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SITE OF SYNTHESIS OF CYTOCHROME P-450 IN LIVER

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SUMMARY The suggestion that a rapidly sedimenting rough endoplasmic reticulum fraction in close association with mitochondria, is the preferred site of cytochrome P-450 synthesis has been examined. The rate of cytochrome P-450 synthesis in the different subcellular fractions has been evaluated *in vivo* and *in vitro*, using the immunoprecipitation technique. The results indicate that the conventional microsomal fraction (100,000 X g sediment) is the major site of cytochrome P-450 synthesis and that the rapidly sedimenting rough endoplasmic reticulum fraction associated with mitochondria is not a preferred site for the hemoprotein synthesis.

In the liver endoplasmic reticulum, Cytochrome P-450 is an important protein of structural and functional significance. Apart from its well known participation in mixed-function oxidase reactions (1), it also constitutes the structural matrix of the endoplasmic reticulum and its involvement in the binding of membranes to ribosomes has been indicated (2,3). An earlier report that the protein is preferentially associated with free polysomes (4) has been contradicted and it has been shown that the protein is present in the membrane and is essentially synthesized by membrane bound polysomes (2,3,5).

The rough endoplasmic reticulum (RER) fraction is highly heterogenous, sedimenting over a wide range of centrifugal force (6). The 640 X g fraction has been shown to consist of an RER fraction in intimate association with mitochondria (RERM) apart from nuclei (7). This RERM fraction, free of nuclei, has been isolated and characterized (8). Heme is essentially synthesized in the mitochondrion and the prosthetic group regulates the synthesis of apo-cytochrome P-450 (9).

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An attractive suggestion has been made that the synthesis of cytochrome P-450 requires the structural interaction between the endoplasmic reticulum and the mitochondria and that the RERM fraction may serve as the primary site of synthesis of this protein (10,11). We have been able to quantitate the synthesis of cytochrome P-450 *in vivo* and in the homologous cell-free system *in vitro*, using the cytochrome P-450 specific antibody (12). In the present study we have evaluated the involvement of the RERM and the conventional microsomal fractions in the synthesis of cytochrome P-450.

EXPERIMENTAL

Cytochrome P-450 was purified from the livers of phenobarbital-treated rats by the procedure described by Ryan *et al* (13). The final preparation had a specific content of 17n moles/mg protein. Antibody was raised in rabbits against the purified cytochrome P-450 preparation and the schedule of antigen injection was as described by Thomas *et al* (14). Monospecific antibody to the protein was prepared on the basis of the general procedure described by McCans *et al* (15). 1 mg of the monospecific antibody was found to precipitate 170 ug of cytochrome P-450 at the equivalence point.

To measure cytochrome P-450 synthesis *in vivo*, 30 μc of ^{14}C-chlorella protein hydrolysate was injected into rats, 6hr. after phenobarbital (80mg/kg) injection. The animals were killed 15min. after the tracer injection. The livers were homogenized in sucrose and the 640 X g pellet (Nuclei-RERM fraction), 10,000 X g pellet (mitochondrialfraction) 100,000 X g pellet (microsomalfraction) and 100,000 X g supernatant (cytosol fraction) were isolated. The RERM fraction free of nuclei was isolated from the 640 X g pellet by the procedure described by Shore and Tata (8). Protein synthesis *in vitro* using the RERM and microsomal fractions was carried out under conditions described by Weinstein (16). The preparations labelled *in vivo* and *in vitro* were solubilized and immunoprecipitated using the monospecific antibody (12). Aliquots were also taken for precipitation of the total proteins with trichloracetic acid.

Radioactivity measurements were made in a Beckman LS-100 liquid scintillation counter with Toluene-PPO or Toluene-Triton-PPO cocktails.

RESULTS AND DISCUSSION

The monospecific cytochrome P-450 antibody has been characterised using Ouchterlony immunodiffusion and immunotritration techniques. The

Table 1. Cytochrome P-450 synthesis in the different sub-cellular fractions of rat liver *in vivo*.

Fraction	Total Protein synthesized (cpm/mg protein)	Cytochrome P-450 synthesized (% of Total Protein radioactivity)	Cytochrome P-450 synthesized (cpm/g liver)
RERM	25992	1.86	4759
Mitochondria	39880	0.09	256
Microsomes	56690	9.69	31141
Post-microsomal supernatant	16932	1.23	7147

^{14}C-chlorella protein hydrolysate (30 μc) was injected 6hr. after phenobarbital injection to rats (60g body wt). The animals were killed 15min. after the tracer administration and the labelling of cytochrome P-450 was evaluated using the immunoprecipitation technique.

ability of the antibody to precipitate cytochrome P-450 labelled *in vivo* and *in vitro* in the homologous cell-free system, has been demonstrated on the basis of SDS-gel electrophoresis of the labelled immunoprecipitates (12).

The rate of labelling of cytochrome P-450 was examined 6hr. after phenobarbital administration. A short labelling period of 15min. was employed so that the primary site showing maximal rate of cytochrome P-450 synthesis can be detected. The results presented in Table 1 indicate that the microsomal fraction accounts for nearly 10% of the total protein radioactivity in cytochrome P-450. The RERM fraction accounts for only 2% of its total protein radioactivity in cytochrome

Table 2. Cytochrome P-450 synthesis by RERM and microsomal fractions *in vitro*.

Fraction	Total Protein synthesized (cpm/mg RNA)	Cytochrome P-450 synthesized (% of Total Protein radioactivity)
RERM	194,860	0.53
Microsomes	1,370,950	2.94

The microsomal or RERM fraction was incubated in a total volume of 0.25ml with a mixture consisting of ATP, 5 X 10^{-4}M; GTP 2.5 X 10^{-5}M; PEP, 2.5 X 10^{-3}M; Pyruvate kinase, 25 μg; aminoacid mixture without leucine, 40 μM; KCl, 60mM; $MgCl_2$, 5mM; 2-mercaptoethanol, 6mM; Post microsomal supernatant, 300 μg protein; ^{3}H-leucine, 25 μc (7c/m mole). The mixture was incubated at 37°C for 30min.

P-450. The microsomal fraction synthesizes about 7 times the amount of cytochrome P-450 synthesized by the RERM fraction, per gram liver, during this labelling period.

These results have been further substantiated by following the synthesis of cytochrome P-450 *in vitro* using the RERM and microsomal fractions. The total protein synthesized by the RERM fraction is significantly lower than that of the microsomal fraction. Immunoprecipitation data indicate that the microsomal fraction accounts for a higher percentage of cytochrome P-450 synthesized than that of the RERM fraction (Table 2).

It is clear from the data presented that cytochrome P-450 is essentially synthesized by the rough endoplasmic reticulum and the association of mitochondria with this fraction does not render it a preferred site for the synthesis of this protein. Meyer and co-workers (10,11,17) have envisaged a special role for the RERM fraction in the biosynthesis of cytochrome P-450

on the basis that this fraction contains a higher proportion of apo-cytochrome P-450 than the microsomal fraction under conditions of enhanced apo-protein synthesis brought about by phenobarbital (18) with concomitant inhibition of heme synthesis brought about by cobalt or lead (10,11,17). It has been shown that addition of heme *in vitro* to the different subcellular fractions isolated from phenobarbital plus cobalt or lead treated rat liver, elicits a greater percentage of reconstitution to holo-cytochrome P-450, as measured spectrally, in the RERM fraction than in the microsomal fraction. However, the results of the present investigation indicate that the reconstitution experiments perhaps reflect the greater depletion of the heme pool in the RERM fraction than that of the microsomal fraction under the experimental conditions employed. At any rate, reconstitution experiments cannot be used as a measure of apo-cytochrome P-450 synthesis. The results of the present study also contradict another brief report of Meyer and Meier (19) that during a 4h. labelling period, apo-cytochrome P-450 is labelled maximally in the RERM fraction. Perhaps, a specific immunoprecipitation procedure has not been employed in these studies and a 4h. labelling period is too long to measure synthetic rates. The results of the present study do not however rule out the suggested role of the RERM fraction in the regulation of the extra-mitochondrial transport of heme and the holo-protein assembly process (10).

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