An enzyme-linked immunoadsorbent assay for measuring cytochrome b_5 and NADPH–cytochrome *P*-450 reductase in rat liver microsomal fractions

Evidence for functionally inactive protein

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(Received 18 May 1983/Accepted 4 October 1983)

Immunoreactive cytochrome b_5 and NADPH–cytochrome *P*-450 reductase (EC 1.6.2.4) from rat liver microsomal fractions were measured by using an enzyme-linked immunoadsorbent assay (e.l.i.s.a.) as a function of age, sex and type of inducer (phenobarbital or 3-methylcholanthrene), and the values were compared with those obtained by spectral measurement (for cytochrome b_5) or enzymic assay (for reductase). In untreated animals, there was more cytochrome b_5 and NADPH–cytochrome *P*-450 reductase when measured by an e.l.i.s.a. than was seen spectrally or enzymically. However, for microsomal preparations from phenobarbital-pretreated animals, spectrally obtained values for cytochrome b_5 and immunoreactive-cytochrome b_5 values were similar. Values from control animals suggest that there is about 20–30% more immunoreactive cytochrome b_5 than that which is spectrally detectable.

The enzymes cytochrome b_5 and NADPH–cytochrome *P*-450 reductase are integral proteins of the endoplasmic reticulum involved in drug metabolism whose activities have been conventionally measured by using the reduced difference spectra of cytochrome b_5 (Omura & Sato, 1964) and, for the reductase, by reduction of an artificial electron acceptor (Masters *et al.*, 1967). The sensitivity of these methods is such that relatively large amounts of cellular material are required for assay, especially when amounts of the enzymes are low. Attempts to measure directly drug-metabolizing proteins at high sensitivity have used the immunological techniques of radial diffusion (Thomas *et al.*, 1979), rocket immunoelectrophoresis (Pickett *et al.*, 1981) and immunoblotting (Guengerich *et al.*, 1982); but all have been concerned with the isoenzymes of cytochrome *P*-450.

Using sheep antibodies against purified rat liver cytochrome b_5 and reductase, we have developed a highly sensitive e.l.i.s.a. for the measurement of these proteins. The e.l.i.s.a. approach is a well-established immunological technique that imparts high sensitivity and selectivity and whose mecha-

nism is relatively well understood (Voller, 1980; Butler *et al.*, 1980a). We have utilized this assay to quantify immunoreactive cytochrome b_5 and NADPH–cytochrome *P*-450 reductase in rat liver microsomal fractions from animals of different strains, age and sex and after treatment with two inducers of drug metabolism, phenobarbital and 3-methylcholanthrene.

Experimental

Materials

Rabbit anti-(sheep IgG) F(ab')₂ fragments and horseradish peroxidase-conjugated rabbit anti-(sheep IgG) were purchased from Cappel Laboratories (Cochranville, PA, U.S.A.). Polystyrene microtitre plates were purchased from Dynatech, Inc. (Alexandria, VA, U.S.A.). All chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) unless otherwise indicated.

Methods

Animals. Sprague–Dawley rats were purchased from King Labs (Oregon, WI, U.S.A.). Okamoto–SHR (spontaneously hypertensive) rats and Wistar–Kyoto (WKY) rats were obtained from an in-

Abbreviations used: e.l.i.s.a., enzyme-linked immunoadsorbent assay; IgG, immunoglobulin G.

bred colony maintained at the University of Iowa by Dr. M. J. Brody. The SHR strain represents an inbred population derived from the Wistar-Kyoto strain (Lais & Brody, 1978). Animals were housed in wire cages in a room free of environmental pesticides. Water and food (Purina Rat Chow, Minneapolis, MN, U.S.A.) were available *ad libitum*. The animals used in these studies were classified either as immature (body wt. 50–90 g) or mature (body wt. 200–240 g).

Purification of cytochrome b_5 and NADPH-cytochrome P-450 reductase and production of antibodies. Cytochrome b_5 was purified from rat liver microsomal fractions to a specific activity of 25 nmol/mg of protein (Omura & Sato, 1964), and an antiserum was produced as described previously (Krieter & Shires, 1980). NADPH-cytochrome P-450 reductase was purified by using a modification of the Yasukochi & Masters (1976) procedure, and an antiserum was produced as described previously (Shawver & Shires, 1983). The IgG fraction from both the cytochrome b_5 and reductase antisera was isolated by column chromatography on a DE-52 DEAE-cellulose column (Whatman, Maidstone, Kent, U.K.). Equivalence points for both antibodies were determined as described by Krieter (1980). For some experiments, cytochrome b_5 and reductase were labelled with ^{125}I by the method of Bolton & Hunter (1973).

Assays for selectivity of antibodies. The selectivity of both antibodies was determined in three ways. By Ouchterlony double diffusion and immunoelectrophoresis, using the same detergent-solubilized microsomal fractions as used for the e.l.i.s.a. (see below), anti-cytochrome b_5 IgG and anti-reductase IgG identified only a single arc of precipitation (Krieter & Shires, 1980; Krieter, 1980; Shawver & Shires, 1983). The antiserum was also tested by using a modification of the immunoblotting method of Towbin *et al.* (1979), where purified protein or a solubilized microsomal fraction was electrophoresed on sodium dodecyl sulphate/polyacrylamide slab gels and transferred to nitrocellulose paper. After blocking of non-specific sites, the primary antibody was added and incubated for 2 h at room temperature with gentle shaking. After washing with phosphate-buffered saline [PBS; 10 mM-potassium phosphate (pH 7.4)/0.9% NaCl] the second antibody-enzyme conjugate was added and incubated for 2 h with gentle shaking. After washing with PBS, the substrate 4-chloro-1-naphthol was added and the reaction was terminated by washing with water. Only one band of precipitation was seen from microsomal protein for either anti-cytochrome b_5 or anti-reductase, and the position of the bands corresponded to the position of immunoprecipitate obtained with purified enzyme protein (Figs. 1 and 2). The antibodies

were also tested in reticulocyte lysates translating total rat liver poly(A⁺) mRNA *in vitro* and poly(A⁺) mRNA isolated from free polyribosomal fractions. Anti-cytochrome b_5 IgG [together with anti-(sheep IgG) as a second precipitating antibody] immunoprecipitated a protein whose relative mobility on sodium dodecyl sulphate/polyacrylamide gels corresponded to that of purified cytochrome b_5 (M_r 17000). Anti-reductase [together with anti-(sheep IgG)] immunoprecipitated only a single translational product (M_r 78000) after incubation in rabbit reticulocyte lysates of poly(A⁺) mRNA species isolated from bound polyribosomal fractions and total liver mRNA, but not of that from free polyribosomal fractions (Shawver & Shires, 1983).

Enzyme-linked immunoadsorbent assay. Liver microsomes from Sprague-Dawley, SHR or WKY rats were solubilized with 1 mg of sodium cholate and 1 μl of Triton X-100/mg of microsomal protein. The solubilized microsomal fractions or purified enzyme were adsorbed to polystyrene microtitre wells (50 μl /well final volume) by incubation at 4°C for 18 h in buffer containing 0.1 M- Na_2CO_3 , 0.1 M- NaHCO_3 and 0.02% NaN_3 at pH 9.6. The wells were then washed four times with 200 μl of PBS containing 0.05% Tween 20/well. The wells were then incubated with 50 μl of the appropriate antibody (see the Figure legends) for 2 h at room temperature with gentle shaking. After washing, the wells were incubated with 50 μl of rabbit anti-(sheep IgG) F(ab')₂ fragments conjugated with β -galactosidase (O'Sullivan & Marks, 1981) for 2 h at room temperature with gentle shaking. After washing, the substrate *o*-nitrophenyl β -D-galactoside at a concentration of 4 mg/ml of buffer containing 50 mM-potassium, pH 7.2, 1.5 mM-MgCl₂ and 100 mM-2-mercaptoethanol was added (50 μl /well) to the wells and incubated for approx. 30 min on a Fisher Minimix (Fischer, Pittsburgh, PA, U.S.A.). The reaction was terminated by the addition of 50 μl of 0.5 M- Na_2CO_3 , and the extent of the reaction was measured by the A_{410} by using a Bio-Tek model 307 EIA reader (Bio-Tek, Burlington, VT, U.S.A.).

Analytical methods. Protein was measured by the method of Lowry *et al.* (1951). The reduced difference spectrum of cytochrome b_5 was determined by the method of Omura & Sato (1964), with an Aminco DW-2 spectrophotometer. The specific activity of NADPH-cytochrome P-450 reductase was determined by the method of Masters *et al.* (1967), with a Beckman 35 spectrophotometer. The radioactivity of iodinated proteins was measured in a Beckman 310 gamma counter. Student Newman-Keuls tests for statistical differences were performed on a Hewlett-Packard model 2648A computer.

Results

In performing e.l.i.s.a. for cytochrome b_5 or NADPH-cytochrome P-450 reductase, antigen is first adsorbed to the well of a microtitre plate, followed in order by binding of the primary antibody (Ab1) and by interaction with anti-(Ab1 IgG) F(ab')₂ conjugated with β -galactosidase. Adsorption of other antigens to the well of microtitre plates ranges from 10 to 80% (Cantarero *et al.*, 1980). The percentage of adsorption to the microtitre well depends, in part, on the specific antigen. Adsorption of cytochrome b_5 and reductase was measured with externally iodinated preparations of each enzyme. After application of the labelled proteins in the carbonate buffer, the wells were rinsed, broken apart and the adsorbed radioactivity was counted. Adsorption was $14 \pm 1.2\%$ for cytochrome b_5 and $12 \pm 0.8\%$ for reductase. These adsorption values are within the ranges observed by others for a large spectrum of antigens (Cantarero *et al.*, 1980). The important aspect of adsorption for e.l.i.s.a. quantification is the constancy of the percentage of adsorbed antigen over the range of antigen levels encountered under assay conditions. It was found that, for both cytochrome b_5 and reductase, the specific percentage adsorption for each protein was constant over the range 0.01–800 ng. Further, it was found that this constancy was not influenced by the presence of other detergent-solubilized microsomal proteins over the range 0.5–1000 ng (results not shown). It should be pointed out that microtitre wells can vary from batch to batch with regard to antigen adsorption capacity (Kricka *et al.*, 1980). Although all experiments reported here were not performed on the same batch of plates, no variability in percentage of antigen adsorbed was observed. Nevertheless, adsorption characteristics of all batches of microtitre plates need to be determined when performing any e.l.i.s.a. type of immunoquantification (Kricka *et al.*, 1980).

The second interaction, the binding of the primary antibody to adsorbed antigen, was performed in considerable antibody excess. The equivalence points of these reactions are 6 μ g of microsomal protein/100 μ l of anti-(cytochrome b_5 IgG) and 160 μ g of microsomal protein/100 μ l of anti-(reductase IgG). It would be expected that, in antibody excess, the antigen-antibody complexes would be stable.

In the third interaction of the e.l.i.s.a., the F(ab')₂-enzyme conjugate is allowed to react with the adsorbed antigen-primary antibody complex. The best combining ratio of conjugate to complex had to be determined empirically for each conjugate. This is necessary for two reasons: the equivalence point varies with each preparation of F(ab')₂-

enzyme conjugate from antiserum IgG, and the β -galactosidase marker activity in each batch of conjugate varies from preparation to preparation.

Non-specific interactions between constituents of the e.l.i.s.a. system may include non-specific binding of the primary antibody to the microtitre well and non-specific binding of the F(ab')₂-enzyme conjugate to either the adsorbed protein or the microtitre well. Measuring β -galactosidase activity in microtitre wells containing all specific e.l.i.s.a. interactants, but minus either adsorbed antigen, primary antibody, or both, showed no evidence for these non-specific interactions. Whereas the overall stoichiometry of the pure e.l.i.s.a. system (comprising only the essential elements of the assay) appears not to be significantly influenced by non-specific interactions among the interactants, performance of the e.l.i.s.a. on preparations of complex protein mixtures, such as solubilized microsomal membranes, may not be as unencumbered. This is indicated by dose-response curves involving absorbance versus microsomal protein concentration, where the curve may not intersect the origin (Fig. 3d). This is a general phenomenon displayed by most e.l.i.s.a. systems (Butler *et al.*, 1980b).

The quantification by e.l.i.s.a. depends on the selectivity of the primary antibody for a single protein. E.l.i.s.a. of cytochrome b_5 and reductase has been tested by using the immunoblotting system, as shown in Figs. 1 and 2. This system, which employs the same antibody combination used in the e.l.i.s.a., mimics most aspects of the e.l.i.s.a. reaction, including incubation conditions, ionic strength and detergent concentrations, and displays the reacting antigen in an electrophoretic pattern. The immunoblot showed a single combining protein band at M_r 17000 for anti-(cytochrome b_5) (Fig. 1) and a single combining protein band at M_r 78000 for anti-reductase (Fig. 2), and both bands corresponded to the position of migration of the purified protein. These results show that, with microsomal preparations, one could expect the e.l.i.s.a. procedure to be selective for these two enzymes.

The quantification in microsomal fractions by the e.l.i.s.a. also depends on establishment of a linear relationship between marker enzyme activity (as measured by absorbance) and the amount of cytochrome b_5 and reductase antigen. As shown in Fig. 3, absorbance was proportional to the concentration of either cytochrome b_5 or reductase over a fairly broad range for both the purified enzymes (Figs. 3a and 3b) and the enzymes in solubilized microsomal preparations (Figs. 3c and 3d). Regression analysis of these data gave correlation coefficients of 0.985, 0.995, 0.999 and 0.995 for data in Figs. 3(a)–3(d) respectively. Quantifying the

Fig. 1. Immunoblot assay to determine specificity of anti-(cytochrome b_5) IgG

Solubilized microsomal fractions or purified cytochrome b_5 were electrophoresed on a sodium dodecyl sulphate/15%-polyacrylamide slab gel, and the protein was transferred to nitrocellulose sheets as described in the experimental section. Sheep anti-(cytochrome b_5) IgG was diluted 1:50 and incubated overnight, followed by incubation with horseradish peroxidase conjugated to rabbit anti-(sheep IgG) (diluted 1:2000). A 0.05% (w/v) solution of 4-chloro-1-naphthol was then added and incubated for 5 min. The reaction was terminated by rinsing with water. (a) Coomassie Brilliant Blue G-250 stain of microsomes electrophoresed on a sodium dodecyl sulphate/15%-polyacrylamide gel. (b) Coomassie Brilliant Blue G-250 stain of purified cytochrome b_5 . (c) Immunological detection of cytochrome b_5 from purified protein. (d) Immunological detection of cytochrome b_5 from microsomal preparation from a mature male Sprague-Dawley rat. (e) Immunological detection of cytochrome b_5 from a microsomal preparation from a mature male Sprague-Dawley rat treated with phenobarbital. Arrows represent migration position of cytochrome b_5 .

amount of cytochrome b_5 or reductase present in microsomal samples was accomplished by preparing a standard curve by using purified enzyme and plotting absorbance versus protein concentration. The amount of each protein in microsomal fractions can then be determined by performing an e.l.i.s.a. on serial dilutions of microsomal fractions and extrapolation of absorbance units to concentration from the standard curve. Standard curves were typically linear within the range 0.2–5 ng. The e.l.i.s.a. of cytochrome b_5 and reductase in microsomal samples was sensitive to at least 4 ng of

Fig. 2. Immunoblot assay to determine specificity of anti-(reductase) IgG

Microsomal fractions of purified reductase were electrophoresed on a sodium dodecyl sulphate/8.5%-polyacrylamide slab gel, and the protein was transferred to nitrocellulose sheets as described in the Experimental section. Sheep anti-(reductase IgG) was diluted 1:50 and incubated overnight, followed by incubation with horseradish peroxidase conjugated to rabbit anti-(sheep IgG) diluted 1:2000. (a) Coomassie Brilliant Blue G-250 stain of microsomal fraction electrophoresed on a sodium dodecyl sulphate/8.5%-polyacrylamide-gel. (b) Coomassie Brilliant Blue G-250 stain of purified reductase. (c) Immunological detection of reductase from purified reductase. (d) Immunological detection of reductase from microsomal preparation from a mature male Sprague-Dawley rat. (e) Immunological detection of reductase from a microsomal preparation from a mature male Sprague-Dawley rat treated with phenobarbital. Arrows represent migration position of NADPH-cytochrome $P-450$ reductase.

microsomal protein for cytochrome b_5 and 1 ng of microsomal protein for reductase.

Assessment of the microsomal content of cytochrome b_5 and NADPH-cytochrome $P-450$ reductase by e.l.i.s.a. was done in side-by-side studies with spectral and enzymic assays respectively, performed on the same microsomal membrane preparations. Reductase in conventional assays is measured as units of enzyme activity rather than absolute content, which does not allow direct comparison with e.l.i.s.a. values. The cytochrome b_5 spectral assay, however, does quantify the amount of holoenzyme present, and therefore can be directly compared with the antigen amounts measured by cytochrome b_5 e.l.i.s.a. This side-by-side comparison of e.l.i.s.a. with conventional assays was made for Sprague-Dawley rats of different ages and sex in four different treatment groups (phenobarbital or 3-methylcholanthrene

Fig. 3. Enzyme-linked immunoadsorbent assay of cytochrome b_5 and NADPH-cytochrome P-450 reductase on purified protein and microsomal protein

(a) E.I.I.S.A. of purified cytochrome b_5 . Sheep anti-(cytochrome b_5) IgG was diluted 1:20, and β -galactosidase-conjugated rabbit anti-[sheep F(ab')₂] fragments were diluted 1:20. (b) E.I.I.S.A. of purified NADPH-cytochrome P-450 reductase. Sheep anti-(reductase IgG) was diluted 1:10, and β -galactosidase-conjugated rabbit anti-[sheep F(ab')₂] fragments were diluted 1:20. (c) E.I.I.S.A. of cytochrome b_5 in microsomal protein. Antibodies were diluted as indicated in (a). (d) E.I.I.S.A. of NADPH-cytochrome P-450 reductase in microsomal protein. Antibodies were diluted as indicated in (b). Although these curves represent microsomal fractions isolated from control immature male rats, all other samples produced similar curves. The absorbance values were always proportional to the microsomal protein concentration.

administration and the respective 0.9% NaCl or corn-oil controls). The results are shown in Table 1. From the ratio of immunoreactive to spectrally active cytochrome b_5 , it can be concluded that immunoreactive cytochrome b_5 content was virtually the same as spectrally determined cytochrome b_5 content in the microsomal fractions from livers of all phenobarbital-treated groups. However, in the control animals (0.9% NaCl-treated), the immunoreactive cytochrome b_5 content was 1.38- and 1.39-fold greater than the spectrally determined content in adult males and females respectively, and 1.38- and 1.34-fold greater in immature males and females. 3-Methylcholanthrene treatment failed to alter significantly the spectrally determined content of cytochrome b_5 in most cases, but the amount of immunoreactive cytochrome b_5 was slightly decreased, as reflected in the slight decline in e.i.i.s.a.-determined to spectrally determined ratios from values with the corn-oil controls. For mature female animals treated with 3-methylcholanthrene, there was an

increase in spectrally determined cytochrome b_5 compared with corn-oil-treated animals, but the ratio of immunoreactive to spectrally determined cytochrome b_5 was 1.21, a value well within the range of ratios in other 3-methylcholanthrene-treated groups. An excess of immunoreactive cytochrome b_5 over spectrally determined cytochrome b_5 was also observed in the microsomal preparations from corn-oil-treated animals in all age and sex groups at a value (1.28–1.38-fold higher) that was about the same as for the 0.9% NaCl-treated rats.

Phenobarbital treatment increased the specific enzymic activity of reductase in adult male Sprague-Dawley-rat liver microsomal fractions, and, as shown in Table 1, the increase was mirrored to a degree by increases in reductase immunoreactivity. The age and sex groups were examined individually for relative increases in reductase amounts after phenobarbital treatment (compared with their 0.9% NaCl controls). Increases were seen in all groups, but there was a

Table 1. Quantification by e.l.i.s.a. and comparison with reduced difference spectra of cytochrome b_5 and enzymic activity of NADPH-cytochrome P-450 reductase in Sprague-Dawley-rat liver microsomal fractions

Spectral or enzymic assays were performed on microsomal preparations from individual animals in each treatment group segregated by sex and age, and e.l.i.s.a. was performed on the same microsomal preparation. Phenobarbital was administered intraperitoneally once a day (80 mg/kg body wt. in 0.9% NaCl) for 4 days. 3-Methylcholanthrene was administered intraperitoneally once a day (25 mg/kg body wt. in corn oil) for 4 days. Spectral-assay values represent mean specific content $\pm$ s.e.m. (determined by reduced difference spectra) from three to five animals. E.l.i.s.a. values represent mean specific content $\pm$ s.e.m. (determined by e.l.i.s.a.) from three to five animals. Three to five points assayed in triplicate were used for each animal. 'Ratio' values represent the mean ratio of e.l.i.s.a. to spectrally determined values $\pm$ s.e.m. Specific activity values represent mean specific activity $\pm$ s.e.m. from three to five animals, with cytochrome c as substrate. Abbreviation used: ND, not determined.

Animals and treatment*	Cytochrome b_5		NADPH-cytochrome P-450 oxidoreductase		
	Spectral assay (nmol/mg of protein)	E.l.i.s.a. (nmol/mg of protein)	Specific activity (nmol/min per mg of protein)	E.l.i.s.a. (nmol/mg of protein)	
Mature males					
0.9% NaCl	0.34 ± 0.03	$0.47 \pm 0.03^*$	319 ± 15	0.29 ± 0.03	
Phenobarbital	0.28 ± 0.01	0.28 ± 0.01	523 ± 46	0.53 ± 0.06	
Corn oil	0.36 ± 0.01	$0.46 \pm 0.12^*$	330 ± 29	0.35 ± 0.04	
3-Methylcholanthrene	0.30 ± 0.04	$0.37 \pm 0.02^*$	251 ± 14	0.20 ± 0.03	
Mature females					
0.9% NaCl	0.33 ± 0.02	$0.46 \pm 0.02^*$	298 ± 15	0.24 ± 0.03	
Phenobarbital	0.27 ± 0.02	0.28 ± 0.01	523 ± 37	0.52 ± 0.03	
Corn oil	0.26 ± 0.01	$0.36 \pm 0.01^*$	263 ± 5	0.25 ± 0.05	
3-Methylcholanthrene	0.43 ± 0.05	$0.52 \pm 0.06^*$	240 ± 16	0.22 ± 0.01	
Immature males					
0.9% NaCl	0.24 ± 0.02	$0.33 \pm 0.02^*$	251 ± 29	0.17 ± 0.02	
Phenobarbital	0.25 ± 0.02	0.27 ± 0.03	423 ± 42	0.45 ± 0.05	
Corn oil	0.26 ± 0.01	$0.34 \pm 0.02^*$	290 ± 3	0.24 ± 0.01	
3-Methylcholanthrene	0.24 ± 0.02	$0.30 \pm 0.02^*$	265 ± 7	0.22 ± 0.01	
Immature females					
0.9% NaCl	0.29 ± 0.01	$0.39 \pm 0.02^*$	343 ± 40	0.33 ± 0.05	
Phenobarbital	0.24 ± 0.06	0.26 ± 0.07	518 ± 28	0.57 ± 0.05	
Corn oil	ND	ND	ND	ND	
3-Methylcholanthrene	0.19 ± 0.05	$0.24 \pm 0.06^*$	311 ± 46	0.29 ± 0.07	

* Significantly greater than spectrally determined values at $P < 0.05$.

greater increase in immunoreactive amounts than in enzymic activity. In adult males, enzymic activity rose 1.6-fold, whereas immunoreactive amounts rose 1.8-fold. For immature males, e.l.i.s.a. values rose 2.6-fold, compared with 1.7-fold for enzymic activity; for immature females, the change was 1.7-fold for e.l.i.s.a. and 1.5-fold for activity; and for mature females, it was 2.2-fold and 1.8-fold for e.l.i.s.a. and enzymic values respectively. Thus phenobarbital stimulated the microsomal content of immunoreactive reductase

to a greater extent that it did with enzymically active reductase. After 3-methylcholanthrene treatment, there was no change in the amounts of immunoreactive or enzymically active reductase (Table 1).

The amounts of immunoreactive cytochrome b_5 and reductase were also determined in different strains of rats and compared with the spectrally determined value for cytochrome b_5 and enzymic assay for reductase. The results for SHR and their genetic control, WKY, are shown in Tables 2 and

Table 2. Quantification by e.l.i.s.a. and reduced difference spectra of cytochrome b_5 in SHR and WKY rat liver microsomal fractions

Assays were performed as described in the legend to Table 1. Spectral-assay values represent mean specific content $\pm$ S.E.M. (determined by reduced difference spectra) from three to five animals. E.l.i.s.a. values represent mean specific content $\pm$ S.E.M. (determined by e.l.i.s.a.) from three to five animals. Three to five points assayed in triplicate were used for each animal. 'Ratio' values represent the mean ratio of e.l.i.s.a. to spectrally determined values $\pm$ S.E.M. Abbreviation used: ND, not determined.

Animals and treatment	Spectral assay (nmol/min per mg of protein)	E.l.i.s.a. (nmol/mg of protein)	Ratio
SHR immature males			
0.9% NaCl	0.35 ± 0.08	$0.45 \pm 0.10^*$	1.29 ± 0.05
Phenobarbital	0.55 ± 0.03	0.54 ± 0.04	0.98 ± 0.12
Corn oil	ND	ND	ND
3-Methylcholanthrene	0.22 ± 0.03	$0.31 \pm 0.06^*$	1.41 ± 0.02
SHR mature males			
0.9% NaCl	0.25 ± 0.06	$0.41 \pm 0.02^*$	1.64 ± 0.03
Phenobarbital	0.29 ± 0.03	0.30 ± 0.02	1.03 ± 0.03
Corn oil	0.25 ± 0.02	$0.38 \pm 0.09^*$	1.52 ± 0.02
3-Methylcholanthrene	0.26 ± 0.02	$0.38 \pm 0.02^*$	1.46 ± 0.04
SHR immature females			
0.9% NaCl	0.47 ± 0.18	$0.65 \pm 0.19^*$	1.38 ± 0.11
Phenobarbital	0.48 ± 0.11	0.49 ± 0.11	1.02 ± 0.06
Corn oil	0.34 ± 0.11	$0.44 \pm 0.10^*$	1.29 ± 0.10
3-Methylcholanthrene	0.40 ± 0.14	$0.50 \pm 0.11^*$	1.25 ± 0.04
SHR mature females			
0.9% NaCl	0.25 ± 0.09	$0.36 \pm 0.08^*$	1.44 ± 0.14
Phenobarbital	0.59 ± 0.05	0.59 ± 0.08	1.00 ± 0.03
Corn oil	ND	ND	ND
3-Methylcholanthrene	0.51 ± 0.08	$0.65 \pm 0.15^*$	1.27 ± 0.10
WKY immature males			
0.9% NaCl	0.35 ± 0.09	$0.47 \pm 0.06^*$	1.34 ± 0.05
Phenobarbital	$0.24 \pm 0.02^{**}$	$0.27 \pm 0.02^{**}$	1.12 ± 0.01
Corn oil	0.27 ± 0.01	$0.38 \pm 0.04^*$	1.41 ± 0.07
3-Methylcholanthrene	0.29 ± 0.03	$0.34 \pm 0.04^*$	1.17 ± 0.02
WKY immature females			
0.9% NaCl	ND	ND	ND
Phenobarbital	0.57 ± 0.07	0.59 ± 0.06	1.04 ± 0.02
Corn oil	0.29 ± 0.05	$0.38 \pm 0.07^*$	1.31 ± 0.02
3-Methylcholanthrene	0.33 ± 0.10	$0.40 \pm 0.10^*$	1.33 ± 0.03
WKY mature females			
0.9% NaCl	0.34 ± 0.03	$0.43 \pm 0.09^*$	1.26 ± 0.05
Phenobarbital	0.45 ± 0.06	0.47 ± 0.08	1.04 ± 0.03
Corn oil	ND	ND	ND
3-Methylcholanthrene	0.41 ± 0.18	$0.54 \pm 0.06^*$	1.31 ± 0.09

* Values significantly greater than spectrally determined values at $P < 0.05$.

** Significantly different from SHR immature phenobarbital-treated males at $P < 0.01$.

3. The same pattern appears in the SHR and WKY animals for differences between immunoreactive cytochrome b_5 and spectrally determined cytochrome b_5 and between immunoreactive reductase and enzymically active reductase as that seen with Sprague-Dawley animals.

Data from Tables 1 and 2 show statistically significant differences between the immunoreactive cytochrome b_5 content and the spectrally determined cytochrome b_5 content in microsomal fractions from animals of the 0.9% NaCl-treated, corn-oil-treated and 3-methylcholanthrene-treated groups of different age, sex or strain. No difference

was observed in immunoreactive and spectrally assayed content of cytochrome b_5 in microsomal fractions from phenobarbital-treated animals regardless of age, sex or strain. The rigorous Student Newman-Keuls procedure was used to analyse these differences at $P < 0.05$ (Tables 1 and 2). When values for the ratios of immunoreactive to spectral ratios of all animals in each treatment group (phenobarbital- or 3-methylcholanthrene-treated animals or 0.9% NaCl or controls) were analysed by analysis of variance, they were shown not to differ within each treatment group, regardless of age, sex or strain ($P < 0.05$). Thus the differ-

Table 3. *Quantification by e.l.i.s.a. and comparison with enzymic activity of NADPH-cytochrome P-450 reductase in SHR and WKY rat liver microsomal fractions*

Assays were performed as described in the legend to Table 1. Specific-activity values represent means $\pm$ S.E.M. from three to five animals, with cytochrome c as substrate. E.l.i.s.a. values represent means $\pm$ S.E.M. from three to five animals. Three to five points assayed in triplicate were used for each animal. Abbreviation used: ND, not determined.

Animals and treatment	Specific activity (nmol/min per mg of protein)	E.l.i.s.a. (nmol/mg of protein)
SHR immature males		
0.9% NaCl	523 $\pm$ 20	0.53 $\pm$ 0.05
Phenobarbital	683 $\pm$ 17	0.66 $\pm$ 0.02
Corn oil	313 $\pm$ 29	0.28 $\pm$ 0.06
3-Methylcholanthrene	231 $\pm$ 31	0.23 $\pm$ 0.03
SHR mature males		
0.9% NaCl	284 $\pm$ 5	0.25 $\pm$ 0.01
Phenobarbital	459 $\pm$ 33	0.44 $\pm$ 0.02
Corn oil	273 $\pm$ 20	0.30 $\pm$ 0.04
3-Methylcholanthrene	250 $\pm$ 16	0.25 $\pm$ 0.01
SHR immature females		
0.9% NaCl	720 $\pm$ 60	0.62 $\pm$ 0.04
Phenobarbital	930 $\pm$ 20	0.91 $\pm$ 0.01
Corn oil	580 $\pm$ 30	0.54 $\pm$ 0.03
3-Methylcholanthrene	684 $\pm$ 17	0.64 $\pm$ 0.03
SHR mature females		
0.9% NaCl	413 $\pm$ 18	0.39 $\pm$ 0.01
Phenobarbital	437 $\pm$ 18	0.43 $\pm$ 0.02
Corn oil	397 $\pm$ 20	0.38 $\pm$ 0.05
3-Methylcholanthrene	320 $\pm$ 45	0.33 $\pm$ 0.07
WKY immature males		
0.9% NaCl	171 $\pm$ 25	0.19 $\pm$ 0.04
Phenobarbital	334 $\pm$ 19	0.44 $\pm$ 0.04
Corn oil	237 $\pm$ 17	0.27 $\pm$ 0.02
3-Methylcholanthrene	275 $\pm$ 10	0.29 $\pm$ 0.02
WKY immature females		
0.9% NaCl	134 $\pm$ 6	0.13 $\pm$ 0.02
Phenobarbital	703 $\pm$ 7*	0.77 $\pm$ 0.01*
Corn oil	207 $\pm$ 65	0.15 $\pm$ 0.02
3-Methylcholanthrene	228 $\pm$ 29	0.27 $\pm$ 0.04
WKY mature females		
0.9% NaCl	357 $\pm$ 7	0.33 $\pm$ 0.01
Phenobarbital	787 $\pm$ 22	0.75 $\pm$ 0.05
Corn oil	ND	ND
3-Methylcholanthrene	128 $\pm$ 17	0.35 $\pm$ 0.05

* Significantly different than SHR immature phenobarbital-treated females at $P < 0.05$.

ences between immunoreactive and spectrally active cytochrome *b*₅ can be viewed as supported by the side-by-side assays of over 30 individuals comprising each treatment group, and the parameters of age, sex and strain, as arranged in these experiments, do not need further consideration. Analysis of variance with individual treatment groups did not include SHR 0.9% NaCl-treated (1.64 ± 0.03) and 3-methylcholanthrene-treated (1.46 ± 0.04) mature males. These two sets of animals (three each) were considerably divergent from other animals in the same treatment group of over 30 animals (1.36 ± 0.02 and 1.27 ± 0.02) respectively, for reasons that are unclear.

To determine if e.l.i.s.a. selectivity for cytochrome *b*₅ or reductase was sustained in the solubilized microsomal preparations from animals in the various sets of each different treatment group, immunoblot analysis was routinely performed on each particular detergent-solubilized preparation. The immunoblot pattern for phenobarbital-treated mature Sprague-Dawley males is shown for cytochrome *b*₅ in Fig. 1(e) and for reductase in Fig. 2(e). Identical patterns were obtained from microsomal preparations from all other sets of male or female, immature or mature and SHR, WKY or Sprague-Dawley animals treated with 0.9% NaCl, corn oil, phenobarbital or 3-methylcholanthrene (results not shown).

Discussion

Cytochrome *b*₅ and NADPH-cytochrome *P*-450 reductase both require prosthetic groups for functionality *in vivo* and spectral or enzymic activity *in vitro* (haem for cytochrome *b*₅, and FAD and FMN for the reductase). Although the synthesis of the apoproteins of these enzymes has been well studied, little is known about the bioformation of the holoenzymes. The mRNA for cytochrome *b*₅ is primarily borne by, and translated on, free polyribosomes (Krieter & Shires, 1980; Gonzalez & Kasper, 1980) and that for the cytochrome *P*-450 reductase on membrane-bound polyribosomes (Gonzalez & Kasper, 1980). Although nascent reductase has direct access to the membrane of the endoplasmic reticulum from the bound polyribosome, in contrast with cytochrome *b*₅, both holoenzymes are found widely distributed on various cytoplasmic membrane types, including the Golgi membranes (Ito & Palade, 1978; Jarasch *et al.*, 1979). Hydrophobic domains of the holoenzymes anchor both the molecules into the outer face of the endoplasmic-reticulum membrane; and the hydrophilic domains, which in both cases contain the prosthetic groups, are exposed at the membrane surface (Black & Coon, 1982; Dailey & Strittmatter, 1980).

In the present study, the higher than expected amounts of immunoreactive cytochrome *b*₅ and NADPH-cytochrome *P*-450 reductase seem best interpreted as being due to the presence of a fraction of the enzymes free of attached prosthetic groups. The case is much stronger for cytochrome *b*₅, because of the direct comparability of the immunoreactive and spectrally determined amounts. It is postulated that both reductase and cytochrome *b*₅, with or without prosthetic groups, would be immunoreactive, whereas only the holoenzymes would be spectrally or enzymically assayable. The case for the cytochrome-*b*₅ e.l.i.s.a. antibodies being indifferent to the presence of haem lies in the absence of any cross-reactivity with other membrane elements, a large proportion of which bear haem groups, for instance the cytochrome *P*-450 group. The chances of haem thus presenting as a haptenic determinant that influences the e.l.i.s.a. determinations seems remote. Since cytochrome *b*₅ does not occur in rat liver in any kind of isoenzymic diversity (Krieter, 1980), but rather as a single polypeptide species (in contrast with cytochrome *P*-450), the effects of regioselectivity of the anti-(cytochrome *b*₅ IgG) for the antigen would be muted. Any variability owing to steric hindrance or selective exposure to the antigen by the antibody would seem to be lessened by the uniform treatment of all microsomal membrane samples by detergent solubilization before e.l.i.s.a. quantification. The argument that higher than expected content values shown by the e.l.i.s.a. method indicates that the e.l.i.s.a. is registering spurious values, perhaps through non-specific interactions, is invalidated by the actual performance of e.l.i.s.a. in the present study, in which, under certain conditions (e.g. phenobarbital treatment), spectrally determined and e.l.i.s.a. values coincide. Further, the patterns of differences between the results for the two methods were reproducible where other variables entered, e.g. sex, strain and age.

Virtually nothing is known about acquisition of haem or FAD and FMN by the apoproteins of cytochrome *b*₅ and NADPH-cytochrome *P*-450 reductase *in vivo*. Hara & Minakami (1970) have suggested that a proportion of microsomal cytochrome *b*₅ might lack prosthetic groups, on the basis of haem-labelling studies. Much more attention has been paid to the haem content of the much more abundant cytochrome *P*-450 group. Haem, whose final biosynthetic steps take place in the mitochondrion, appears to exist within the cell in a small but reactive pool that is postulated to provide the source for haemoprotein prosthetic groups and perhaps regulates rate-limiting enzymic steps in the haem biosynthetic pathway. Speculation regarding the character of this pool, especially

about the mechanism by which newly formed haem makes it way to newly formed apoproteins, has included suggestions that haem apoprotein synthesis takes place only on the mitochondrially associated rough endoplasmic reticulum (Meyer *et al.*, 1980) or that there are special haem-carrier molecules in the cytoplasm that transport the ligand to appropriate intracellular locations (see, e.g., Ketterer *et al.*, 1976; Davies *et al.*, 1982). In the present study, cytochrome *b₅* was measured only in microsomal membranes. It thus still remains uncertain whether some cytochrome *b₅* apoprotein is synthesized and inserted into the membranes without attached haem, or whether, once holoenzyme is inserted, the prosthetic group may be dissociated. Phenobarbital is a well-known inducer of haem synthesis (Giger & Meyer, 1981), and the presumably heightened availability of the ligand might explain the apparent saturation of this microsomal apoprotein observed in phenobarbital-treated rats.

We thank Dr. J. E. Butler, from the Department of Microbiology, and Dr. G. R. Dutton and Mr. Tony Gard, from this Department, for their helpful discussion. We also thank Dr. M. J. Brody for generously supplying the SHR and WKY animals used in these studies. This research was supported by N.I.H. grants GM07069, GM12675 and NS13121.

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