Biochemical Pharmacology, Vol. 34, No. 10, pp. 1671–1681, 1985.
Printed in Great Britain.

0006-2952/85 $3.00 + 0.00

A MONOCLONAL ANTIBODY RAISED TO RAT LIVER CYTOCHROME P-448 (FORM C) WHICH RECOGNISES AN EPITOPE COMMON TO MANY OTHER FORMS OF CYTOCHROME P-450

ALAN R. BOOBIS*†, JAMIE MCQUADE†, DOROTHEA SESARDIC†, RICHARD T. ROBSON‡, CHRIS HAYWARD‡, EDWARD A. LOCK§, CLIFF R. ELCOMBE§, MICHAEL S. ROSE‡ and DONALD S. DAVIES†

† Department of Clinical Pharmacology, Royal Postgraduate Medical School, London W12 0HS; ‡ Corporate Bioscience Group, Imperial Chemical Industries PLC, Runcorn, Cheshire WA7 4QE; and §Central Toxicology Laboratory, Imperial Chemical Industries PLC, Alderley Park, near Macclesfield, Cheshire SK10 4TJ, U.K.

(*Received* 30 *August* 1984; *accepted* 13 *November* 1984)

Abstract—A murine monoclonal antibody has been raised against a partially purified preparation of hepatic cytochrome P-448 (form c) from β-naphthoflavone-treated rats. The monoclonal origin of the antibody was established by limiting dilution culture and isoelectricfocusing. The antibody has been designated 3/4/2. It reacts with apparently homogeneous cytochrome P-448 from rat liver in solid phase assay. It also cross reacts with a number of other cytochromes P-450, from rat and rabbit. In addition, a positive reaction was obtained with microsomal fractions from a variety of species, including man. None of the species tested was negative. The antibody does not react appreciably with purified haemoproteins other than cytochromes P-450.

Antibody 3/4/2 is not inhibitory, either in reconstituted systems or with intact microsomal fraction. However, evidence was obtained that the antibody does cause some perturbation of the tertiary structure of the apoprotein at or near the haem.

The cytochrome P-450‖ dependent monooxygenase system plays an essential role in the metabolism of a wide variety of both exogenous [1] and endogenous [2] substrates. It is responsible, not only for determining the duration of action of a wide variety of drugs [3], but also for the activation of a number of compounds to highly reactive electrophilic intermediates which can cause tissue damage [4]. The resultant effect may be cytotoxicity, mutagenicity or carcinogenicity.

It is becoming increasingly apparent that many oxidative reactions involving endogenous substrates are catalysed by a cytochrome P-450 dependent monooxygenase system. Obvious examples include the 25- [5] and the 1α-hydroxylation [6] of vitamin D, many of the oxidation steps involved in steroid biosynthesis [7] and degradation [8], bile acid synthesis [9] and the oxidation of fatty acids [10]. Recently, evidence has been presented that the enzymes catalysing the synthesis of both thromboxane A_2 [11] and prostacyclin [12] are unusual forms of cytochrome P-450.

The substrate specificity of the monooxygenase system resides in the cytochrome P-450 component [13]. There are multiple forms of cytochrome P-450 within a given tissue from a given species [14]. Most, if not all, of these forms are regulated by separate genes [15]. They are subject to differential induction [16] and inhibition [17]. They also show marked differences in development [17], tissue distribution [18], species distribution [19] and sex distribution [20]. Thus each form of cytochrome P-450 is, potentially, selectively regulated both by genetic and environmental factors and will have a unique profile of substrate specificity. The consequences of this diversity of enzymes for the toxicity and carcinogenicity of foreign compounds has been the subject of several recent reviews [21–23].

In view of the possibility that interindividual differences in the complement of cytochromes P-450 are responsible for differences in susceptibility to the toxicity or carcinogenicity of foreign compounds [23], it is important to be able to quantitate the amount of the different forms of this haemoprotein in different individuals. In addition, the substrate specificity of each form needs to be defined to enable predictions to be made from the metabolism of one substrate to that of others. Previously this problem has been tackled by purifying various isozymes of cytochrome P-450 to apparent homogeneity and investigating their substrate specificity in recon-

* To whom all correspondence and reprint requests should be addressed.

‖ There are multiple forms of cytochrome P-450 and as yet there is no accepted nomenclature for the different isozymes. The term cytochrome P-450 is used here in a generic sense to denote any or all forms of the haemoprotein. The term cytochrome P-448 is used to denote the major form (designated form c by Ryan *et al.*, *J. biol. Chem.* **254**, 1365, 1979) in rat liver increased in response to treatment of the animals with polycyclic aromatic hydrocarbons.

stituted enzyme systems [24, 25]. The disadvantage of this approach is that is is necessary to purify all of the forms of cytochrome P-450 before the major form contributing to any one oxidative reaction can be determined.

Another possibility is to raise antibodies against different forms of cytochrome P-450 and to investigate what activities are inhibited in the intact microsomal fraction by such antibodies [26]. This approach does have its merits but suffers from the disadvantage that a pure antigen is required before an appropriate antibody can be raised. An additional problem has recently come to light in the discovery of forms of cytochrome P-450 differing in only a very small number of amino acids [27]. Such forms of cytochrome P-450 are not distinguishable by conventional polyclonal antibodies [28].

We [29], and other groups [30–33], have started to generate monoclonal antibodies against cytochrome P-450 as a means of overcoming many of these problems. The monoclonality of the cells producing the antibody ensures that the final product interacts with only a single epitope [34]. That is not to say that this epitope is not common to a variety of proteins. However, it should be possible to obtain monoclonal antibodies which react with epitopes unique to the protein of interest. We have raised antibodies against rabbit cytochrome P-450 LM4 [29] which appear to meet these criteria.

We now report the production of a mouse monoclonal antibody directed against rat liver cytochrome P-448. This antibody, in marked contrast to those reacting with a unique epitope, appears to react with an epitope common to a number of different forms of cytochromes P-450. Despite this cross-reactivity however, this antibody may have valuable applications which are discussed.

MATERIALS AND METHODS

Materials. Chemicals and their sources were as follows: most of the reagents used in sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE)*, Amido Black 10B, 4-chloro-1- naphthol, DEAE Affi-Gel Blue, and nitrocellulose membrane (0.45 μm) were purchased from Bio-Rad Laboratories Ltd (Watford, Herts). Bovine serum albumin (fraction V), chloramine T, sorbitol, Coomassie Brilliant Blue R, bromphenol blue, normal rat, rabbit and mouse sera, mouse IgG, cholic acid, deoxycholic acid, HEPES, protein A, DTT, benzo[*a*]pyrene, β-naphthoflavone, 3-methylcholanthrene (practical grade), *o*-phenylenediamine, guanidine hydrochloride, pristane (2,6,10,14-tetramethylpentadecane), molecular weight standards for SDS-PAGE, NADPH (tetrasodium salt, type 1), adenosine 5′-monophosphate, haemoproteins (see Table 3) and Freund's complete adjuvant were all obtained from Sigma Chemical Co. Ltd (Poole, Dorset). Phenobarbitone sodium was from McArthur's (London, U.K.). Miles Laboratories Ltd (Slough, U.K.) provided rabbit immunoglobulin fraction of anti-mouse IgG (lyophylised fraction), rabbit anti-mouse IgG1 (lyophylised serum), horseradish peroxidase conjugated rabbit anti-mouse IgG and GelBond flexible film. Horseradish peroxidase conjugated sheep anti-mouse Igs (see Table 1) were purchased from Serotec Ltd (Bicester, Oxon). 3,3′,5,5′-Tetramethylbenzidine, Tween 20, ammonium sulphate, Triton X-100, sodium dithionite and hydrogen peroxide were purchased from BDH Chemicals Ltd (Poole, Dorset). Photographic materials for autoradiography were obtained fron Kodak Ltd (Hemel Hempstead, Herts). Pharmacia Fine Chemicals Ltd (Milton Keynes, U.K.) supplied agarose IEF, Pharmalyte pH 5-8, Sephadex IEF, Sephadex G25 (fine), Sephadex G50 (fine), IEF calibration kit standards, pH range 5–10.5, 2′,5′-ADP-Sepharose 4B, cyanogen bromide activated Sepharose CL-4B and octyl-Sepharose CL-4B. [^{125}I], carrier free for protein iodination, 100 mCi/ml, sheep anti-mouse Ig, [^{125}I]-labelled F(ab′)$_2$ fragment, donkey anti-rabbit Ig, [^{125}I]-labelled F(ab′)$_2$ fragment, L-[U-^{14}C]-leucine, >300 mCi/mmole, in sterile aqueous solution and [^{14}C]-methylated protein mixture for molecular weight calibration of autoradiographs of SDS-PAGE gels were obtained from Amersham International PLC (Amersham, U.K.). Materials required for the selection and culturing of the hybridoma cells were purchased from Gibco Ltd (Uxbridge, Middlesex). Koch-Light Ltd (Haverhill, Suffolk) supplied polyethyleneglycol 1540. Cooke 96-well polystyrene microtitre plates for use in solid phase ELISA assays were obtained from Dynatech Ltd (Billinghurst, Sussex).

Cells and media. The mouse myeloma cell line P3.NS1.1–Ag4–1 (NS1 cells) was routinely maintained in the Dulbecco's modification of minimal essential medium supplemented with L-glutamine, sodium pyruvate, and 10% (v/v) horse serum (full growth medium, FGM).

For hybridoma selection and cloning, cells were grown in FGM further supplemented with 10% (v/v), foetal calf serum, HAT, penicillin and streptomycin (HAT medium).

Preparation of antigen. Male Alderley Park rats were injected daily with β-naphthoflavone intraperitoneally at a dose of 100 mg/kg in corn oil for 4 days. Hepatic microsomal fraction was prepared [35]

* Abbreviations used are: 2-AAF, 2-N-acetylaminofluorene; AHH, aryl hydrocarbon (benzo[*a*]pyrene) hydroxylase; BNF, β-naphthoflavone; CO, carbon monoxide; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; EROD, ethoxyresorufin *O*-deethylase; F(ab′)$_2$ immunoglobulin fraction containing only the antigen binding sites, after pepsin digestion of Ig; FGM, full growth medium; HAT, hypoxanthine, aminopterin, thymidine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; IEF, isoelectricfocusing; Ig, immunoglobulin; IgG, immunoglobulin class G; i.p., intraperitoneal; MAb, monoclonal antibody; 3-MC, 3-methylcholanthrene; PB, phenobarbitone sodium; PBS, phosphate buffered saline (10 mM potassium phosphate buffer, pH 7.4, containing 0.15 M sodium chloride); PBST, phosphate buffered saline containing 0.05% (v/v) Tween 20; RAM, rabbit anti-mouse; SDS, sodium dodecyl sulphate; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TCA, trichloroacetic acid; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.

on the fifth day and resuspended in 0.1 M potassium pyrophosphate buffer containing 1 mM EDTA and 23 μM DTT. The microsomal fraction was solubilised with sodium cholate and a partially purified preparation of cytochrome P-448 was prepared by hydrophobic chromatography on octyl–Sepharose 4B [36]. The final preparation had a specific content of approximately 4 nmole cytochrome P-450/mg protein. The cytochrome P-450 fraction used to immunize the mice was active in a reconstituted system comprising the cytochrome P-450 fraction, rat liver NADPH-cytochrome c reductase and phosphatidylcholine with benzo[*a*]pyrene as substrate.

Immunization and fusion. A male Balb/c mouse received a primary intraperitoneal injection of 0.4 mg protein in the presence of Freund's complete adjuvent, followed by two i.p. boosts of 0.4 mg protein in phosphate buffered saline (PBS) at 3-weekly intervals. Four days after the final injection the mouse was killed and the spleen was asceptically removed. A monodispersed cell suspension was prepared by pressing the spleen through a 250 μm nylon mesh sieve. Spleen cells (10^8) and NS1 myeloma cells (10^7) were fused together in the presence of polyethylene glycol, mole wt. 1540, dissolved in serum free medium (50% w/v) for 1 min at 37°, followed by continuous dilution of the cells into 25 ml of FGM over a period of 5 min. The cells were plated out into 48 × 3 ml culture wells (Linbro wells) and after 24 hr the FGM was replaced with HAT medium. Hybrid growth was observed in all 48 wells, and 12 days after fusion spent medium from all the cultures was tested in an immunoradiometric assay to detect the presence of antigen-specific antibody.

Immunoassay and cloning. The partially purified cytochrome P-448 antigen was coated on to individual polystyrene microtitre wells (Dynatech) at a concentration of 10 μg/ml in bicarbonate buffer, pH 9.3, for 4 hr at room temperature. Specific adsorption of antibody from samples of the tissue culture supernatants was then determined essentially as described previously [29]. The serum of the mouse used for the fusion was incorporated into the assay as a positive control. Thirty-seven positive cultures were identified. Clones from the positive cultures were generated by the technique of limiting dilution into 96-well microtitre trays. Clones positive against the partially purified cytochrome P-448 preparation were recloned in the same way, to ensure monoclonality. One of the subsequent hybridomas was designated 3/4/2.

Production and purification of the antibody. Balb/c mice were pretreated with 0.5 ml pristane i.p. 2–4 weeks prior to injection of 5×10^6 3/4/2 hybridoma cells i.p. After 2–3 weeks ascites fluid was collected by syringe and centrifuging at 2000 *g* for 10 min at 4° to obtain the clear supernatant. The immunoglobulin fraction was precipitated by saturation of the supernatant to 50% (w/v) with ammonium sulphate. Ammonium sulphate was removed by extensive dialysis against PBS at 4°. Some of the ammonium sulphate fractionated immunoglobulin was further purified on DEAE Affi-Gel Blue [37]. The immunoglobulin fraction in 25 mM Tris hydrochloride buffer, pH 7.2, was applied to the column and after adsorption onto the gel, was washed with several volumes of this buffer. The immunoglobulin was specifically eluted as a sharp peak by including 25 mM sodium chloride in the buffer.

Cells were also grown in bulk culture, by Celltech Ltd, (Slough, Bucks) to obtain a large amount of the antibody (800 mg). The immunoglobulin was harvested from the tissue culture supernatant by polyethylene glycol precipitation.

Solid phase immunoassay. Wells of polystyrene microtitre plates were coated with microsomal fraction from BNF-, PB-treated or control rats, at a protein concentration of 0.1 mg/ml (100 μl/well) or with partially purified or apparently homogeneous preparations of cytochrome P-450 (at a concentration of 0.1 nmole/ml, 100 μl/well). The assay was performed as described previously [29], with [^{125}I]-labelled RAM IgG or horseradish peroxidase conjugated RAM IgG as a second antibody. After each stage of the assay the wells of the plates were washed extensively with PBS containing 0.05% Tween 20. In recent experiments the buffer containing the second antibody incorporated 1% (v/v) normal rat serum to reduce non-specific binding.

Labelling of proteins. Microsomal proteins, solubilized with sodium cholate were labelled with [^{125}I] using chloramine T [38]. All procedures involving the use of [^{125}I] were performed in a suitably designated "hot laboratory" in a lead-shielded, well-ventilated fume cupboard.

The iodinated proteins were separated from unreacted [^{125}I], and other reactants, by gel filtration on Sephadex G50 (fine) equilibrated in PBS.

SDS-polyacrylamide gel electrophoresis. Proteins were separated by SDS-polyacrylamide gel electrophoresis by the method of Laemmli [39] as previously described [40]. Slab gels were 1.5 × 100 × 140 mm and contained 10% (w/v) acrylamide. Electrophoresis was performed using a Model 220 cell (Bio-Rad Laboratories Ltd, Watford, Herts) driven by a Model 500/200 power source (Bio-Rad). Gels were destained overnight in a Model 222 charcoal diffusion destainer (Bio-Rad) and dried under vacuum on a Model 224 slab gel drier (Bio-Rad). Standards, with their subunit molecular weights were as follows: bovine serum albumin, 69,000; catalase, 58,000; glutamic dehydrogenase, 53,000; ovalbumin, 43,000; aldolase, 40,000.

Western blotting. In some experiments the separated proteins were electrophoretically transferred to pure nitrocellulose membranes, 0.45 μm, 15 × 9.2 cm, using a Model 160/1 Trans-Blot cell and Model 250/2.5 power supply (Bio-Rad Laboratories Ltd, Watford, Herts), using a suitable gel holder fitted with 6-mm sponge pads and blot adsorbent filter paper. The method was essentially as described by Towben *et al.* [41], with suitable modification for the transfer of proteins in the presence of SDS. Proteins thus transferred were visualized by staining with 1% (w/v) Amido Black [42]. Specific antibody interaction was detected by incubating strips of the membrane with the monoclonal antibody followed by horseradish peroxidase conjugated RAM IgG [41]. The presence of bound second antibody was detected by incubating with hydrogen peroxide, the substrate for the conjugated peroxidase, and 4-chloro-1-naphthol as cosubstrate, 2.2 mM in 20%

(v/v) methanol, 80% (v/v) 0.025 M Tris hydrochloride buffer, pH 7.4, containing 0.14 M sodium chloride.

Isoelectricfocusing. Isoelectricfocusing of monoclonal antibody was performed using a Model 3000 flat-bed apparatus (Pharmacia (Great Britain) Ltd, Milton Keynes, Bucks) with a Model ECPS 3000/150 electrophoresis constant power supply (Pharmacia). The methods used were modified from those published by Pharmacia Fine Chemicals ("A Workshop on Electrophoretic Techniques" and the "Pharmacia Instruction Manual"). Flat-bed analytical IEF was performed in agarose gels comprising 1% (w/v) agarose IEF, 12% (w/v) sorbitol and Pharmalyte pH 5–8 diluted 1 in 16 prior to use, cast on 0.1 mm flexible plastic laminate (GelBond film). Gels were then fixed, dried in a current of warm air and then stained with 0.2% (w/v) Coomassie Brilliant Blue R-250. Destaining was accomplished in 35% (v/v) ethanol, 10% (v/v) acetic acid. IEF-calibration kit standards for the determination of the isoelectric points of proteins in the range pH 5–10.5 were run on each gel.

Preparative IEF was performed with Sephadex IEF containing Pharmalyte 5–8. Gels were run for 5 hr at 45–50 W. The region of interest was scraped from the plate, packed into a 10 cm column and proteins eluted from the Sephadex with PBS. Pharmalyte was removed from the proteins by gel filtration on a 10 cm column of Sephadex G25 (fine) equilibrated with PBS.

Immunoprecipitation and autoradiography. Mouse IgG was covalently coupled to cyanogen bromide activated Sepharose 4B. This gel was then used to affinity purify polyclonal rabbit anti-mouse IgG for use as the precipitating antibody in immunoprecipitation experiments. Monoclonal antibody was incubated at various dilutions with labelled microsomal fractions for 16 hr at 4° and then with a variety of anti-mouse immunoglobulins for a further 4 hr at 4°. Any immunoprecipitate was collected by centrifugation and washed as described previously [29]. The pellet was finally resuspended in SDS-PAGE sample preparation buffer and subjected to SDS-PAGE.

After separation of [^{125}I]-labelled microsomal proteins by SDS-polyacrylamide gel electrophoresis the gels were dried under vacuum and then exposed to Kodak X-Omat R8 X-ray film, 10 × 24 cm, in X-Omatic cassettes installed with intensifying screens [43] for 1–3 days at −80°. [^{14}C]-Methylated protein mixture (CFA.626 from Amersham International, Amersham, U.K.) was used for molecular weight calibration. The films were developed under a type GBX safelight filter in Kodak DX80 developer, diluted 1 in 4, and fixed in Kodak FX-40 X-ray liquid fixer, diluted 1 in 4, as described in the manufacturer's instructions.

MAb sub-class identification. Sub-class of the monoclonal antibody was determined by ELISA. Wells of microtitre trays were coated with MAb 3/4/2, obtained by tissue culture, at a concentration of 10 μg/ml (100 μl per well). The wells were washed with PBST and then incubated with horseradish peroxidase conjugated, sub-class specific, sheep anti-mouse antibodies.

Assay of haem. The presence of haem in immunoprecipitates was detected using the method of Thomas *et al.* [44] with 3,3′,5,5′-tetramethylbenzidine as cosubstrate and hydrogen peroxide. The assay is sensitive to 1 pmole of cytochrome P-450. This assay was also used to detect the presence of haemoproteins bound to monoclonal antibody in a solid phase assay.

The wells of polystyrene microtitre plates were coated with 100 μl of 50 μg/ml solution of RAM Ig. Unbound material was removed and the wells washed with PBST as described above. Serial dilutions of 3/4/2 were prepared and added to the coated wells. After incubation, any unbound monoclonal antibody was removed and the wells washed as before. Cholate solubilised microsomal proteins from liver of BNF-treated rats were then added to the wells, which were then incubated and washed as before. The presence of bound haemoprotein was determined by adding 95 μl of the tetramethylbenzidine reagent (0.125 M acetate buffer, pH 5.0 containing 3.15 mM tetramethylbenzidine and 50% (v/v) methanol) to the wells and incubating them for 1 hr. Hydrogen peroxide, 5 μl of 0.6 M, to give a final concentration of 30 mM, was added and after 30 min further incubation absorbance at 690 nm was determined.

Reconstitution of cytochrome P-450. In reconstitution experiments activity was determined as described by Lu and Levin [45] using NADPH-cytochrome c reductase prepared by affinity chromatography on 2′,5′-ADP-Sepharose 4B [46]. After reconstitution of cytochrome P-450, NADPH-cytochrome c reductase and posphatidylcholine, the following reagents were added to give the final concentrations shown: sodium deoxycholate, 120 μM; HEPES buffer, pH 7.4, 50 mM; magnesium chloride, 6 mM; NADPH, 1.2 mM. Aryl hydrocarbon hydroxylase activity was determined as previously described [35]. 7-Ethoxyresorufin *O*-deethylase [47], aldrin epoxidase [48] and 2-acetylaminofluorene hydroxylase [49] activities were all determined by published procedures. Protein concentration and time of incubation were selected to be in the linear range for each assay.

Induction of animals and preparation of microsomal fractions. Induction by phenobarbitone was accomplished by treating rats daily for 4 days with 80 mg/kg phenobarbitone sodium in normal saline by intraperitoneal injection. The first dose was administered as 2 × 40 mg/kg 8 hr apart and the animals were killed 24 hr after the last dose. Induction with 3-methylcholanthrene was accomplished by administering a single dose of 80 mg/kg of the hydrocarbon in corn oil by i.p. injection, 48 hr prior to killing the animals. TCDD-treated animals received a single dose of 50 μg/kg TCDD in dioxane by i.p. injection, 5 days prior to sacrifice.

The source and conditions of maintenance of Wistar rats, rabbits, guinea pigs, dogs, baboons, pigs, hamsters and mice were as previously described [50]. Fischer and DA strains of rat were obtained from Bantin and Kingman (Hull, Yorkshire).

Human liver samples were obtained, with local Research Ethics Committee permission, as described elsewhere [35].

Microsomal fractions from liver of all species were prepared by differential centrifugation [35].

Other assays. Protein concentration was determined by the method of Lowry *et al.* [51] with crystalline bovine serum albumin (fraction V) as standard. Cytochrome P-450 content was measured by reduced CO-difference spectroscopy [52] on a Model 555 split-beam scanning spectrophotometer with microprocessor-controlled background correction (Perkin-Elmer Ltd, Beaconsfield, Bucks). An extinction coefficient of 91 per mM/cm was assumed for reduced CO-cytochrome P-450 between 450 and 480 nm [52].

RESULTS

The monoclonal antibody described is secreted by a hybridoma that has been designated 3/4/2. This clone was derived from the fusion of spleen cells obtained from a mouse immunized with partially purified cytochrome P-448 with NS1 myeloma cells.

3/4/2 is IgG, sub-class 1, as determined by ELISA using sub-class specific antibodies (Table 1).

Evidence for the monoclonal origin of antibody 3/4/2

The original subculture from the 37 positive cultures was cloned by limiting dilution and only wells containing individual colonies were selected for further study. Clone 3/4/2 was subcloned, again by limiting dilution and all of the subclones were positive by solid phase assay. In addition, the well containing subclone 3/4/2 contained only a single colony, determined by visual inspection under a microscope.

Isoelectricfocusing of 3/4/2 gave a cluster of 2 major and 4 minor bands with pI values in the range 7.5–8.0 (Fig. 1) indicating possible monoclonality of the antibody. After preparative isoelectricfocusing the bands were removed, eluted with PBS and subjected to SDS-PAGE. Only bands corresponding in molecular weight to the heavy and light chains of immunoglobulin were observed.

Cells producing 3/4/2 were grown in culture medium containing [^{14}C]-leucine for 24 hr. The supernatant was purified by ammonium sulphate fractionation and the resultant immunoglobulin fraction subjected to SDS-PAGE followed by autoradiography. Labelled proteins were observed with mole wt 60,000 and 20,000, corresponding in molecular weight to the heavy and light chains of 3/4/2, respectively. Control NS1 myeloma cells secreted no labelled immunoglobulin into the medium.

Fig. 1. Isoelectricfocusing of various preparations of MAb 3/4/2. The agarose gel was prepared and run as described under Materials and Methods. Lane 1, crude ascites fluid fraction; lane 2, DEAE Affi-Gel Blue purified fraction of MAb 3/4/2. Fifty micrograms of each preparation were applied to the gel. The cathode is at the top of the figure. pI values of the standards (lane 3) were as follows: 1, 8.65; 2, 8.45; 3, 8.15; 4, 7.35; 5, 6.55; 6, 5.85; 7, 5.20.

Table 1. Sub-class identification of MAb 3/4/2

Sub-class specific antibody against	Absorbance at 492 nm
IgG, A, M (heavy and light chain)	1.3
IgM	0.2
IgA	0.2
IgG1	1.2
$IgG2_A$	0.15
$IgG2_B$	0.2

The wells of microtitre trays were coated with MAb 3/4/2 as described under Materials and Methods. Horseradish peroxidase, sub-class specific, sheep anti-mouse antisera were added to the wells and specific interaction quantitated spectrophotometrically after addition of hydrogen peroxide and *o*-phenylenediamine, absorbance being determined at 492 nm.

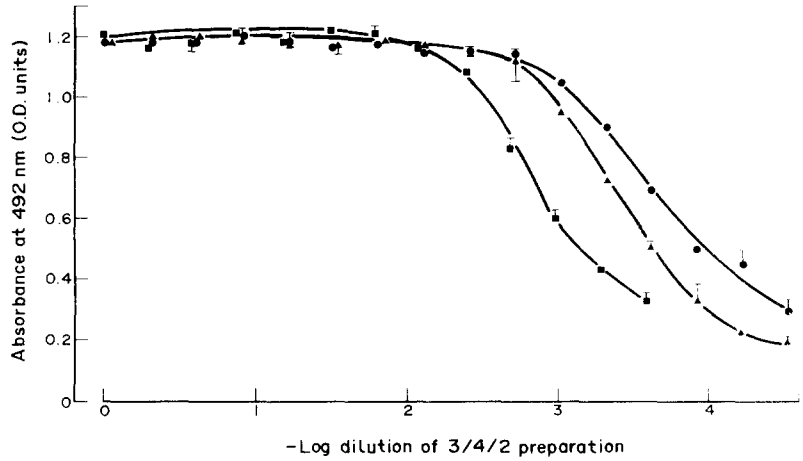

Fig. 2. Immunotitration of 3/4/2 purified by DEAE Affi-Gel Blue chromatography. MAb 3/4/2 was purified from mouse ascites fluid by DEAE Affi-Gel Blue chromatography. Serial, 2-fold dilutions of the MAb were assayed by solid phase ELISA (●). A crude fraction of 3/4/2 from ascites fluid (▲) and a fraction from clone 3/4/2 tissue culture fluid, partially purified by PEG fractionation (■), were similarly assayed. For each preparation the starting concentration of protein was adjusted to 0.5 mg/ml (log dilution = 0). Values are means ± S.D. of triplicate determinations.

Titration of 3/4/2

Solid phase ELISA assay against partially purified rat liver cytochrome P-448 was performed with serial, 2-fold dilutions of 3/4/2 from tissue culture fluid and from mouse ascites fluid (Fig. 2). The titration curves have very similar shapes, the PEG fractionated antibody from tissue culture fluid having only 20% of the activity of the DEAE Affi-Gel Blue purified ascites fluid fraction. Purification by DEAE Affi-Gel Blue gave a purer fraction than ammonium sulphate fractionation. This was apparent from both Fig. 2 and from SDS-gel electrophoresis of the different fractions (data not shown). Radial immunodiffusion of the different monoclonal antibody fractions confirmed that the ammonium sulphate fractionated material contained only 40% of the immunoglobulin content of the DEAE Affi-Gel Blue purified material and that the PEG fractionated culture fluid contained only 20% of the immunoglobulin that the DEAE fraction contained (data not shown).

Titration curves of 3/4/2 against protein A were also obtained (Fig. 3). These revealed that the monoclonal antibody bound only very weakly to protein A. Hence, it would not be possible to use protein A to purify 3/4/2.

Autoradiography

Hepatic microsomal fraction from BNF-treated rats was solubilized with cholate and labelled with [^{125}I]. The labelled proteins were incubated with 3/4/2. Immune complexes were precipitated by the

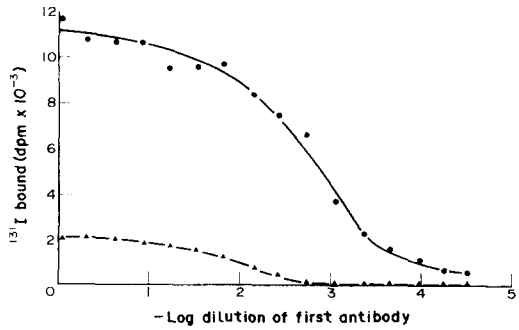

Fig. 3. Reactivity of MAb 3/4/2 with protein A. Individual polystyrene microtitre wells were coated with protein A and then incubated with serial, 2-fold dilutions of 3/4/2 (▲), at an initial protein concentration of 0.5 mg/ml, or with rabbit anti-mouse IgG 1, whole serum (●), at an initial protein concentration of 0.13 mg/ml, as a positive control. Quantitation was by the addition of [^{125}I]-labelled anti-mouse or anti-rabbit F(ab')$_2$ fraction as described under Materials and Methods.

Table 2. Reactivity of MAb 3/4/2 with purified and partially purified preparations of cytochrome P-450 in solid phase ELISA

Antigen	Absorbance at 492 nm Without 3/4/2	With 3/4/2
Rabbit		
Form 2	0.13 ± 0.05	0.46 ± 0.02
Form 3b	0.13 ± 0.03	0.41 ± 0.03
Form 3c	0.19 ± 0.01	0.35 ± 0.02
Form 4	0.27 ± 0.02	0.71 ± 0.01
Form 6	0.11 ± 0.02	0.72 ± 0.01
Rat		
Microsomal fraction*	0.30 ± 0.01	1.03 ± 0.01
Cytochrome P-448†	0.25 ± 0.01	0.92 ± 0.02
Human		
Microsomal fraction	0.20 ± 0.06	0.57 ± 0.01
Cytochrome P-450‡	0.19 ± 0.03	0.67 ± 0.06

* Hepatic microsomal fraction from BNF-treated rats.
† Partially purified preparation of cytochrome P-448 from liver of BNF-treated rats.
‡ Partially purified preparation of cytochrome P-450 from human liver.

addition of affinity-purified rabbit anti-mouse IgG and subjected to SDS-polyacrylamide gel electrophoresis followed by autoradiography. The use of large amounts of precipitating antibody tended to decrease the apparent mole wt of the labelled proteins. This was due presumably to a "snowplough effect". When the antibody concentrations were suitably adjusted, labelled proteins were detectable only in the molecular weight range 53–58 K (data not shown). However, the resolution of the autoradiographs was not sufficient to enable the number of bands recognised by 3/4/2 to be determined in this experiment.

Specificity of 3/4/2

In solid phase haem assay, 3/4/2 specifically adsorbed haem-positive material from cholate solubilised microsomal fraction from BNF-treated rats.

Solid phase ELISA assays were performed with homogeneous preparations of rat cytochrome P-448 (form c), rabbit cytochromes P-450 forms 2, 3b, 3c, 4 and 6 and partially purified preparations of cytochrome P-450 from phenobarbitone treated rats and from humans. Results are shown in Table 2. The antibody showed strong cross-reactivity with all of the forms of cytochrome P-450 tested. With the other haemoproteins investigated there was negligible cross-reactivity (Table 3).

Western blot of proteins from 3-methylcholanthrene-treated rats and rabbits revealed that 3/4/2 recognized proteins only in the molecular weight region of 52–58 K (Fig. 4). With hepatic microsomal fraction from 3-MC treated rats a single protein band of molecular weight approximately 57 K was detected, whereas with rabbit two proteins of mole wt 55 K and 57 K, corresponding to forms 4 and 6, were recognized. 3/4/2 also recognized a single protein in blot transferred hepatic microsomal fraction from PB-treated rat. This protein had a mole wt of 56 K and did not correspond to either forms b or e, the major PB-inducible forms in rat liver. The MAb reacted with a single protein in human hepatic microsomal fraction of mole wt 54 K.

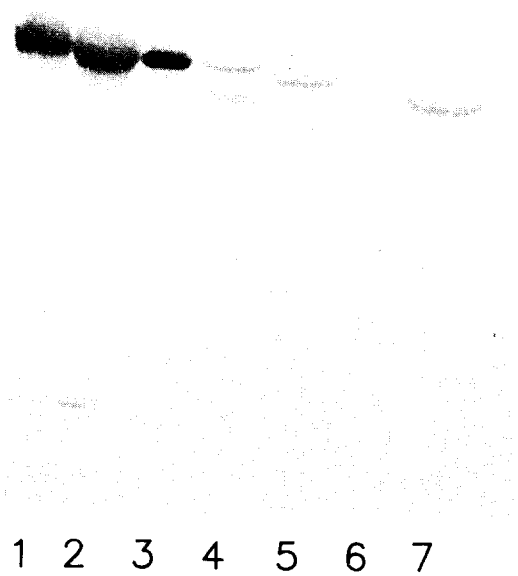

Fig. 4. Western blot of MAb 3/4/2 with microsomal proteins from rats, rabbit and human. Hepatic microsomal fraction from BNF-treated (lane 1) and 3-MC-treated (lane 2) rats, 3-MC-treated rabbit (lane 4), PB-treated (lane 5) and control (lane 6) rats and human (lane 7) and homogeneous rat cytochrome P-448 (lane 3), were subjected to SDS-PAGE followed by electrophoretic blot transfer on to nitrocellulose membrane. The reactivity of the separated proteins with MAb 3/4/2 was then assessed as described under Materials and Methods. The heaviest proteins are at the top of the figure.

Table 3. Immunoreactivity of MAb 3/4/2 with various haemoproteins in solid phase immunosorbant radiometric assay

Haemoprotein	cpm [^{125}I] × 10^{-3} Control*	With 3/4/2
Cytochrome P-448†	3.4	38.0
Cytochrome P-450‡	1.4	15.5
Microsomal fraction from control rat	2.8	11.0
Cytochrome c (horse)	1.0	0.9
Cytochrome c (pigeon)	1.0	1.2
Cytochrome c (tuna)	0.8	1.2
Haemoglobin (human)	0.8	1.2
Myoglobin (horse)	0.7	1.0
Haemocyanin (keyhole limpit)	0.8	1.9
Catalase (bovine)	1.3	3.0
Horseradish peroxidase	4.3	4.1

* Controls were incubated with spent tissue culture fluid from NS1 myeloma cells grown under the same conditions as clone 3/4/2.

† Partially purified preparation from liver of BNF-treated rats, purified as described under Materials and Methods.

‡ Partially purified preparation from liver of PB-treated rats, purified as described under Materials and Methods.

The reactivity of the antibody against hepatic microsomal fractions from a variety of different species was also investigated by ELISA. The results are shown in Table 4. 3/4/2 reacted positively with all of the microsomal fractions tested.

Recognition site for 3/4/2

Because of the cross-reactivity of 3/4/2 the possible effects of this MAb on the binding spectrum of cytochrome P-450 were investigated.

Preincubation of 3/4/2 with a purified preparation of cytochrome P-448 had no effect on the reduced CO-difference spectrum of the haemoprotein (data not shown).

Table 4. Cross-reactivity of MAb 3/4/2 with hepatic microsomal fractions from various species in ELISA

Species	Treatment	Absorbance reading*
Wistar rat	None	7
Wistar rat	PB†	8
Wistar rat	3-MC‡	8
ICI Wistar rat	BNF§	8
Fischer rat	None	6
Fischer rat	PB	7
Fischer rat	3-MC	7
DA rat	None	7
DA rat	PB	8
DA rat	3-MC	7
AKR mouse	None	6
AKR mouse	PB	8
AKR mouse	3-MC	6
Balb/c mouse	None	6
Balb/c mouse	PB	8
Balb/c mouse	3-MC	7
NZW rabbit	3-MC	5
Dunkin-Hartley guinea pig	None	8
Syrian hamster	None	4
Pig (blue)	None	4
Baboon	None	4
Human	None	5 ± 1 (N = 9)
Control (no microsomal protein)‖	—	0

Microtitre wells were coated with microsomal fraction from each species at the same protein concentration (50 μl of 0.1 mg/ml). Results are means of duplicate determinations, which were within 10% of each other 90% of the time.

* Absorbance at 492 nm in solid phase ELISA was determined on a Titretek Multiscan plate reader (Dynatech), operating in matrix mode. Values were expressed as an integral number of absorbance increments from 0 to 9, with a value of 10 assigned to absorbance 0.60.

† Animals were treated for 4 days with i.p. injections of 80 mg/kg phenobarbitone sodium in saline. The first dose was administered as 2 × 40 mg/kg, 8 hr apart. Animals were sacrificed 24 hr after the last dose.

‡ Animals recieved a single i.p. injection of 80 mg/kg 3-methylcholanthrene in corn oil 48 hr prior to sacrifice.

§ Rats were treated with 100 mg/kg β-naphthoflavone in corn oil by i.p. injection daily, for 4 days. The animals were killed 24 hr after the last injection.

‖ Controls, in which the MAb was omitted, were also run for each microsomal fraction. An additional control involved incubation of a non-cytochrome P-450 directed MAb with microsomal fraction from BNF-treated rat. In all cases the absorbance reading in matrix mode was between 0 and 1.

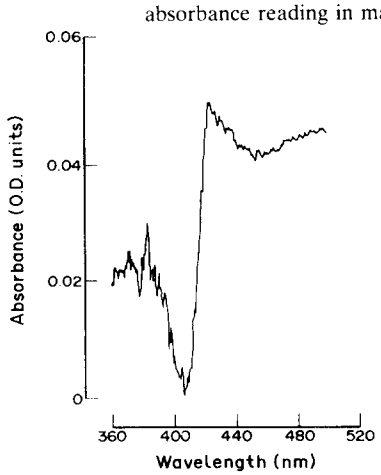

Fig. 5. Binding spectrum of MAb 3/4/2 with cytochrome P-450. Hepatic microsomal fraction from BNF-treated rat was preincubated with 3/4/2 for 1.5 hr at 4°. The difference spectrum of the microsomal fraction was then recorded against microsomal fraction to which 3/4/2 was added immediately prior to spectroscopy.

The addition of 3/4/2 to microsomal suspensions from BNF-treated rats produced a binding spectrum that took at least 1 hr to develop, at 4° (Fig. 5). The effects of other monoclonal antibodies against rat cytochrome P-448, produced in a separate fusion, were investigated. Even at protein concentrations greater than those used for 3/4/2 these antibodies produced no binding spectrum. The spectrum produced by 3/4/2 was similar to that of a type 2 compound such as aniline, and was concentration-dependent. However, 3/4/2 did not prevent the binding of aniline to cytochrome P-450.

Effect of 3/4/2 on monooxygenase activity

Incubation of 3/4/2 with hepatic microsomal suspensions from BNF- or PB-treated rats, TCDD-treated rabbits or humans had no effect on monooxygenase activity (Table 5). Apparently homogeneous cytochrome P-448, 4 pmole was incubated with up to 0.4 mg of DEAE Affi-Gel Blue purified 3/4/2 for 30 min at room temperature. The cyto-

Table 5. Effects of MAb 3/4/2 on monooxygenase activity

Monooxygenase activity	Concn of 3/4/2 tested (μg/ml)	% Control activity*	Ig control† (% control)
Rat cytochrome P-448‡			
AHH	200	96	69
	400	73	
EROD	100	86	
	200	75	65
Microsomal fraction from BNF-treated rats			
EROD	250	99	101
Microsomal fraction from PB-treated rats			
Aldrin epoxidase	250	113	105
Microsomal fraction from TCDD-treated rabbits			
2-AAF N-hydroxylase	1300	89	
2-AAF 1-hydroxylase	1300	114	
2-AAF 7-hydroxylase	1300	124	
Human cytochrome P-450§			
AHH	500	75	55
Aldrin epoxidase	100	64	5.2

* Activity is expressed as a percent of control values, determined in the absence of any antibody.

† Activity was determined with an equivalent amount of mouse serum immunoglobulin fraction and expressed as a percent of the same controls as above.

‡ Activity was determined in a reconstituted system containing apparently homogeneous rat liver cytochrome P-448.

§ Activity was determined in a reconstituted system containing a partially purified preparation of human hepatic cytochrome P-450.

chrome P-448 was then reconstituted with purified NADPH-cytochrome c reductase and phosphatidylcholine and aryl hydrocarbon hydroxylase activity determined. The antibody had only a very slight effect on reconstituted monooxygenase activity, indicating that it had no marked inhibitory effect on cytochrome P-450. Similarly, 3/4/2 had almost no effect on 7-ethoxyresorufin O-deethylase activity of reconstituted cytochrome P-448.

DISCUSSION

Although antibodies against cytochrome P-450 are often monospecific [53] there are a number of reports of such antibodies cross-reacting with, presumably, analogous forms of cytochrome P-450 in other species. This is true of both polyclonal [54] and monoclonal [55] antibodies. In the present report we have described the generation of a monoclonal antibody against rat hepatic cytochrome P-448 (form c) which appears to cross react with forms of cytochrome P-450 in a wide variety of other species, including man, and after induction by a variety of compounds.

The amino acid sequence of several forms of cytochrome P-450 has now been elucidated [27] as has the nucleotide sequence of a structural gene [56]. Such studies have revealed so-called "conserved regions" on cytochrome P-450. These are sequences of up to 15 or 20 amino acids which are similar, if not identical, in the forms of cytochrome P-450 so far studied [57]. It seems quite possible that the monoclonal antibody reported here reacts with some such conserved region in cytochrome P-450. It is obvious that this region cannot be associated with the catalytic activity of the enzyme, since the antibody was non-inhibitory. This would exclude the active site of the cytochrome and the cytochrome c reductase binding site, but other possible areas include the site of haem attachment or a hydrophobic region which maintains the orientation of the enzyme within the membrane [58]. We have not yet been able to determine which of these sites, if any, is the recognition determinant for 3/4/2.

The monoclonal antibody did cause a perturbation of the absolute spectrum of cytochrome P-450, as determined by difference spectroscopy, due presumably to an alteration in spin state. The binding was very similar to that occurring in the presence of aniline, a compound known to bind to the sixth ligand of the haem iron [59]. However, binding of the antibody did not prevent aniline binding or *vice versa*. Indeed, the binding of aniline and 3/4/2 were additive. Nor was the antibody inhibitory, either in a reconstituted system or in intact microsomal fractions.

It was apparent from blotting experiments that the monoclonal antibody 3/4/2 reacted with cytochrome P-448 to a much greater extent than with the cytochromes present in control or phenobarbitone induced animals. This was not readily apparent from normal ELISA assay. From these experiments it appears that 3/4/2 recognises an epitope common to certain hydrocarbon-inducible forms of cytochrome P-450. This would include rat form c, rabbit forms 4 and 6 and a human form of cytochrome P-450, but

not rat form d. It is thus tempting to conclude that 3/4/2 reacts with a form of cytochrome P-450 in human liver analogous to rat form c.

The cross-reactivity of 3/4/2 in ELISA was somewhat in disagreement with the results of Western blotting. Thus, the MAb blotted only forms 4 and 6 in rabbit liver but reacted with forms 2, 3b, 3c, 4 and 6 in ELISA. There are several possible explanations for this. The ELISA assay was optimised to detect any reactivity of the MAb, and as such is more sensitive than blotting. Thus, 3/4/2 might react weakly with forms 2, 3b and 3c due to the existence of a similar, but not identical, epitope to that found on forms 4 and 6. Alternatively, linearization of the proteins for blotting might disrupt the epitope on forms 2, 3b and 3c, while leaving that on forms 4 and 6 intact. This seems a less likely possibility.

MAb 3/4/2 binds to a variety of different forms of cytochrome P-450 but does not inhibit their activity. Such an antibody might be particularly useful in immunopurification of cytochrome P-450, as the antibody–antigen complex can be used to reconstitute catalytic activity in the presence of cytochrome c reductase [60]. Such antibodies will also find application in immunocytochemical localisation of cytochrome P-450, inter- and intra-species comparisons of cytochrome P-450 structure and in isozyme quantitation.

Acknowledgements—This work was supported by a Joint Research Scheme Award from Imperial Chemical Industries PLC. We are extremely grateful to the following people all of whom contributed to the work reported here. Mr. Tim Green and Ms. Elizabeth Howard provided skilled assistance in the generation and maintenance of the hybridoma cell line and Ms. Patricia Canning provided excellent technical assistance during the harvesting of the antibody and in its characterization. Dr. Katherine M. Lewis prepared the partially purified preparation of cytochrome P-448 with which the mouse was immunised. Dr. Lewis also provided the partially purified preparation of cytochrome P-450 from PB-treated rats. Ms. Susan Baker purified rat cytochrome P-448 and NADPH-cytochrome c reductase to apparent homogeneity, and also provided a partially purified preparation of human cytochrome P-450. Dr. E. F. Johnson (LaJolla, CA) kindly supplied samples of rabbit liver cytochromes P-450 forms 2, 3b, 3c, 4 and 6. Dr. S. S. Thorgeirsson, Dr. M. E. McManus and Ms. D. M. Schwartz (Bethesda, MD) gave advice and help on the assay of 2-AAF oxidation.

REFERENCES

1. R. T. Williams, *Detoxication Mechanisms*, Chapman & Hall, London (1973).
2. J. W. Bridges, in *Environmental Chemicals, Enzyme Function and Human Disease*, pp. 5–24. Excerpta Medica, Amsterdam (1980).
3. H. Remmer, *Am. J. Med.* **49**, 617 (1970).
4. R. Snyder, D. V. Parke, J. J. Kocsis, D. J. Jollow, C. G. Gibson and C. W. Witmer (Eds) *Biological Reactive Intermediates—II. Chemical Mechanisms and Biological Effects*. Plenum Press, New York (1982).
5. E. E. Delvin, A. Arabian and F. H. Glorieux, *Biochem. J.* **172**, 417 (1978).
6. J. G. Ghazarian, C. R. Jefcoate, J. C. Knutson, W. H. Orme-Johnson and H. F. DeLuca, *J. biol. Chem.* **249**, 3026 (1974).
7. W. R. Butt, *Hormone Chemistry*, 2 edn. Vol. 2, pp. 16–59. Ellis Horwood Ltd, Chichester (1976).
8. J.-A. Gustafsson and M. Ingelman-Sundberg, *J. biol. Chem.* **250**, 2451 (1975).
9. G. S. Boyd, A. M. Grimwade and M. E. Lawson, *Eur. J. Biochem.* **37**, 334 (1973).
10. I. Bjorkhem and M. Hamberg, *Biochem. biophys. Res. Commun.* **47**, 333 (1972).
11. D. L. Cinti and M. B. Feinstein, *Biochem. biophys. Res. Commun.* **73**, 171 (1976).
12. V. Ullrich, L. Castle and P. Weber, *Biochem. Pharmac.* **30**, 2033 (1981).
13. A. Y. H. Lu, W. Levin, S. B. West, M. Jacobson, D. Ryan, R. Kuntzman and A. H. Conney, *J. biol. Chem.* **248**, 456 (1973).
14. F. P. Guengerich, G. A. Dannan, S. T. Wright, M. V. Martin and L. S. Kaminsky, *Biochemistry* **21**, 6019 (1982).
15. D. W. Nebert and M. Negishi, *Biochem. Pharmac.* **31**, 2311 (1982).
16. D. E. Ryan, P. E. Thomas and W. Levin, *J. biol. Chem.* **255**, 7941 (1980).
17. S. A. Atlas, A. R. Boobis, J. S. Felton, S. S. Thorgeirsson and D. W. Nebert, *J. biol. Chem.* **252**, 4712 (1977).
18. H. H. Liem, U. Muller-Eberhard and E. F. Johnson, *Molec. Pharmac.* **18**, 565 (1980).
19. F. P. Guengerich, P. Wang, P. S. Mason and M. B. Mitchell, *Biochemistry* **20**, 2370 (1981).
20. H. Chao and L. W. K. Chung, *Molec. Pharmac.* **21**, 744 (1982).
21. D. W. Nebert, *J. natl. Cancer Inst.* **64**, 1279 (1980).
22. *Microsomes, Drug Oxidations, and Drug Toxicity* (Eds. R. Sato and R. Kato), Japan Scientific Societies Press, Tokyo and Wiley-Interscience, New York (1982).
23. A. R. Boobis and D. S. Davies, *Xenobiotica* **14**, 151 (1984).
24. A. W. Wood, D. E. Ryan, P. E. Thomas and W. Levin, *J. biol. Chem.* **258**, 8839 (1983).
25. D. E. Ryan, S. Iida, A. W. Wood, P. E. Thomas, C. S. Lieber and W. Levin, *J. biol. Chem.* **259**, 1239 (1984).
26. N. A. Elshourbagy and P. S. Guzelian, *J. biol. Chem.* **255**, 1279 (1980).
27. A. Kumar, C. Raphael and M. Adesnik, *J. biol. Chem.* **258**, 11280 (1983).
28. G. P. Vlasuk, J. Ghrayab, D. E. Ryan, L. Reik, P. E. Thomas, W. Levin and F. G. Walz Jr., *Biochemistry* **21**, 789 (1982).
29. A. R. Boobis, M. B. Slade, C. Stern, K. M. Lewis and D. S. Davies, *Life Sci.* **29**, 1443 (1981).
30. S. S. Park, A. V. Persson, M. J. Coon and H. V. Gelboin, *FEBS Lett.* **116**, 231 (1980).
31. P. E. Thomas, L. M. Reik, D. E. Ryan and W. Levin, *J. biol. Chem.* **259**, 3890 (1984).
32. I. Reubi, K. J. Griffin, J. L. Raucy and E. F. Johnson, *J. biol. Chem.* **259**, 5887 (1984).
33. F. Letawe-Goujon, P. Kremers, P. Beaune, M. Pave and J. E. Gielen, *Biochem. Pharmac.* **119**, 744 (1984).
34. G. Kohler and C. Milstein, *Nature* **256**, 495 (1975).
35. A. R. Boobis, M. J. Brodie, G. C. Kahn, D. R. Fletcher, J. H. Saunders and D. S. Davies, *Br. J. clin. Pharmac.* **9**, 11 (1980).
36. P. Beaune, P. Dansette, J. P. Flinois, S. Columelli, D. Mansuy and J. P. Leroux, *Biochem. biophys. Res. Commun.* **88**, 826 (1979).
37. C. Bruck, D. Portelle, C. Glineur and A. Bollen, *J. Immun. Meth.* **53**, 313 (1982).
38. P. J. McConahey and F. J. Dixon, *Int. Archs Allergy* **29**, 185 (1966).
39. U.K. Laemmli, *Nature* **227**, 680 (1970).
40. A. R. Boobis, D. W. Nebert and J. S. Felton, *Molec. Pharmac.* **13**, 259 (1977).

41. H. Towbin, T. Staehelin and J. Gordon, *Proc. natn. Acad. Sci. U.S.A.* **76**, 4350 (1979).
42. W. F. Glass II, R. C. Briggs and L. S. Hnilica, *Science* **211**, 70 (1981).
43. R. Swanstrom and P. Shank, *Analyt. Biochem.* **86**, 184 (1978).
44. P. E. Thomas, D. Ryan and W. Levin, *Analyt. Biochem.* **75**, 168 (1976).
45. A. Y. H. Lu and W. Levin, *Biochem. biophys. Acta* **344**, 205 (1974).
46. Y. Yasukochi and B. S. S. Masters, *J. biol. Chem.* **251**, 5337 (1976).
47. M. D. Burke and R. T. Mayer, *Drug Metab. Dispos.* **2**, 583 (1974).
48. T. Wolff, E. Deml and H. Wanders, *Drug. Metab. Dispos.* **7**, 301 (1979).
49. M. E. McManus, R. F. Minchin, N. D. Saunders, P. J. Wirth and S. S. Thorgeirsson, *Carcinogenesis* **4**, 603 (1983).
50. G. M. Pacifici, A. R. Boobis, M. J. Brodie, M. E. McManus and D. S. Davies, *Xenobiotica* **11**, 73 (1981).
51. O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. biol. Chem.* **193**, 265 (1951).
52. T. Omura and R. Sato, *J. biol. Chem.* **239**, 2370 (1964).
53. P. E. Thomas, D. Korzeniowski, D. Ryan and W. Levin, *Archs. Biochem. Biophys.* **192**, 524 (1979).
54. F. P. Guengerich, P. Wang, P. S. Mason and M. B. Mitchell, *Biochemistry* **20**, 2370 (1981).
55. T. Fujino, S. S. Park, D. West and H. V. Gelboin, *Proc. natn. Acad. Sci. U.S.A.* **79**, 3682 (1982).
56. Y. Mizukami, K. Sogawa, Y. Suwa, M. Muramatsu and Y. Fujii-Kuriyama, *Proc. natn. Acad. Sci. U.S.A.* **80**, 3958 (1983).
57. S. D. Black, G. E. Tarr and M. J. Coon, *J. biol. Chem.* **257**, 14616 (1982).
58. J. K. Leighton, B. A. DeBrunner-Vossbrinck and B. Kemper, *Biochemistry* **23**, 204 (1984).
59. Y. Yoshida and H. Kumaoka, *J. Biochem.* **78**, 455 (1975).
60. K. M. Lewis, A. R. Boobis, M. B. Slade and D. S. Davies, *Biochem. Pharmac.* **31**, 1815 (1982).