Quantification of NADPH:cytochrome *P*-450 reductase in liver microsomes by a specific radioimmunoassay technique

Elizabeth A. SHEPHARD,* Ian R. PHILLIPS, Richard M. BAYNEY, Susan F. PIKE and Brian R. RABIN

Department of Biochemistry, University College London, Gower Street, London WC1E 6BT, U.K.

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We have developed a specific radioimmunoassay to quantify NADPH:cytochrome *P*-450 reductase. The assay is based on the use of ¹²⁵I-labelled NADPH:cytochrome *P*-450 reductase as the radiolabelled antigen and can detect quantities of this protein in amounts as low as 30 pg. The results of the radioimmunoassay demonstrate that the 2.7-fold increase in enzyme activity in rat liver microsomal membranes after phenobarbital treatment is due to increased amounts of the protein. β -Naphthoflavone treatment, however, did not alter the activity or the quantity of this enzyme in microsomes. The quantification of NADPH:cytochrome *P*-450 reductase in the microsomes isolated from control and phenobarbital- and β -naphthoflavone-treated animals permits the calculation of the ratio of this protein to that of total cytochromes *P*-450. A molar ratio of 15:1 (cytochromes *P*-450/NADPH:cytochrome *P*-450 reductase) was calculated for control and phenobarbital-treated animals. This ratio increased to 21:1 after β -naphthoflavone treatment. Thus the molar ratio of these proteins in liver microsomes can vary with exposure of the animals to particular xenobiotics.

NADPH:cytochrome *P*-450 reductase (EC 1.6.2.4) is present in liver microsomal membranes and is thought to be closely associated with, and to transfer electrons to, cytochromes *P*-450 (for review, see Sato & Omura, 1978; Masters, 1980). Together, these proteins play a major role in the detoxification of drugs and xenobiotics, the activation of pro-carcinogens and the metabolism of endogenous substrates (Conney, 1967; Gelboin, 1967; Estabrook & Lindenlaub, 1979; Guengerich, 1979; Coon *et al.*, 1980; Lu & West, 1980). The liver contains several cytochrome *P*-450 variants, some of which are induced by the drug phenobarbital (Thomas *et al.*, 1979, 1981; Pickett *et al.*, 1981; Phillips *et al.*, 1981; Mitani *et al.*, 1982). Phenobarbital treatment also causes an increase in the measured activity of NADPH:cytochrome *P*-450 reductase (Orrenius & Ernster, 1964; Orrenius *et al.*, 1965). It is clearly important to define the extent to which this increase is due to changes in the quantity of enzyme protein. It is also important to know whether xenobiotics inducing different cyto-

chrome *P*-450 variants have similar effects to phenobarbital on the expression of genes coding for NADPH:cytochrome *P*-450 reductase.

To investigate these points we developed a specific radioimmunoassay for the quantification of NADPH:cytochrome *P*-450 reductase. The data obtained with this technique have enabled us to calculate the molar ratio of cytochromes *P*-450 to NADPH:cytochrome *P*-450 reductase in microsomal membranes and to determine whether this ratio is changed after treatment with different xenobiotics.

Materials and methods

Animals

Male Sprague–Dawley rats (180–200 g) bred at University College Animal facility were used in these experiments. The rats were fed (diet GR3 EK; Dixons and Sons, Ware, Herts., U.K.) and watered *ad libitum* under controlled lighting conditions of 12 h light/12 h dark. Rats treated with sodium phenobarbital were fed a 0.1% (w/v) solution for 4 days and injected intraperitoneally on day 5 with a 4% (w/v) solution of sodium phenobarbital in 0.9% (w/v) NaCl at a dose of 40 mg/kg body wt. Rats treated with β -naphthoflavone were injected daily for

Abbreviations used: PB-inducible cytochrome *P*-450, the major phenobarbital-inducible cytochrome *P*-450 of mol.wt. 52000; SDS, sodium dodecyl sulphate; PB, phenobarbital; NF, β -naphthoflavone.

* To whom reprint requests should be sent.

3 days with β -naphthoflavone in corn oil at a dose of 40 mg/kg body wt. Control animals received injections of 0.9% (w/v) NaCl (saline) or corn oil. No difference in the activity or the amount of NADPH:cytochrome *P*-450 reductase was observed when animals were treated with saline or corn oil. Animals were starved overnight before use and killed by cervical dislocation.

Isolation and solubilization of microsomal membrane vesicles

The following procedures were carried out at 4°C. Total microsomal membrane vesicles were isolated from liver as described by van der Hoeven & Coon (1974). The microsomal membrane pellet was resuspended (at a final protein concentration of about 20 mg/ml) in 10 mM-potassium phosphate buffer (pH 7.25) containing 20% (v/v) glycerol/1 mM-EDTA, then flushed with N₂ and stored at -78°C. Microsomal membrane vesicles were thawed, flushed with N₂, homogenized briefly and solubilized by a modification of the procedure of Imai (1976; Shephard *et al.*, 1983). Solubilized microsomal vesicles were stored in portions at -78°C.

Purification of NADPH:cytochrome P-450 reductase and PB-inducible cytochrome P-450

NADPH:cytochrome *P*-450 reductase was purified from both phenobarbital- and β -naphthoflavone-treated rats by n-octylamine-Sephadex 4B and adenosine 2',5'-bisphosphate-agarose column chromatography (Guengerich & Martin, 1980). PB-inducible cytochrome *P*-450 was purified from the livers of phenobarbital-treated rats by n-octylamine-Sephadex 4B and DEAE-cellulose column chromatography (Guengerich & Martin, 1980).

Assays

NADPH:cytochrome *P*-450 reductase activity was determined by the method of Vermilion & Coon (1974) and protein concentrations were determined by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The specific content of total cytochromes *P*-450 was measured by the method of Omura & Sato (1964).

Preparation of antibodies

Antibodies were raised in male New Zealand White or Red rabbits (Phillips *et al.*, 1983). Pure antigen was always mixed 1:1 (v/v) with Freund's complete adjuvant before injection. Rabbits were initially injected, in the subscapular space, with 50 μ g of antigen. After 3 weeks the rabbits were re-injected with half this dose. After a further 5 weeks animals were given a second booster injection of 12.5 μ g of antigen. At 7 days after the final booster injection blood was withdrawn from the marginal ear vein and

allowed to clot at room temperature for 30 min. The clot was broken, left at 4°C overnight and then centrifuged at 10000g for 15 min at 4°C. Anti-serum was decanted and stored in portions at -20°C.

Ouchterlony double-immunodiffusion analysis

This was carried out as described by Thomas *et al.* (1981).

Iodination of NADPH:cytochrome P-450 reductase

Purified NADPH:cytochrome *P*-450 reductase in 30 mM-potassium phosphate (pH 7.7)/0.1 mM-EDTA/20% (v/v) glycerol/0.4 mM-phenylmethanesulphonyl fluoride was brought to final concentrations of 0.4% (w/v) recrystallized sodium cholate and 0.2% (w/v) Lubrol PX (Sigma). The sample was dialysed overnight at 4°C against 500 vol. of 0.1 M-potassium phosphate (pH 7.5)/0.4% (w/v) recrystallized sodium cholate/0.2% (w/v) Lubrol PX (buffer A) with one change of dialysis buffer. Approx. 5 μ g of the protein in 20 μ l of buffer A was added to 0.5 mCi of dried Bolton and Hunter reagent [*N*-succinimidyl-3-(4-hydroxy-5-[¹²⁵I]iodophenyl)-propionate} (sp. radioactivity approx. 2000 Ci/mmol; Amersham International) (Bolton & Hunter, 1973). The sample was gently agitated and left for 1 h at 4°C with occasional shaking. The reaction was terminated by adding 0.5 ml of 0.2 M-glycine in buffer A and incubating for a further 2 min. ¹²⁵I-labelled protein was separated from unchanged Bolton and Hunter reagent by Sephadex G-75 column chromatography. The column (0.9 cm $\times$ 30 cm) had been previously equilibrated with buffer A at room temperature, washed with 100 mg of bovine serum albumin (Sigma; fraction V, RIA grade) and re-equilibrated with buffer A. The iodinated protein was eluted with buffer A containing 0.25% (w/v) gelatin. Fractions (1 ml) were collected into tubes containing 100 μ l of bovine serum albumin at a concentration of 60 mg/ml. The fractions containing iodinated NADPH:cytochrome *P*-450 reductase were made 1% with respect to both Triton X-100 and sodium deoxycholate, phenylmethanesulphonyl fluoride was added to a final concentration of 0.4 mM and samples were stored in portions at -78°C. PB-inducible cytochrome *P*-450 was iodinated by the same method, except that the iodination reaction was carried out at room temperature (Phillips *et al.*, 1983).

Radioimmunoassay of NADPH:cytochrome P-450 reductase

All radioimmunoassays were carried out in a final volume of 300 μ l in 50 mM-Tris/HCl (pH 7.4) containing 1% (w/v) Triton X-100/1% (w/v) sodium deoxycholate/150 mM-NaCl/5 mM-EDTA/0.02% (w/v) NaN₃/0.25% (w/v) bovine serum albumin/

0.6 mM-phenylmethanesulphonyl fluoride. Samples containing 2×10^4 c.p.m. were mixed with various quantities of the detergent-solubilized sample being analysed. An amount of NADPH:cytochrome *P*-450 reductase antiserum sufficient to precipitate 50% of the precipitable iodinated protein in the absence of any competitor was added to each sample. The samples were incubated at 4°C overnight. Fixed *Staphylococcus aureus* cells (10 µl of a 10% suspension) were added and the samples were incubated at room temperature for 1 h. The cells were pelleted by centrifugation at 1700 *g* for 30 min at 4°C. The supernatants were removed by aspiration and the radioactivity in the pellets was determined by using an LKB Wallac 1270 Rack gamma II instrument. Radioiodinated NADPH:cytochrome *P*-450 reductase was less stable than radiolabelled PB-inducible cytochrome *P*-450 (Phillips *et al.*, 1983) but was usable in the radioimmunoassay for 1–2 months. All radioimmunoassays were performed in duplicate. Duplicates varied by less than 0.5%.

Staphylococcus aureus cells

Fixed *Staph. aureus* cells were obtained from Michelle Ginsberg, Imperial Cancer Research Fund Laboratories, London WC2A 3PX, U.K., and were prepared for use as described previously (Phillips *et al.*, 1981), except that sodium deoxycholate was present in the first wash buffer at a final concentration of 1% (w/v) and methionine was omitted from both wash buffers.

SDS/polyacrylamide-gel electrophoresis

Proteins were analysed on 10% polyacrylamide slab gels containing SDS as described previously (Phillips *et al.*, 1981).

Results

Purification of NADPH:cytochrome *P*-450 reductase

NADPH:cytochrome *P*-450 reductase was purified to homogeneity from the livers of phenobarbital-treated and β -naphthoflavone-treated rats to specific activities of 63.8 and 63.5 $\mu\text{mol} \cdot \text{min}^{-1} \cdot \text{mg}^{-1}$ respectively. The protein, isolated from either source, migrated on SDS/polyacrylamide gels as a single band of mol.wt. 76 000 (Fig. 1, tracks *a* and *b*).

Ouchterlony double-diffusion analysis

Antibodies raised to NADPH:cytochrome *P*-450 reductase isolated from PB-treated rats gave single immunoprecipitation lines of similar intensity on reaction with either of the purified antigens (Fig. 2). A single precipitin line was formed between the antiserum and solubilized total liver microsomal membrane proteins isolated from phenobarbital- or

Fig. 1. SDS/polyacrylamide-gel electrophoresis of NADPH:cytochrome *P*-450 reductase purified from PB- or NF-treated rats

NADPH:cytochrome *P*-450 reductase was purified from PB- or NF-treated rats as described in the Materials and methods section. SDS, 2-mercaptoethanol and glycerol were added to the purified proteins to give final concentrations of 2, 1 and 15% respectively. Samples were heated at 100°C for 3 min and then electrophoresed on a 10% polyacrylamide gel containing SDS. The gel was stained with Kenacid Blue. (a) NADPH:cytochrome *P*-450 reductase purified from PB-treated rats (1 µg); (b) NADPH:cytochrome *P*-450 reductase purified from NF-treated rats (1 µg); (c) molecular-weight markers.

β -naphthoflavone-treated rats. As shown in Fig. 2 the precipitin lines formed a pattern of fusion for both purified reductase preparations and solubilized microsomes. The same pattern of precipitin lines was obtained using antiserum raised to NADPH:cytochrome *P*-450 reductase isolated from NF-treated rats (results not shown). These results show that NADPH:cytochrome *P*-450 reductase isolated from the livers of both phenobarbital- and β -naphthoflavone-treated rats possess similar antigenic determinants.

Fig. 2. Ouchterlony double-immunodiffusion analysis using an antibody raised to NADPH:cytochrome P-450 reductase isolated from PB-treated rats

Antiserum (70 mg/ml) was placed in the centre well. NADPH:cytochrome P-450 reductase purified from PB- or NF-treated rats (0.2 mg/ml) was placed in wells (a) and (b) respectively. Solubilized microsomes isolated from PB-treated (9.5 mg/ml) or NF-treated (9.5 mg/ml) rats were placed in wells (c) and (d) respectively. All samples were added in a volume of 12 μ l. Plates were incubated overnight in a moist chamber, at room temperature.

Immunotitrations of NADPH:cytochrome P-450 reductase activity

The antiserum to NADPH:cytochrome P-450 reductase was further characterized by its ability to inhibit NADPH:cytochrome c (P-450) reductase activity. NADPH:cytochrome P-450 reductase purified from PB-treated rats was incubated with increasing amounts of anti-(NADPH:cytochrome P-450 reductase) serum and the activity of the enzyme was assayed as described in the Materials and methods section. As shown in Fig. 3 the antiserum was capable of inhibiting up to 92% of the NADPH:cytochrome c (P-450) reductase activity. Increasing amounts of non-immune serum had no effect on the activity of the enzyme.

Effect of phenobarbital or β -naphthoflavone pre-treatment on the activity of NADPH:cytochrome P-450 reductase in rat liver microsomes

Treatment of rats with phenobarbital increased the specific activity of NADPH:cytochrome P-450 reductase approx. 2.7-fold (from 0.12 to 0.33 μ mol $\cdot$ min $^{-1}$ $\cdot$ mg $^{-1}$). However, treatment of rats with β -naphthoflavone did not alter the specific activity of the enzyme. To determine whether the observed increase in NADPH:cytochrome P-450 reductase activity on treatment with phenobarbital is due to an alteration in the activity state of the enzyme or in the amount of the protein present in microsomal membranes we developed a radio-immunoassay technique to quantify this enzyme. The assay utilizes 125 I-labelled NADPH:cyto-

Fig. 3. Immunotitration of NADPH:cytochrome c (P-450) reductase activity

NADPH:cytochrome P-450 reductase purified from PB-treated rats was incubated together with increasing amounts of anti-(NADPH:cytochrome P-450 reductase) serum (●) or non-immune serum (○). NADPH:cytochrome c (P-450) reductase activity was measured as described in the Materials and methods section.

chrome P-450 reductase as the radiolabelled antigen.

Radioiodination of NADPH:cytochrome P-450 reductase

When NADPH:cytochrome P-450 reductase purified from PB-treated rats was iodinated with Bolton and Hunter reagent, 18% of the radioactivity was incorporated into the protein giving a specific radioactivity of about 14 μ Ci/ μ g.

Specificity of the interaction between 125 I-labelled NADPH:cytochrome P-450 reductase and NADPH:cytochrome P-450 reductase antiserum

A maximum of approx. 54% of the 125 I-labelled NADPH:cytochrome P-450 reductase was precipitable by NADPH:cytochrome P-450 reductase antiserum (Fig. 4). The inability of the antiserum to precipitate all the iodinated antigen is probably due to blocking of antigenic sites by the Bolton and Hunter reagent or to radiation damage. This finding is not unusual and is similar to results obtained with radioiodinated cytochrome P-450 (Phillips *et al.*, 1983). 125 I-labelled NADPH:cytochrome P-450

Fig. 4. Specificity of interaction between ^{125}I -labelled NADPH:cytochrome P-450 reductase and anti-(NADPH:cytochrome P-450 reductase) serum

(a) Specificity of ^{125}I -labelled NADPH:cytochrome P-450 reductase. ^{125}I -labelled NADPH:cytochrome P-450 reductase (2×10^4 c.p.m.) was incubated with the indicated amounts of NADPH:cytochrome P-450 reductase antiserum (●) or non-immune serum (○). (b) Specificity of NADPH:cytochrome P-450 reductase antiserum were incubated with 2×10^4 c.p.m. of ^{125}I -labelled NADPH:cytochrome P-450 reductase serum. The individual amounts of NADPH:cytochrome P-450 reductase (●) or ^{125}I -labelled PB-inducible cytochrome P-450 (○). Antibody-antigen complexes were precipitated with *Staph. aureus* cells. The radioactivity in the pellet was expressed as the percentage of total radioactivity.

Fig. 5. Specificity of NADPH:cytochrome P-450 reductase radioimmunoassay

^{125}I -labelled NADPH:cytochrome P-450 reductase (2×10^4 c.p.m.) was mixed with the indicated amounts of unlabelled NADPH:cytochrome P-450 reductase purified from PB-treated (●) or NF-treated rats (○) or with unlabelled purified PB-inducible cytochrome P-450 (■). Protein mixtures were incubated with an amount of antiserum, raised to NADPH:cytochrome P-450 reductase isolated from PB-treated rats, that was sufficient to precipitate 50% of the total immunoprecipitable radioactivity in the absence of competitor. Antibody-antigen complexes were precipitated with *Staph. aureus* cells. After subtracting the amount immunoprecipitated by non-immune serum, radioactivity in the pellet was expressed as a percentage of that immunoprecipitated in the absence of competitor.

precipitation reaction is therefore specific for both NADPH:cytochrome P-450 reductase and its antibody.

Specificity of NADPH:cytochrome P-450 reductase radioimmunoassay

Radioiodinated NADPH:cytochrome P-450 reductase (from PB-treated rats) was mixed with various amounts of unlabelled NADPH:cytochrome P-450 reductase, and the amount of antiserum that precipitated 50% of the total precipitable radioactivity in the absence of any competitor protein was added. Samples were processed as described in the Materials and methods section. From Fig. 5 it can be seen that NADPH:cytochrome P-450 reductase isolated from PB-treated rats competes for essentially all the antibody-binding sites that recognize the radioiodinated NADPH:cytochrome P-450 reductase. PB-inducible cytochrome P-450 (one of the most abundant liver microsomal membrane proteins in phenobarbital-treated rats) did not compete up to an amount of $2 \mu\text{g}$ with the ^{125}I -labelled NADPH:cytochrome P-450 reductase for antibody binding sites (Fig. 5). The radioimmunoassay was capable of detecting NADPH:cytochrome P-450 reductase in amounts

reductase was not immunoprecipitated by non-immune serum (Fig. 4a), and NADPH:cytochrome P-450 reductase antiserum was unable to precipitate ^{125}I -labelled PB-inducible cytochrome P-450 (the major phenobarbital-inducible cytochrome P-450; Phillips *et al.*, 1983) (Fig. 4b). The immuno-

as low as 30pg and was sensitive up to approx. 100ng.

Radioimmunoassay of NADPH:cytochrome P-450 reductase in microsomal membranes

Before assaying the amount of NADPH:cytochrome P-450 reductase present in the microsomes of PB- and NF-treated and untreated rats, it was necessary to ascertain that NADPH:cytochrome P-450 reductase isolated from PB- and NF-treated rats compete equally, on a weight-for-weight basis, with ^{125}I -labelled NADPH:cytochrome P-450 reductase (from PB-treated rats) for the antibody binding sites. Radioiodinated NADPH:cytochrome P-450 reductase (from PB-treated rats) was mixed

Fig. 6. Radioimmunoassay of NADPH:cytochrome P-450 reductase in liver microsomal membrane vesicles isolated from PB-treated and NF-treated rats and control rats

^{125}I -labelled NADPH:cytochrome P-450 reductase (2×10^4 c.p.m.) was mixed with the indicated amounts of unlabelled NADPH:cytochrome P-450 reductase purified from PB-treated rats (●), or solubilized microsomal membranes isolated from the livers of PB-treated (○), NF-treated (▲) or control rats (△). Mixtures were incubated with an amount of NADPH:cytochrome P-450 reductase antiserum sufficient to precipitate 50% of the total immunoprecipitable radioactivity. Antibody-antigen complexes were precipitated with *Staph. aureus* cells. After subtracting the amount immunoprecipitated by non-immune serum, radioactivity in the pellets was expressed as the percentage of that immunoprecipitated in the absence of competitor.

with various amounts of NADPH:cytochrome P-450 reductase purified from PB- or NF-treated animals, and the amount of antiserum that precipitated 50% of the total precipitable radioactivity in the absence of any competitor protein was added. Samples were processed as described in the Materials and methods section. As shown in Fig. 5 NADPH:cytochrome P-450 reductase from either PB- or NF-treated rats competes equally with ^{125}I -labelled NADPH:cytochrome P-450 reductase (from PB-treated rats) for antibody binding sites. These data indicate that NADPH:cytochrome P-450 reductases isolated from PB- and NF-treated rats, are, if not the same protein, very similar and are immunochemically indistinguishable by this assay.

Thus it was possible to use the radioimmunoassay to determine the amount of NADPH:cytochrome P-450 reductase in solubilized liver microsomal membrane vesicles isolated from PB- and NF-treated and untreated animals (Fig. 6). Taking the points on the curves corresponding to 50% competition, and using the competition curve generated by NADPH:cytochrome P-450 reductase purified from PB-treated animals as a standard, the amount of NADPH:cytochrome P-450 reductase was calculated (Table 1). The amount of NADPH:cytochrome P-450 reductase increased from 7 to $18 \mu\text{g}/\text{mg}$ of microsomal protein (i.e. 2.6-fold) on treatment with PB, but remained unaltered when rats were treated with NF.

The ratio of total cytochromes P-450 to NADPH:cytochrome P-450 reductase in rat liver microsomal membranes

Using the radioimmunoassay data on the amounts of NADPH:cytochrome P-450 reductase and the amount of total cytochromes P-450 (determined spectrally) it is possible to calculate the molar ratio of cytochromes P-450/NADPH:cytochrome P-450 reductase in liver microsomal membranes. The data in Table 1 show that the ratio is 15:1 in control animals and remains unchanged when animals are treated with PB. However, this ratio increases to 21:1 after NF treatment.

Table 1. The ratio of total cytochromes P-450 to NADPH:cytochrome P-450 reductase in rat liver microsomes
Each determination was made in duplicate on microsomal vesicles isolated from pools of four to 15 rat livers.

Treatment	Reductase* [$\mu\text{g} \cdot (\text{mg of microsomal protein})^{-1}$]	Reductase [nmol $\cdot (\text{mg of microsomal protein})^{-1}$]	Total cytochromes P-450† [nmol $\cdot (\text{mg of microsomal protein})^{-1}$]	Cytochrome P-450/ NADPH:cytochrome P-450 reductase molar ratio
Control	7	0.092	1.37	14.9:1
PB	18	0.236	3.54	15:1
NF	7	0.092	1.94	21.1:1

* Measured by radioimmunoassay. Duplicate points did not vary by more than 0.5%.

† Measured by CO-reduced difference spectral analysis of solubilized liver microsomal membrane vesicles.

Discussion

We have purified NADPH:cytochrome *P*-450 reductase from the liver microsomes of both PB- and NF-treated rats. The two proteins have identical molecular weights and are immunochemically indistinguishable. By using radioiodinated purified NADPH:cytochrome *P*-450 reductase and antibodies specific for this protein we developed a radioimmunoassay technique that allows, for the first time, the direct quantification of this protein in microsomal membranes isolated from animals treated with different xenobiotics. The results obtained by the radioimmunoassay show that the amount of NADPH:cytochrome *P*-450 reductase present in rat liver microsomal membranes increased by the same degree as the activity of the enzyme after treatment with PB. Thus, the PB-induced increase in NADPH:cytochrome *P*-450 reductase activity observed by ourselves and others (Orrenius & Ernster, 1964; Orrenius *et al.*, 1965) can be accounted for entirely by an increase in the amount of the protein and not to any change in its activity state. This result is supported by the findings that both the rate of synthesis of the protein and the amount of its mRNA are increased by PB treatment (Kuriyama *et al.*, 1969; Gonzalez & Kasper, 1980, 1982; Shephard *et al.*, 1982). NF treatment neither increased the activity nor the amount of NADPH:cytochrome *P*-450 reductase. The translatable level of the mRNA coding for this protein is also not affected by treatment with NF (Shephard *et al.*, 1982). Therefore, in addition to inducing different cytochrome *P*-450 variants (Phillips *et al.*, 1983), PB and NF have differential effects on NADPH:cytochrome *P*-450 reductase gene expression.

The molecular arrangement and interaction of NADPH:cytochrome *P*-450 reductase and cytochromes *P*-450 in the microsomal membrane has long been a point of interest. Although the results of reconstitution experiments *in vitro* indicate that the ratio of a cytochrome *P*-450 and NADPH:cytochrome *P*-450 reductase required for optimal mono-oxygenase activity is 1:1 (Miwa *et al.*, 1978; French *et al.*, 1980) it has been suggested that these proteins are not present in an equimolar ratio in microsomal membranes (Estabrook *et al.*, 1971; Omura, 1978; Estabrook & Werringloer, 1978). The rigorous quantification by radioimmunoassay of NADPH:cytochrome *P*-450 reductase has enabled us to determine accurately the molar ratio in microsomal membranes of total cytochromes *P*-450 and NADPH:cytochrome *P*-450 reductase. Whereas the ratio of 15:1 found in control animals remained constant after treatment with PB, it increased substantially on treatment with NF. These findings raise some interesting points regarding the relationships between the protein components of

liver microsomal membrane mixed-function mono-oxygenase systems. The 2.6-fold increase in the amount of NADPH:cytochrome *P*-450 reductase after PB treatment is accompanied by a 43-fold induction of PB-inducible cytochrome *P*-450 (Phillips *et al.*, 1983). Thus although both these microsomal membrane proteins are induced by PB the large difference in the extent of their induction suggests that different control processes may be involved. However, the net effect of phenobarbital treatment is to maintain a constant ratio between total cytochromes *P*-450 and NADPH:cytochrome *P*-450 reductase. If this constancy is not fortuitous the relative induction of the two proteins by PB may involve a finely balanced co-ordinate control mechanism.

The fact that NF fails to induce increased levels of NADPH:cytochrome *P*-450 reductase suggests, but does not prove, that the cytochrome *P*-450 variants induced by this ligand may utilize an alternative electron donor. Indeed there is evidence that electron donors other than NADPH:cytochrome *P*-450 reductase can function in cytochrome *P*-450-mediated systems. Thus West *et al.* (1974) showed that cytochrome *P*-448, isolated from 3-methylcholanthrene-treated rats, catalysed the hydroxylation of benzo[3,4]pyrene more efficiently in the presence of cytochrome *b*₅ reductase and cytochrome *b*₅, than in the presence of NADPH:cytochrome *P*-450 reductase. Studies on the inhibition of various mono-oxygenase-catalysed reactions with monospecific antibodies to cytochrome *b*₅, cytochrome *b*₅ reductase and NADPH:cytochrome *P*-450 reductase also indicate that different mono-oxygenase reactions may employ different electron donors (Noshiro & Omura, 1978; Omura, 1978).

Alternative rationalizations of our data are possible but the results reported in the present paper indicate that the relationship between the induction of NADPH:cytochrome *P*-450 reductase and the spectrum of cytochromes *P*-450 present after different xenobiotic treatments is complex. Further experiments on the detailed molecular arrangement and functional association of the proteins that comprise the microsomal membrane mixed-function mono-oxygenase system are needed to elucidate this problem.

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