrome P-450 IgG (10–18 mg) in 0.5 ml of 0.1 M potassium phosphate buffer (pH 7.25) was added and the mixture was incubated at 20°C for 1 h and then kept overnight at 4°C. As a control experiment, nonimmunized rabbit IgG and goat IgG against rabbit Fab fragment (kindly supplied by Dr. S. Sasaki in our laboratory) were added instead of anti-cytochrome P-450 IgG. These immunoprecipitates were successively washed with 1 ml of 0.1 M Tris-HCl (pH 8.0) containing 1% DOC and three times with 1 ml of 0.1 M Tris-HCl (pH 8.0). The washed precipitates were

treated with 5% TCA at 90°C for 15 min and then with ethanol-ether (3:1), and were finally dissolved in 0.5 ml of solouene 350 (Packard Instrument Co., Inc., Downers Grove, Ill.) at 50°C for 1 h for assay of the radioactivity.

### *Puromycin Treatment and Protease*

#### *Digestion of Rough Microsomes*

The rough microsomes (4 mg/ml) prepared as described above were incubated with either [<sup>3</sup>H]puromycin (for cold microsomes) or cold puromycin (for <sup>3</sup>H-labeled microsomes) in TK<sub>750</sub>M buffer at 25°C for 15 min and then were layered over 3 ml of 0.5 M sucrose in TKM buffer to separate the membranes by centrifugation at 40,000 rpm for 60 min in a Hitachi RP 40 rotor. The membrane fractions (7 mg/ml) thus obtained were digested with a mixture of trypsin and chymotrypsin (174 μg/ml each) in 0.1 M Tris-HCl (pH 8.0) containing 0.25 M sucrose at 25°C for 60 min. Subsequently diisopropyl-fluorophosphonate was added to a concentration of 1 mM to stop the proteolytic digestion. After the incubation was carried out at 25°C for 15 min and then on ice for 30 min, the microsomal membranes were dissolved by adding sodium cholate (pH 8.0, 1%) and Emulgen 913 (0.4%). After standing on ice for 15 min, the reaction mixtures were centrifuged at 40,000 rpm for 2 h in a Hitachi 65 T rotor, and then an aliquot (0.3 ml) of the supernate was immunoprecipitated either with anti-P-450 IgG (2.0 mg) and purified cytochrome P-450 (0.2 nmol) added as a carrier or with rabbit IgG to rat albumin and rat serum albumin (5 μg) as a carrier. As a control, IgG from nonimmunized rabbits (15 μg) and sheep IgG to rabbit Fab were added in another aliquot of the supernate to form immunoprecipitates. The radioactivities trapped in the control immunoprecipitates were ~30 cpm for the in vivo labeling experiment with <sup>3</sup>H-amino acid mixture and 100 cpm for the in vitro labeling experiment with [<sup>3</sup>H]puromycin, respectively. These values were subtracted from the figures in Table III.

#### *SDS-Polyacrylamide Gel Electrophoresis*

Washed immunoprecipitates from solubilized microsomes were dissolved in 50 μl of sample buffer containing 10 mM Tris acetate buffer (pH 9.0), 0.025 mM EDTA, 1% SDS, and 0.1% β-mercaptoethanol and boiled for 2 min. The treated samples were analyzed in SDS-polyacrylamide gel (7.5% gel concentration) according to the procedure of Hinman and Phillips (17). After the run, the gel column was cut into thin slices (2 mm in thickness), and the slices were processed with 1 ml of solouene 350 at 40°C overnight.

#### *Determination of Radioactivity*

The radioactivity of these immunoprecipitates and gel slices dissolved in solouene 350 was determined in a toluene scintillator system using a Packard model 3330

liquid scintillation spectrometer. Counting efficiencies were checked using an external standard. For measurement of the radioactivity in the total microsomal protein, the microsomal fractions were precipitated in 10% TCA and then washed with 5% TCA. The washed microsomes were successively treated in 5% TCA (at 90°C for 15 min), with ethanol-ether (3:1), and then with ether. The dried samples (~0.5 mg) were dissolved in 0.5 ml of solouene 350 for assay of the radioactivity.

#### *Measurement of Protein, RNA, and Cytochrome P-450 Content*

Protein was assayed according to the method of Lowry et al., with bovine serum albumin as a standard (22). RNA was estimated as described previously using yeast RNA as a standard (10), and the amount of ribosomes was determined using  $E_{260}^{1\%1\text{cm}} = 135$  (47). The cytochrome P-450 content in microsomes and the solubilized preparations were determined from the CO-difference spectrum of dithionite-treated microsomes as described by Omura and Sato (32), and Imai and Sato (18).

## RESULTS

### *Detection of Nascent Cytochrome P-450 Peptides on Loosely Bound, Tightly Bound, and Free Ribosomes by Immunoprecipitation*

We have previously demonstrated that cytochrome P-450 molecules are synthesized mainly on the membrane-bound ribosomes (29). First of all, we studied on which type of the bound ribosomes, "loosely bound" or "tightly bound" ribosomes, the cytochrome is synthesized. Membrane-bound ribosomes separated from free ribosomes by sucrose density gradient centrifugation were further divided into two subclasses, the tightly bound and the loosely bound ribosomes, by treatment of the rough microsomes with high concentrations of KCl as described in Materials and Methods. About 15% of the bound ribosomes was released from the rough microsomes by treatment with 0.5 M KCl (TK<sub>500</sub>M) and recovered as loosely bound ribosomes, mainly in the form of polysomes (data not shown). Free, loosely bound, and tightly bound ribosomes (0.25–0.7 mg/ml) were incubated separately with 10 M excess of [<sup>3</sup>H]puromycin at 25°C for 15 min in the presence of 750 mM KCl to label nascent peptides on ribosomes in vitro as described previously (1, 29). After dialysis of the reaction mixtures (2 ml each) against 0.1 M Tris-HCl (pH 8.0) for 10 h in the cold, the anti-cytochrome P-450 IgG (1.5 mg) was

added to the aliquots of the reaction mixtures together with purified cytochrome P-450 (0.2 nmol) as a carrier. The immunoprecipitates were extensively washed as described in Materials and Methods, and the radioactivity in the precipitates was determined. Immunoprecipitation of sheep antibody against normal rabbit Fab fragment and normal rabbit IgG (10  $\mu$ g) was carried out as a control to assess nonspecific adsorption of radioactive materials in the immunoprecipitates. The specificity of the antibody precipitation reaction is apparent from Fig. 2 which demonstrates that only one major polypeptide was precipitated by the antiserum.

As shown in Table II,  $^3\text{H}$  radioactivity was preferentially found in the immunoprecipitate from the tightly bound ribosomes, while about one-fifth or less of the radioactivity was precipitated from the free and the loosely bound ribosomes. Since the ratio of membrane-bound ribosomes to free ribosomes in rat liver is reported to be about 3 (3), we conclude that cytochrome P-450 is synthesized predominantly on the tightly bound ribosomes.

In the tightly bound ribosomes, the  $^3\text{H}$  radioactivity in the immunoprecipitates of cytochrome P-450 was one-tenth to one-twentieth of the total radioactivity in the 10% TCA-insoluble proteins. Although these two values of  $^3\text{H}$  radioactivity could not be directly compared with each other, the result may indicate that roughly 5% of the nascent peptides synthesized on the tightly bound ribosomes are the nascent peptides of cytochrome P-450.

#### *Protease Digestion of Nascent Cytochrome P-450 Peptides after Release from Ribosome by Puromycin*

We next examined the fate of the nascent peptides of cytochrome P-450 after release from the ribosomes. The nascent peptides were labeled either in vitro with [ $^3\text{H}$ ]puromycin or in vivo with  $^3\text{H}$ -amino acids.

Isolated rough-surfaced microsomes were incubated with [ $^3\text{H}$ ]puromycin in high salt buffer of  $\text{TK}_{750}\text{M}$  and the reaction mixture (6 ml) was layered over 0.5 M sucrose (3 ml) in TKM in a Hitachi RP-40 rotor and centrifuged at 40,000 rpm for 60 min to obtain the membrane fraction as a pellet. About 80% of the 10% TCA-insoluble radioactivity in the reaction mixture was recovered in the membrane fraction, indicating that most of

the nascent peptides on the membrane-bound ribosomes were attached to the membrane fraction after release from the ribosomes by the puromycin treatment. Even more quantitative recovery ( $\sim 90\%$ ) of the radioactivity in the pellet was obtained with the nascent cytochrome P-450 peptides precipitated by anti-cytochrome P-450 IgG (data not shown).

To determine the distribution of the puromycin-released nascent peptides of cytochrome P-450 in the membrane fractions, the membrane fractions (5 mg/ml) were digested by a mixture of trypsin and chymotrypsin (25  $\mu$ g of each per mg membrane protein) at 25°C for 1 h. As shown in Table III, approximately one-half of the 10% TCA-insoluble radioactivity of the total microsomal proteins was lost during the proteolytic digestion, as reported previously (9, 39). The labeled nascent peptides of cytochrome P-450 were digested to an even greater extent by the same treatment, only  $\sim 10\%$  of the radioactivity remaining as a resistant part.

Since it has been reported that neither trypsin nor chymotrypsin can cross the membrane of microsomal vesicles, their action is limited to the outer surface of the membrane (20). The digestibility by proteases of nascent cytochrome P-450 peptides released from ribosomes suggests that a large part of their molecules is exposed on the outer surface of the membrane.

A similar kind of experiment was carried out using rough-surfaced microsomes labeled in vivo with a  $^3\text{H}$ -amino acid mixture for a very short time (2 min). Digestibility of the nascent peptides of albumin by the proteases was tested as a control because these nascent peptides, when released from the membrane-bound ribosomes into the cavity of microsomal vesicles (37), were protected from digestion with proteases by the membrane structures.

About 90% of the radioactivity in the rough microsomes was recovered in the membrane fraction when the microsomes were treated with puromycin and high salt. More than one-half of the radioactivity was probably in nascent peptides, because 65% of the total radioactivity incorporated into the rough microsomes was shown to be associated with the membrane-bound ribosomes under the present conditions and because most of the radioactivity ( $\sim 80\%$ ) was transferred to the membrane by the treatment with puromycin in high salt buffer ( $\text{TK}_{750}\text{M}$ ) as described above.

When the puromycin-treated microsomes were

subsequently incubated with trypsin and chymotrypsin, approximately one-half of the total radioactivity was digested as shown in Table III, and ~85% of the  $^3\text{H}$  radioactivity incorporated into the nascent peptides of cytochrome P-450 was lost during the treatment, in agreement with the results of the *in vitro* labeling experiment with [ $^3\text{H}$ ]puromycin.

On the other hand, nascent peptides of albumin were effectively protected from the digestion as reported previously (12, 30); ~80% of nascent albumin peptide was recovered after trypsin and chymotrypsin treatment (Table III). Low susceptibility of nascent albumin to the proteolytic digestion was not due to the nature of the peptides *per se*, because when the membranes were dissolved beforehand with a detergent (DOC, sodium cholate, or Emulgen 913), the nascent albumin became digestible by the proteases, as reported previously (30).

These results suggest that after release from membrane-bound ribosomes, the nascent cytochrome P-450 peptides are not transported into the intracisternal cavity as predicted by the vectorial transport model for the secretory proteins such as albumin but, rather, are incorporated directly onto or into the microsomal membrane.

#### *Distribution of the Newly Synthesized Molecules of Cytochrome P-450 between Rough and Smooth Microsomes*

In liver cells, the ER membrane exists as two morphologically different entities, the rough-surfaced ER membrane with attached ribosomes and the smooth-surfaced ER membrane without them. These two types of ER membrane are frequently observed by electron microscopy to be interconnected with each other (34). Subfractionation of the ER membrane into rough-surfaced and smooth-surfaced microsomes shows that cytochrome P-450 is more or less evenly distributed between these two types of microsomal membranes. We examined the time-course of distribution of  $^{14}\text{C}$  radioactivity incorporated into the molecules of cytochrome P-450 in the rough and the smooth microsomes after administration of [ $^{14}\text{C}$ ]leucine.

Fig. 1 shows the time-course of radioactivity in the immunoprecipitates of cytochrome P-450 from the rough and the smooth microsomes after the injection of [ $^{14}\text{C}$ ]leucine. At early time points after the injection of [ $^{14}\text{C}$ ]leucine, the specific

radioactivity of cytochrome P-450 (cpm per nmol P-450) was clearly higher in the rough microsomes than in the smooth. The radioactivity in the rough microsomes reached a peak value around 10 min and then decreased, while that of the counterpart from the smooth microsomes continued to increase slowly until these two values became equal at ~60 min after the labeling.

Fig. 2 shows the results of SDS-gel electrophoresis of the immunoprecipitates of cytochrome P-450 at various time points. A single main peak of radioactivity was obtained at the position corresponding to the mobility of the purified cytochrome P-450, although it sometimes appeared to have a tailing or shoulder at the trailing edge. The radioactivities of the main peaks were >90% of the total when the background levels were subtracted.

These results suggest that the P-450 molecules newly synthesized on membrane-bound ribosomes appear first in the rough membranes nearest their site of synthesis (the membrane-bound ribosomes) and subsequently in the smooth, and the movement of the new protein molecules from the rough microsomes to the smooth resulted in their equilibrated partition between these two membrane systems.

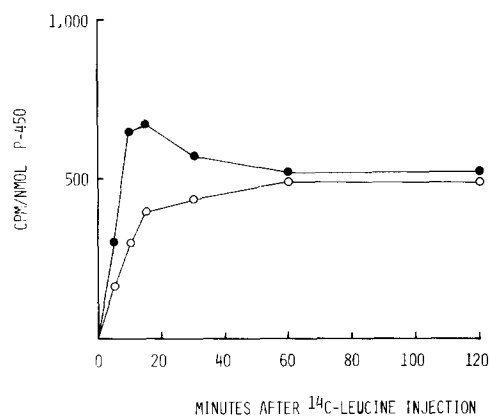


FIGURE 1 Incorporation of [ $^{14}\text{C}$ ]leucine into cytochrome P-450 in the rough and smooth microsomes. ●, rough microsomes; ○, smooth microsomes. Three rats were used at given time points after the injection of [ $^{14}\text{C}$ ]leucine (20  $\mu\text{Ci}/100$  g of rat weight). The rough and the smooth microsomes were prepared from liver and solubilized. Cytochrome P-450 (1.7–2.0 nmol) in the supernate was immunoprecipitated, washed, and processed for assay of radioactivity as described in Materials and Methods. The radioactivities in control immunoprecipitates were always below 20 cpm and were subtracted.

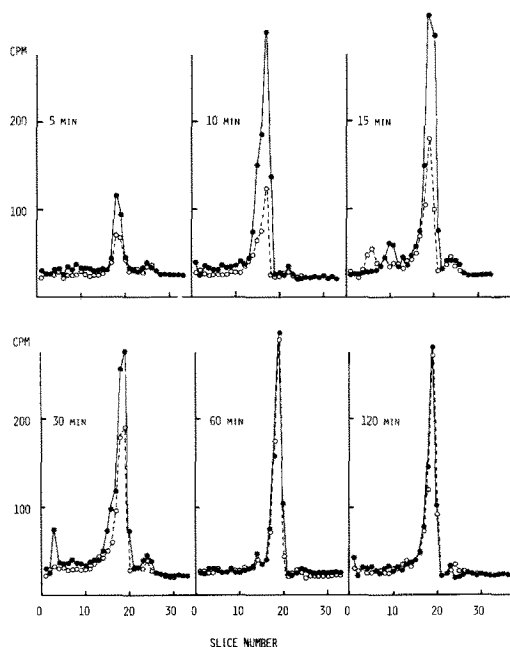


FIGURE 2 Acrylamide gel electrophoreses of the immunoprecipitates of cytochrome P-450 from the rough and smooth microsomes. ●, rough microsomes; ○, smooth microsomes. The washed immunoprecipitates of cytochrome P-450 prepared as described in the legend to Fig. 1 were analyzed by polyacrylamide gel electrophoresis according to Hinman and Phillips (17) as described in Materials and Methods.

The movement of the cytochrome molecules between these two types of microsomes could be brought about by either of the following mechanisms: (a) attachment-detachment of ribosomes to and from microsomal membranes accompanying the ribosomal cycle for protein synthesis, (b) lateral movement of the protein molecules per se on the membranes, or (c) both. To test these alternatives, we followed the change in the radioactivity of cytochrome P-450 in the rough and the smooth microsomes after injection of cycloheximide. Cycloheximide is an inhibitor of protein synthesis which blocks the elongation step of peptide synthesis so that the polysome integrity is well preserved as if it were frozen and the nascent peptides remain attached on the structure (35). Attachment-detachment of ribosomes accompanying the ribosomal cycle, therefore, is blocked, leaving membrane-bound ribosomes as they are.

As shown in the inset of Fig. 3, >95% of protein synthesis activity was immediately inhibited within 2 min of intravenous injection of the drug. Accordingly, cycloheximide was intra-

venously injected into rats at 10 min after administration of the radioactive amino acid, when the incorporation of radioactivity into cytochrome P-450 in the rough microsomes attained a maximum, and thereafter rough and smooth microsomes were separated at the time intervals given in Fig. 3 to determine the radioactivity incorporated into the immunoprecipitates of cytochrome P-450 from the two types of microsomes.

As shown in Fig. 3, the ratios of the specific

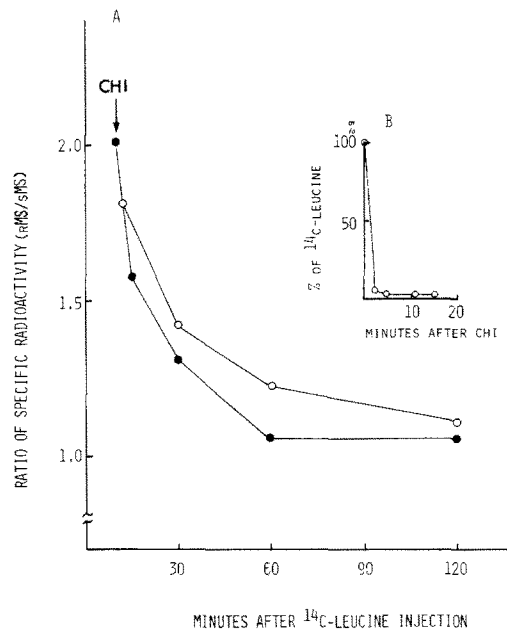


FIGURE 3 (A) Change in the ratios of the radioactivity in the immunoprecipitates of cytochrome P-450 from rough to smooth microsomes after the treatment with cycloheximide. ●, control; ○, treated with cycloheximide. 10 min after intravenous injection of [<sup>14</sup>C]leucine (20 mCi/100 g rat weight), cycloheximide (1.2 mg/100 g rat weight) was administered intravenously through a caudal vein. The radioactivities in the immunoprecipitates from the rough and the smooth microsomes were counted separately as described and, after subtraction of the radioactivities in the control immunoprecipitates, the ratios of the radioactivities between the immunoprecipitates from the rough to the smooth microsomes were calculated at indicated time points. (B) Incorporation of [<sup>14</sup>C]leucine into microsomal proteins after cycloheximide treatment. At indicated time points after intravenous injection of cycloheximide (1.2 mg/100 g rat weight, 10 mg/ml of saline), rats were given [<sup>14</sup>C]leucine (5  $\mu$ Ci/100 g body weight) intravenously through a caudal vein. After 5 min of labeling, the rats were sacrificed to prepare microsomes. Radioactivity of the microsomes was counted as described in Materials and Methods.

radioactivities in cytochrome P-450 from the rough and the smooth microsomes decreased rapidly and approached 1 even during the treatment with the drug. Thus, the time-course was essentially similar to that in the case with non-treated rats, except that the rate was somewhat slowed. These results may indicate that translocation of the new molecules of cytochrome P-450 from the rough to the smooth microsomal membrane is mainly due to the lateral movement of these molecules in the membrane rather than the attachment-detachment of ribosomes during the ribosomal cycle for protein synthesis.

## DISCUSSION

Membrane structures have a hydrophobic nature within them which may serve as a barrier to molecules of a hydrophilic nature, thus establishing an asymmetric arrangement of membrane proteins along the transverse plane of the membrane (38, 43). It is plausible to suppose, therefore, that differences in the topography of membrane proteins are somehow related to the sites of the biosynthesis and subsequent integration of these proteins into the membrane.

In the present report, we investigated the biosynthetic processes of microsomal cytochrome P-450 in rat liver using the antibody monospecific to a phenobarbital-inducible P-450. Cytochrome P-450 is one of the major protein constituents of microsomal membranes and has recently been reported to exist in multiple forms (8, 15, 48). The antibody used in this study was raised in rabbits against the purified cytochrome P-450 from phenobarbital-treated rats. From inhibition experiments of drug-metabolizing activities and Ouchterlony agar double diffusion, it was shown that the antibody is monospecific for a phenobarbital-inducible type of cytochrome P-450 and displays little cross-reactivity with a methylcholanthrene-inducible one.

Using this antibody, we have previously observed that the cytochrome is predominantly synthesized on the membrane-bound ribosomes (29). In this report, we have further studied the site of synthesis of the cytochrome in the hepatocytes with special reference to the loosely bound and tightly bound ribosomes, which were discriminated by treatment with 0.5 M KCl (TK<sub>500</sub>M). A possibility has been raised that there exist functional differences between these two types of membrane-bound ribosomes as to the synthesis of membrane proteins (13, 38).

As shown in Table II, the cytochrome P-450 is

preferentially synthesized on tightly bound ribosomes whose release from membranes requires treatment with puromycin and a high concentration of KCl, suggesting that the nascent peptides of cytochrome P-450 on ribosomes somehow interact with membrane structures and thus anchor the ribosomes to the membrane as previously discussed (1, 2, 9). The situation is somewhat different from that with other microsomal enzymes, NADPH-cytochrome *c* reductase and cytochrome *b*<sub>5</sub>. These microsomal enzymes are reported to be synthesized on the loosely bound ribosomes or sometimes on free ribosomes (12, 13). Although the reason for the difference in the mode of binding to the membrane of ribosomes synthesizing these microsomal proteins is not yet known, it may be in part due to hydrophobic interaction of their extending nascent peptides on ribosomes with membrane structures or due to topological localization of their nascent peptides extending from ribosomes in the membrane. In this regard, it is interesting to note that, while cytochrome *b*<sub>5</sub> and NADPH-cytochrome *c* reductase have a hydrophobic segment only at their C-terminals (28, 33), the N-terminal region of nascent cytochrome P-450 peptide on ribosomes has hydrophobic nature to interact with the membrane, as has recently been reported by sequence analysis of the cytochrome (14).

Upon treatment of the rough microsomes with puromycin and high salt, the nascent polypeptides of cytochrome P-450 released from ribosomes were quantitatively found associated with microsomal membrane, together with most of other nascent polypeptides (>80%) on bound ribosomes. These results suggest that the nascent P-450 peptides have some affinity for the microsomal membranes.

When the microsomal membranes loaded with the released nascent polypeptides were incubated

TABLE II  
*Localization of Nascent Cytochrome P-450 Peptides*

	TCA-insoluble	P-450
	cpm/OD <sub>260</sub>	
Tightly bound ribosomes	4,500	396
Loosely bound ribosomes	—	50
Free ribosomes	3,500	84

The radioactivity in the control immunoprecipitates was ~35 cpm/OD<sub>260</sub>, which had been subtracted from the figures in the table.



TABLE III  
Protease Digestion of Nascent Peptides in Microsomal Vesicles

Protease treatment	<sup>3</sup> H-amino acid label in vivo		<sup>3</sup> H-puromycin label in vitro	
	-	+	-	+
	<i>cpm per ml of reaction mixture</i>			
Total membrane protein	17,200	9,430	4.50 × 10 <sup>5</sup>	2.19 × 10 <sup>5</sup>
P-450	246	25	9.25 × 10 <sup>3</sup>	1.36 × 10 <sup>3</sup>
Albumin	2,900	2,350	-	-

with a mixture of trypsin and chymotrypsin, ~90% of nascent cytochrome P-450 peptides were lost, together with approximately one-half of the total nascent peptides. On the contrary, most of albumin nascent chain was not digested (Table III), in agreement with the previous report from our laboratory (30).

Exogenous proteases cannot penetrate into the cavities of microsomal vesicles, and their action is, therefore, limited to the outer surface of the microsomes. The susceptibility of nascent cytochrome P-450 peptides to the proteolysis clearly indicates that the nascent cytochrome P-450 peptides are probably incorporated directly onto or into the outer surface of the membranes.

The different transport route of protein molecules immediately after release from bound ribosomes as observed with cytochrome P-450 and albumin may determine their asymmetric distribution in microsomal vesicles because it seems thermodynamically unlikely to suppose the translocation of completed and folded protein molecules across the hydrophobic membrane structures. Synthesis of the cytochrome by membrane-bound polysomes and the direct disposition of the cytochrome on the ER surface may ensure the outside location of the molecule or at least a part of the molecule in microsomal membranes as revealed by enzymic iodination (51), immunochemical techniques (49, 52), and immunoelectron microscopy (26, 27).

The mechanism, which discriminates certain proteins, synthesized on bound ribosomes for direct disposition on the outer surface of the ER, from others for vectorial discharge across the ER membrane into the cavity remains to be solved.

From the kinetic study of labeling of cytochrome P-450 molecules of rough and smooth microsomes (Fig. 1), it was shown that the newly synthesized molecules appeared first in the rough microsomes and subsequently in the smooth microsomes and attained apparently an even distribution between these two membrane systems in ~1 h after labeling, indicating that transfer of

the protein molecules from the rough to the smooth membrane is not unidirectional but that these molecules in the rough and the smooth microsomes are in a dynamic equilibrium with each other in vivo. Essentially the same situations have already been reported with other microsomal protein components of cytochrome *b*<sub>5</sub> and NADPH-cytochrome *c* reductase (31) and the bulk of microsomal proteins of new-born rats (6, 7).

Since cycloheximide, known to inhibit protein biosynthesis by blocking the translocation step and leaving polysomes frozen as they are (35), did not affect significantly the transfer process of the newly synthesized cytochrome P-450 from the rough to the smooth membrane; lateral movement of the protein components per se in the plane of the membranes appears to be the most likely or a main factor for the transfer of the molecules.

The cytochrome P-450 molecules spread in this way are distributed over almost every microsomal vesicle derived from ER membrane, but their distribution in each microsomal vesicle, however, does not appear to be homogeneous, sometimes forming clusters or patches as revealed by our recent immunoelectron microscope observations (26, 27).

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