Biochemical Pharmacology, Vol. 41, No. 10, pp. 1505–1512, 1991
Printed in Great Britain.

0006-2952/91 $3.00 + 0.00

METABOLISM OF 7,12-DIMETHYLBENZ[*a*]ANTHRACENE IN HEPATIC MICROSOMAL MEMBRANES FROM RATS TREATED WITH ISOENZYME-SELECTIVE INDUCERS OF CYTOCHROMES P450

VALERIE M. MORRISON,*† ALAN K. BURNETT‡ and JOHN A. CRAFT*§

* Department of Biological Sciences, Glasgow College, Glasgow G4 0BA, U.K. and
‡ Leukaemia Research Fund Laboratories, Department of Haematology, Glasgow Royal Infirmary, Glasgow G4 0SF, U.K.

(*Received* 13 *December* 1990; *accepted* 15 *December* 1990)

Abstract—Previous work has shown that member(s) of the cytochrome P450IIC sub-family play significant roles in the formation of diols of 7,12-dimethylbenz[*a*]anthracene (DMBA) and are particularly important in formation of the proximate carcinogen (DMBA-3,4-diol). To further characterize the role of members of this subfamily in DMBA-diol formation and to assess the part played by other P450s, DMBA metabolism has been investigated in microsomes prepared from animals pre-treated with isoenzyme selective inducers. The rates of formation of DMBA-diols in membranes from phenobarbital-treated rats were very low when NADH was used as reductant and rates were not altered when NADPH and NADH were used in combination rather than using NADPH alone. This suggests that cytochrome b_5 is not involved in DMBA-diol formation in these membranes. Treatment of animals with clofibrate, pyrazole and dexamethasone produced regio-selective alterations in the rates of formation of DMBA-diols at the -3,4-, -5,6- and -8,9- positions. However, none of the inducers caused increases in the rates of DMBA-diol formation of any great magnitude suggesting that the isoforms which are the major induced proteins (P450IVA1, P450IIE1 and P450IIIA1) do not play a significant role in diol formation. The content of other P450s in these membrane are also altered and these were investigated by Western blot using antibodies to P450IIC6, P450IIB1 and P450IIIA1. The results of the Western blots show that the effects of the inducing agents on DMBA-diol formation can be explained by alterations of members of the P450IIC and P450IIB subfamilies.

Treatment of Long-Evans rats by four or more bi-weekly administrations of 7,12-dimethylbenz[*a*]anthracene (DMBA)‖ causes an erythroleukaemia in a high proportion of animals [1]. This model of chemical leukaemogenesis is of particular interest since pretreatment of the animals with the azo dye, Sudan III [1-(4-phenylazo-phenylazo)-2-napthol] prior to the administration of DMBA protects the animals from disease induction [2]. The Long-Evans model is also distinct in that administration of DMBA to other rodents causes tumours in other tissues [3, 4]. These species and tissue-selective effects elicited by DMBA may relate to a differential in metabolic capacity to produce the ultimate carcinogen.

The carcinogenic properties of (DMBA) are dependent on metabolic activation to an ultimate carcinogen [5] which can mutate cellular proto-oncogenes [6–8]. Studies of DMBA–DNA adduct formation suggest that the ultimate carcinogen is the bay region diol-epoxide DMBA-3,4-diol-1,2-oxide [9–12]. This is formed by a sequential pathway initiated by the formation of DMBA-3,4-oxide, catalysed by a cytochrome P450 (P450) or cytochromes P450 isoenzymes, and this intermediate may then be hydrolysed by epoxide hydrolase. The resulting *trans*-DMBA-3,4-diol (the proximate carcinogen) undergoes a terminal, P450 catalysed mono-oxygenation to form the ultimate carcinogen. In the rat DMBA can also be converted to other *trans*-dihydrodiols at the -5,6- and -8,9-positions and these metabolites have much reduced carcinogenic and mutagenic potential. The outcome of exposure to DMBA may therefore be determined by the relative capacity of animals to form these diol products.

The identity of the specific P450s forming individual DMBA-metabolites is being investigated by the use of agents which induce and/or suppress the expression of specific P450 isoforms. For instance, Sudan III causes a shift in the regio-selective metabolism of DMBA to products which are without toxic or carcinogenic properties and the animals become less susceptible to leukaemogenesis. Sudan III elicits responses similar to those resulting from the administration of 3-methylcholanthrene (MC) and causes an increased level of hepatic P450IA1 and consequent increased rates of formation of -5,6- and -8,9-diols [13, 14]. The formation of -5,6- and -8,9-diols in membranes from Sudan-treated animals

† Present address: Department of Veterinary Physiology and Pharmacology, The Texas Veterinary Medicinal Centre, College of Veterinary Medicine, Texas A and M University, TX 77843-4466, U.S.A.

§ To whom correspondence should be sent.

‖ Abbreviations: DMBA, 7,12-dimethylbenz[*a*]anthracene; P450, cytochrome P450; PB, phenobarbital; MC, 3-methylcholanthrene; PCN, pregnenalone-16α-carbonitrile; HPLC, high performance liquid chromatography; SDS–PAGE, sodium dodecyl sulphate–polyacrylamide gel electrophoresis.

can be quantitatively inhibited by an antibody to P450IA1 [14].

The regioselective metabolism of DMBA is also altered by pretreatment of the animals with phenobarbital (PB) and the rates of formation of DMBA-3,4-diol and DMBA-5,6-diol are increased [14, 15]. Phenobarbital causes an increase in the hepatic content of at least three P450-gene subfamilies [16–18]. Members of the P450IIC group are marginally increased by PB, while members of the P450IIB are greatly increased. Isoforms in the P450IIIA sub-family are greatly induced by pregnenalone-16α-carbonitrile (PCN) but are marginally increased by PB. Antibody inhibition studies and correlation between the expression of P450IIC in membranes from animals treated with various xenobiotics and rates of formation of DMBA-3,4-diol demonstrate that P450IIC plays a major role in the formation of the proximate carcinogen [14, 19]. Antibody and metyrapone inhibition studies [14] suggest that P450IIB may be important in the formation of the 5,6-diol.

Cytochrome P450 catalysed reactions are dependent on a source of electrons which in most cases is supplied by NADPH via NADPH-cytochrome P450 reductase [20]. However, some reactions are dependent on NADH as the reducing source and many NADPH-dependent reactions have increased rates when NADH is also present. Waxman and Walsh [21], studied various reactions catalysed by a purified P450IIC isoform in a reconstituted system and found a marked stimulation of ethoxycoumarin-*O*-deethylation, acetanilide hydroxylation and the oxidation of toluene and *p*-tolyl ethyl sulphide when cytochrome b_5 and NADH were also present. Because of the involvement of P450IIC in the formation of DMBA-diols and the apparent role of cytochrome b_5 in other P450IIC-mediated reactions [21], the ability of NADH to support DMBA-diol formation has been investigated.

Although 90% of the production of 3,4-diol can be accounted by P450IIC in membranes from PB-treated animals other isoforms are likely to be significant in membranes from untreated animals [19]. One method of addressing this problem is by treatment of animals with xenobiotics which produce characteristic and selective changes of the hepatic content of constitutively expressed P450s. PCN is an inducer of P450IIIA [22] and members of this gene family can also be induced by treatment with other synthetic glucocorticoids such as dexamethasone [23]. Clofibrate increases the amount of a P450 which functions in the ω and ω-1 hydroxylation of medium length fatty acids and causes a 20-fold induction of P450IVA1 [24]. Treatment of rats with pyrazole [25] increases the membrane content of P450IIE1 which is involved in the metabolic activation of the procarcinogen *N*-nitrosodimethylamine and in the metabolism of various hydrocarbons such as benzene [26] and pentane [27]. The effect of treatment of animals with dexamethasone, clofibrate and pyrazole on the P450 content of hepatic microsomes provides a means of investigating the role of several P450s in the metabolism of DMBA. The effects produced by these agents on DMBA-metabolism and P450 expression have been compared to the previously characterised effects produced by phenobarbital and Sudan III.

MATERIALS AND METHODS

Chemicals. DMBA, glucose-6-phosphate sodium salt, glucose-6-phosphate dehydrogenase (yeast Type VII), NADH and NADP-Na were obtained from the Sigma Chemical Co. (Poole, U.K.). [G^{3}H]DMBA (87.4 Ci/mmol) was obtained from Amersham International (Amersham, U.K.). The radiochemical purity of this material was confirmed by normal-phase HPLC (97%). Solvents were of HPLC grade and were obtained from FSA Laboratory Supplies (Loughborough, U.K.). Acetone was redistilled over potassium permanganate. All other reagents were of analytical grade.

Enzyme and antibody preparation. Antisera to cytochromes P450 (P450IIB1, P450IIC6 and P450IIIA1) were generously provided by Dr C. R. Wolf. The cytochromes were purified from adult, male, Wistar rats which were pretreated with phenobarbital and antisera produced in rabbit as previously described [28–30]. The specificity of these antibodies when used in Western blots and inhibition studies have been described [14, 31]. Anti-P450IIB1 reacts with P450IIB1 and IIB2, anti-P450IIC6 recognises several proteins of the IIC2 subfamily and anti-P450IIIA1 recognises a single antigen.

Animal treatment and preparation from liver. Female Long-Evans rats taken from a random-bred colony maintained on commercial diet A1 (John Stewart, Larbert, Stirlingshire) aged 40–44 days were used. Treated animals received either; 0.1% (w/v) PB in drinking water for 7 days; 7.5 mg/kg Sudan in corn oil (o.g.) 24 hr prior to killing; pyrazole (200 mg/kg) was dissolved in saline (0.9% w/v) and was administered as a single injection (i.p.) 24 hr prior to killing; dexamethasone (200 mg/kg), suspended in corn oil, was given (i.p.) for 3 days prior to killing; clofibrate was via the diet (0.5% w/w) for 10 days prior to killing. The clofibrate diet was prepared by mixing clofibrate dissolved in acetone with powdered Biosure diet. The acetone was allowed to evaporate overnight.

Microsomes were prepared as previously described (32) in Tris–HCl (0.05 M, pH 7.4 at 4°), KCl (1.15% w/v), EDTA (1 mM). The microsomal pellets were resuspended in potassium phosphate buffer (0.2 M, pH 7.0), EDTA (1 mM) to a protein concentration of between 10–20 mg/mL. For the metabolic studies the membranes were used immediately or were stored at −70° but for no longer than one week. Membrane protein was determined by the procedure of Bradford [33] using a bovine serum albumin standard, P450 by the method of Omura and Sato [34] and benzphetamine demethylase by determination of formaldehyde by the method of Nash [35].

Metabolism of DMBA with membrane samples. [^{3}H]DMBA (10 μCi, 50 μM) was incubated with rat microsomal samples (1 mg/mL) at 37° in the presence of an NADPH-regenerating system containing $NADP^+$ (0.4 mM), glucose-6-phosphate (4.0 mM) and glucose-6-phosphate dehydrogenase (4 units) in Tris–HCl buffer (0.07 M, pH 7.5 37°) containing

Table 1. Electron transport systems involved in the metabolism of DMBA in hepatic microsomes

Electron source	Rate of diol formation (pmol/min/mg microsomal protein) DMBA-3,4-diol	DMBA-5,6-diol	DMBA-8,9-diol
NADPH	15.57 ± 2.23	30.48 ± 3.25	11.29 ± 3.07
NADH	1.10 ± 0.22*	4.47 ± 1.02*	1.93 ± 1.06*
NADPH + NADH	17.99 ± 3.46	34.38 ± 0.88	13.16 ± 0.86

[^{3}H]DMBA was incubated with microsomal membranes (1 mg protein) from PB-treated animals in a total volume of 1 mL TKME. Reactions were initiated either by the addition of the NADPH regenerating mixture as described in Materials and Methods or the addition of NADH (0.4 mM) or both reductants were added simultaneously. Reactions were terminated after 20 min and initial rates of formation of DMBA-diols determined as described in Materials and Methods. Data was obtained with four separate microsomal preparations (duplicate determinations for each preparation) and show mean ± SD for the pooled data. The significance of the addition of NADH on formation of DMBA-diols was assessed by Student's *t*-test.

* $P < 0.001$.

potassium chloride (215 mM), EDTA (0.1 mM) and magnesium chloride (14 mM) in a final volume of 1 mL. Reactions were continued for 30 min or for 20 min when membranes from untreated animals or treated animals were used respectively. Under these conditions, the formation of all three diols studied is linear with respect to time but formation of the hydroxymethyl-MBA metabolites are not linear and these have not been investigated in this study. The reactions were stopped by the addition of 1 mL of ice-cold acetone and the aqueous phase was extracted twice with 2 mL of ethyl acetate. The combined organic phases were dried and evaporated to dryness prior to HPLC. The recovery of input radioactivity was 95% and the residual radioactivity remained in the aqueous phase or was associated with proteins [14].

Analysis of metabolites. Analytical HPLC was performed using a Gilson/Apple system (Gilson Medical Electronics F.A., France/Apple Computers, Hemel Hempstead) with a normal-phase silica column (Zorbax-SIL HPLC Technology, Macclesfield) as previously described [13, 14]. The dihydrodiols of DMBA have been identified by comparison of their retention times on two different chromatographic systems to authentic reference dihydrodiols and spectral properties of the eluted materials are in accord with published data [13, 14]. Typical retention times of the diols were: DMBA-3,4-diol, 28 min; DMBA-5,6-diol, 48 min; DMBA-8,9-diol, 65 min. The column eluate was collected in 1 mL fractions, the radioactivity determined in a liquid scintillation spectrometer and the initial rates of formation of the individual diols determined. Recovery of radioactivity loaded to the column was better than 85%.

Determination of the P450 components of rat liver membranes. Microsomal proteins were resolved by SDS–polyacrylamide gel electrophoresis [36] on 10% gels prior to electrophoretic transfer to nitrocellulose filters (Schleicher and Schull) as described by Towbin *et al.* [37]. The P450s were visualized by immunostaining, using antibodies to P450s, by the method of Domin *et al.* [38] using 4-chloro-1-naphthol as substrate.

RESULTS

The possible involvement of cytochrome b_5 in the formation of DMBA-diols has been investigated by a study of the role of NADH in these oxidation reactions. NADH and NADPH, alone and in combination, were tested as reductants in reactions containing [^{3}H]DMBA and microsomal membranes prepared from PB-treated animals. The results, shown in Table 1, confirm that formation of DMBA-diols requires the presence of NADPH and that reaction rates were very low when NADH was used as the sole source of electrons. In the presence of NADH alone, all three diols were detected but the rate of formation of the -3,4-diol, -5,6-diol and -8,9-diol were only 7, 15 and 17%, respectively, of the rates observed in the presence of NADPH. Inclusion of both NADPH and NADH resulted in only a marginal increase (~15%) in the rate of formation of DMBA-diols relative to NADPH alone but this was not statistically significant.

The regio-selective metabolism of DMBA has been investigated with microsomal membranes prepared from the livers of untreated and xenobiotic-treated rats. The membranes were initially characterised by their content of total P450 and by their capacity to demethylate benzphetamine which is a reaction catalysed by several PB-inducible isoforms. The results of these determinations are shown in Table 2. Treatment with Sudan did not appreciably alter the P450 content although a shift of the CO-difference spectra from 450 to 447 nm was observed (data not shown). PB caused a 2.8-fold increase in the content of P450 while treatment with dexamethasone, pyrazole and clofibrate also increased the P450 content of the respective microsomal preparations by ~1.5-fold in each case. The rates of benzphetamine N-demethylation were also altered by some of the animal treatments. PB caused a 5.3-fold increase in demethylation compared to untreated while dexamethasone and clofibrate increased this activity by 2.3-fold and 2.6-fold, respectively. The treatments with Sudan and pyrazole did not effect catalysis with this substrate.

The rates of formation of the DMBA-diols were

Table 2. Effect of various inducers of P450 enzymes on P450 content and benzphetamine-N-demethylation by rat hepatic microsomes

Animal treatment	P450 (nmol/mg protein)	Benzphetamine-*N*-demethylase (nmol/min/mg protein)
Untreated	0.65 ± 0.05	2.52 ± 0.06
Sudan	0.69 ± 0.04	2.92 ± 0.09
Phenobarbital	1.81 ± 0.05*	13.37 ± 0.99*
Dexamethasone	0.94 ± 0.10*	5.70 ± 0.40*
Clofibrate	1.00 ± 0.17*	6.43 ± 0.80*
Pyrazole	0.92 ± 0.07*	2.60 ± 0.21

Microsomal membranes (1 mg protein) were prepared from untreated animals and animals pre-treated with PB, Sudan, clofibrate, pyrazole or dexamethasone. The P450 content and rates of N-demethylation of benzphetamine were determined as described in Materials and Methods. Data was obtained with four microsomal preparations for each animal treatment (triplicate determinations for each preparation) and show mean ± SD for the pooled data. The significance of animal treatment on P450 content and benzphetamine demethylation was assessed by Student's *t*-test.

* $P < 0.001$.

determined using the membranes from the untreated and xenobiotic-treated rats. As previously reported DMBA is converted to three major diol products; DMBA-3,4-diol, DMBA-5,6-diol and DMBA-8,9-diol (Table 3). Animals receiving Sudan III or phenobarbital had altered capacities to form these diols as previously observed. Administration of Sudan suppressed the rate of formation of the -3,4-diol but caused increased rates of formation of the -5,6-diol (6-fold) and the -8,9-diol (16-fold). Treatment of animals with PB increased the rate of formation of the 3,4-diol (2-fold), the 5,6-diol (4-fold) and the -8,9-diol (2-fold). The other inducing agents also had regio-selective effects on the rates of formation of DMBA-diols. The formation of DMBA-3,4-diol was decreased when microsomal membranes from animals treated with dexamethasone, clofibrate and pyrazole were used, to 21, 58 and 38%, respectively of control values. Membranes from clofibrate treated animals had increased rates of formation of the -5,6-diol (3-fold) and the -8,9-diol (2-fold) but the formation of these diols by membranes from animals receiving dexamethasone or pyrazole were unaltered.

The effect of treating animals with xenobiotics on the microsomal content of individual P450 isoenzymes was investigated by Western blotting. The membrane proteins were separated by SDS–PAGE, transferred to nitrocellulose and probed with antibodies raised to P450IIIA1, P450IIC6 and P450IIB1 (Fig. 1). The antibodies produce single, major immunostained bands but minor bands with much lower staining intensity were also observed when anti-P450IIIA1 and anti-P450IIB1 were used. The minor bands may result from cross-reactivity of the antibodies with other proteins in the same gene subfamily but the intensity of immunostaining of these was not altered by animal treatment. Anti-P450IIIA1 produced a dominant staining band which was observed in each of the membrane preparations. The intensity of the major immunostained band was increased in membranes from dexamethasone-treated animals and to a lesser extent in membranes from PB-treated animals. The intensity of immunostain of the major band identified by anti-P450IIIA1 with membranes from Sudan, clofibrate and pyrazole-treated rats was not changed in comparison to that observed with membranes from the untreated animals. Anti-P450IIC6 reacted with a protein band present in each of the preparations and the intensity of staining of this was increased (~2-fold) when microsomal membranes from PB-treated animals were used. When membranes from Sudan, dexamethasone and pyrazole-treated animals were investigated the intensity of staining of the anti-P450IIC6-reactive material appeared to be decreased in comparison with membranes from untreated animals. The protein band reacting with anti-P450IIC6 with membranes from clofibrate-treated animals was unchanged in comparison to membranes from untreated animals. When anti-P450IIB1 was used with membranes from untreated animals two bands with differing staining intensity but of very similar molecular size were observed. The immunoreactive band of higher molecular weight was found to be greatly increased in the membranes from PB-treated animals and was increased to a lesser extent in membranes from clofibrate-treated and dexamethasone-treated animals. Sudan III or pyrazole treatment did not effect the immunostain intensity of either band detected by anti-P450IIB1.

DISCUSSION

Previous studies have provided evidence for the role of specific P450s in the regio-selective formation of DMBA-diols in rat-liver, microsomal membranes [13, 14, 19]. In membranes from Sudan-treated animals P450IA1 is responsible for the formation of most of the -5,6- and -8,9-diols. Other P450s are important in membranes from PB-treated animals and a member (or members) of the P450IIC family account for up to 90% of DMBA-3,4-diol and 50% of DMBA-5,6-diol formation [19]. In these membranes the remainder of the -5,6-diol is formed by member(s) of the P450IIB family while P450IIC also plays a significant role in reactions leading to

Table 3. Effect of various inducers of P450 enzymes on the rate of formation of DMBA-diols by hepatic microsomes

Animal treatment	Rate of diol formation (pmol/min/mg protein) DMBA-3,4-diol	DMBA-5,6-diol	DMBA-8,9-diol
Untreated	11.2 ± 2.0	11.7 ± 1.5	9.7 ± 1.1
Sudan	6.7 ± 1.2	71.7 ± 14.5*	159.2 ± 5.0*
Phenobarbital	19.5 ± 7.4*	46.4 ± 8.2*	19.3 ± 3.9*
Dexamethasone	2.3 ± 0.7*	7.4 ± 0.6	11.7 ± 0.9
Clofibrate	6.5 ± 1.4	34.1 ± 6.7*	20.2 ± 3.6*
Pyrazole	4.3 ± 1.1*	7.5 ± 2.3	8.9 ± 1.6

Microsomes (1 mg) prepared from untreated animals or pretreated as above were incubated in the presence of [^{3}H]DMBA and intial rates of formation of DMBA-diols determined as described in Material and Methods. Data was obtained from three different membrane preparations (duplicate determinations for each preparation) and show mean ± SD for the pooled data.

* $P < 0.001$.

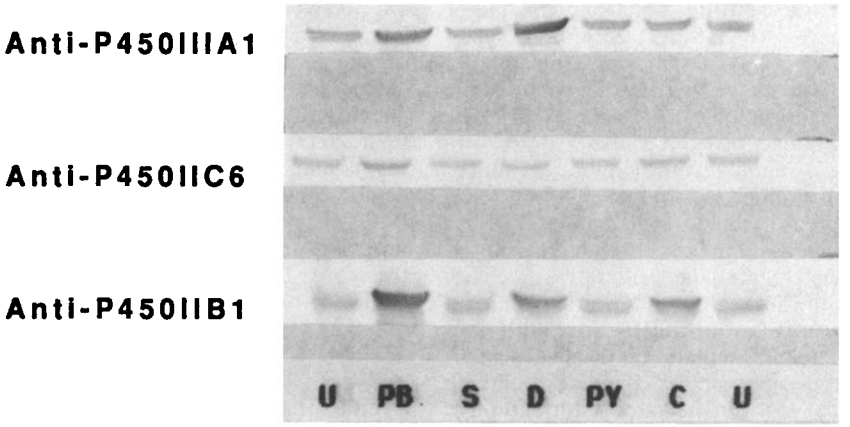

Fig. 1. Immunoblot analysis of P450 isoenzymes in microsomal membranes from rats treated with a variety of xenobiotics. Protein components of microsomal membranes from untreated animals (U) or animals treated with phenobarbital (PB), Sudan (S), dexamethasone (D), pyrazole (PY) or clofibrate (C) were resolved by SDS–PAGE and transferred to nitrocellulose as described in Materials and Methods. These were immunostained using either anti-P450IIIA1, anti-P450IIC6 or anti-P450IIB1. Protein loading to each lane was 10 μg microsomal protein.

formation of the -8,9-diol. Although P450IA2, P450IIB2 and members of the P450IIC subfamily are expressed constitutively the results of these previous studies may have been influenced by the use of microsomes from Sudan or PB-induced animals. This study was undertaken to identify other P450s which may catalyse DMBA-diol formation, especially those isoenzymes which are expressed constitutively, and to further characterize the P450IIC isoform involved.

Waxman and Walsh [21] found that the catalytic activity of a purified P450IIC (termed P450-PB1) in a reconstituted system was enhanced by the inclusion of cytochrome b_5. If the P450IIC involved in the formation of DMBA-diols is identical to this isoform it might be expected that inclusion of NADH would increase the formation of these diols. Although inclusion of NADH with the DMBA-reaction mixture did cause a slight increase in the formation of DMBA-diols, this was within experimental error (Table 1). From the results presented in Table 1 there does not appear to be a dependence on cytochrome b_5 in the formation of DMBA-diols in these membranes. The P450IIC family is extensive in the rat and probably contains more than 10 genes [39] and proteins of at least five of these have been isolated [21, 22, 40, 41]. With the exception of the P450IIC isolated by Waxman and Walsh, none of these isoenzymes has a dependence on the NADH-cytochrome b_5 reductase/cytochrome b_5 system. The P450IIC isolated by Waxman and Walsh was also stimulated by metyrapone [21] but this agent has no stimulatory effect of DMBA-diol formation in microsomes [14]. These observations may indicate that the isoform isolated by Waxman and Walsh is not the P450IIC involved in DMBA metabolism. Alternatively, it may be that the catalytic properties of membrane-embedded P450IIC is modified by the bilayer environment and unlike the reconstituted system does not have a requirement for cytochrome

b_5 and is not stimulated by metyrapone. Differences in turnover number and regio-selectivity during DMBA metabolism when reconstituted systems are compared to microsomal membranes, have previously been noted [14, 42].

Treatment of animals with Sudan and PB produces characteristic regio-selective alterations in the metabolism of DMBA [13, 14, 19] and these are shown in Table 3. The effect of treatment of animals with inducers of specific P450s was investigated to identify other individual P450 isoenzymes which may be involved in the metabolism of DMBA. This included treatment of animals with clofibrate, pyrazole and dexamethasone which induce P450IVA1 [24], P450IIE1 [25] and P450IIIA1 [22] respectively. Typically these agents cause marked increases in the membrane content of these isoforms and increase catalytic rates associated with the oxidation of isoform-selective substrates. None of the inducers caused increases in the rates of DMBA-diol formation of any great magnitude (Table 3) suggesting that the isoforms which are the major induced proteins (P450IVA1, P450IIE1 and P450IIIA1) do not play a significant role in diol formation. However, the agents did cause some changes in diol formation and these appear to be associated with effects on other P450s.

Treatment of animals with clofibrate induces P450IVA1 by as much as 20-fold [24] but also appears to cause an induction of member(s) of the P450IIB sub-family. This is indicated both by the increased benzphetamine demethlyase activity (Table 2) and the increase on Western blots of a protein detected by anti-P450IIB1 in membranes from clofibrate-treated animals (Fig. 1). The increase of P450IIB in these membranes correlates well with the observed increase in the rate of formation of the -5,6-diol (Table 3) whose production has been attributed to the action of P450IIB. The clofibrate-induced increase of P450IIB reported here is in contrast to the findings of Sharma *et al.* [43] who found that this treatment caused a decrease in benzphetamine-N-demethylation and ethoxyresorufin-O-deethylation. In the work reported by Sharma *et al.* [43], Wistar rats were used and the differences between the results could therefore be due to strain difference.

Treatment of rats with pyrazole causes an increase of P450IIE protein [25] and nitrosodimethylamine demethylase activity [44]. P450IIE1 has been shown to be involved in the metabolism of some hydrocarbons [26, 27] and therefore the effect of treatment of animals with pyrazole on the metabolism of DMBA has been investigated. However, the effects of pyrazole are not simple since this agent can act as a suicide inhibitor of P450s and treatment of animals with this agent *in vivo* causes reduced rates of oxidation of some substrates [44, 45]. In this study pyrazole was found to reduce the rate of formation of the -3,4-diol (Table 3) suggesting that a member of the P450IIC sub-family may be affected by the pyrazole treatment. This is supported by a slightly decreased content of P450IIC proteins in the microsomal membranes from pyrazole-treated animals (Fig. 1) while immunoblots of these membranes with anti-P450IIB1 or anti P450IIIA1 indicated that these isoforms were unaffected by pyrazole. Previous work in this laboratory has indicated that a PB-inducible isoform other than P450IIB may be more susceptible to suicide inhibition by pyrazole than are other isoforms (V. M. Morrison and J. A. Craft, unpublished data).

In rats, dexamethasone induces P450IIIA1 but also suppresses the expression of P450IIC and induces P450IIB [46]. Previous work using an antibody to P450IIIA1 has indicated that this isoform is not involved in DMBA-diol formation and that conclusion is now supported directly by rate determination data with membranes from dexamethasone-treated animals (Table 3). However, the concomitant induction of P450IIIA1 and suppression of P450IIC by treatment of rats with dexamethasone is intriguing and provides a means for investigating further the role of P450IIC in the metabolism of DMBA. The rate of formation of DMBA-3,4-diol was reduced in microsomal membranes from dexamethasone-treated rats by 80% (Table 3) but Western blots of these membranes when immunostained with anti-P450IIC6 showed a much smaller reduction in the content of P450IIC when compared to microsomal membranes from untreated animals (Fig. 1). Meehan *et al.* [46] found that treatment of rats with dexamethasone caused a slight induction of P450IIC-related mRNA, as demonstrated by Northern blots, but a reduction of P450IIC-related protein on Western blots. It was proposed that the effect of dexamethasone may therefore be complex and may induce some member(s) of the P450IIC family while suppressing the formation of others. The P450IIC which is involved in DMBA-diol formation appears to be one which is sensitive to dexamethasone suppression but this P450 may constitute only a small part of the total anti-P450IIC6-reactive protein. Because of the role of P450IIC in formation of the -5,6-diol it might be expected that this metabolite would also be formed at much reduced rates in the membranes from dexamethasone-treated animals. However, the rate of formation of the -5,6-diol was only slightly reduced (<30%) in membranes from dexamethasone-treated rats and the effect was very much less marked than for the -3,4-diol. Increased rates of benzphetamine demethylation (Table 2), and increased immunostaining with anti-P450IIB1 with these microsomes indicate an increase in P450IIB sub-family (Fig. 1) which is also involved in DMBA-5,6-diol formation. The effect of an increase in P450IIB member(s) may thus cancel out the effect of decreased P450IIC member(s). Other studies have reported a dexamethasone-induced increase of P450IIB protein [46, 47] and mRNA [48].

The results of this work provide further indications that a member(s) of the P450IIC sub-family is involved in the formation of DMBA-3,4-diol and that both P450IIC and P450IIB are involved in the formation of DMBA-5,6-diol. Treatment of rats with xenobiotics which cause large increases in the membrane content of specific P450s, enabled the elimination of P450IVA1, P450IIE1 and P450IIIA1 as playing major roles in DMBA-diol formation and the effects of these agents on DMBA-diol formation

appear to be mediated by alterations of P450IIC and P450IIB content.

Acknowledgements—We thank Dr C. R. Wolf for generously providing gifts of antisera used, the Wellcome Trust for the provision of equipment and the Leukaemia Research Fund for financial support. V. M. M. was an SERC Research Student.

REFERENCES

1. Huggins CB, *Experimental Leukaemia and Mammary Cancer*. University of Chicago Press, Chicago, Illinois, 1979.
2. Huggins CB, Ueda N and Russo A, Azo dyes prevent hydrocarbon induced leukaemia in the rat. *Proc Natl Acad Sci USA* **75**: 4524–4527, 1978.
3. Bianafiori C, Bonser GM and Cashera F, Ovarian and mammary tumours in intact C3 Hb virgin mice following a limited dose of four carcinogenic chemicals. *Br J Cancer* **15**: 270–283, 1961.
4. Huggins C, Grand LC and Brillantes FP, Mammary cancer induced by a single feeding of polynuclear hydrocarbons and its suppression. *Nature* **189**: 204–207, 1987.
5. Grover PL, Pathways involved in the metabolism and activation of polycyclic hydrocarbons. *Xenobiotica* **16**: 915–931, 1986.
6. Quintanilla M, Brown K, Ramsden M and Balmain A, Carcinogen specific mutation and amplification of the Ha-ras during mouse skin carcinogenesis. *Nature* **322**: 78–80, 1986.
7. Balmain A, Ramsden M, Bowden GT and Smith J, Activation of the mouse skin cellular Harvey-ras gene in the chemically induced benign skin papillomas. *Nature* **307**: 658–660, 1984.
8. Dandekar S, Saraswaki S, Zarbl H, Young LJT and Cardiff RB, Specific activation of the cellular Harvey-ras oncogene in dimethylbenz(*a*)anthracene-induced mouse mammary tumours. *Mol Cell Biol* **6**: 4104–4108, 1986.
9. Vigny P, Brunissen A, Phillips DH, Cooper CS, Hewer A, Grover PL and Sims P, Metabolic activation of 7,12-dimethylbenz(*a*)anthracene in rat mammary tissue; fluorescence spectral characteristics of hydrocarbon DNA adducts. *Cancer Lett* **26**: 51–59, 1985.
10. Sawicki JT, Moscell RC and Dipple A, Involvement of both *syn* and *anti* dihydrodiol epoxides in the binding of 7,12-dimethylbenz(*a*)anthracene to DNA in mouse embryo cell cultures. *Cancer Res* **43**: 3212–3218, 1983.
11. DiGiovanni J, Nebzydoski AP and Decina PC, Formation of 7-hydroxymethyl-12-methyl-benz(*a*)-anthracene-DNA adducts from 7,12-dimethyl benz(*a*)-anthracene in mouse epidermis. *Cancer Res* **43**: 4221–4226, 1983.
12. Cheng SC, Prakash AS, Pigott MA, Hilton BD, Lee H, Harvey RG and Dipple A, A metabolite of the carcinogen 7,12-dimethylbenz(*a*)anthracene that reacts predominantly with adenine residues in DNA. *Carcinogenesis* **9**: 1721–1723, 1988.
13. O'Dowd JJ, Burnett AK, Weston A, Bullied NJ and Craft JA, Alterations in the metabolism of 7,12-dimethylbenz(*a*)anthracene and various xenobiotics by rat hepatic microsomes following Sudan III treatment *in vivo*. *Carcinogenesis* **6**: 469–472, 1985.
14. McCord A, Burnett AK, Wolf CR, Morrison V and Craft JA, Role of specific cytochrome P450 isoenzymes in the regio-selective metabolism of 7,12-dimethylbenz(*a*)anthracene in microsomes from rats treated with phenobarbital or Sudan III. *Carcinogenesis* **9**: 1485–1491, 1988.
15. Christou M, Wilson NM and Jefcoate CR, The role of secondary metabolism in the metabolic activation of 7,12-dimethylbenz(*a*)anthracene by rat liver microsomes. *Carcinogenesis* **5**: 1239–1247, 1984.
16. Adesnik M and Atchison M, Genes for cytochrome P450 and their regulation. *CRC Crit Rev Biochem* **19**: 247–305, 1986.
17. Whitlock JP, The regulation of cytochrome P450 gene expression. *Annu Rev Pharmacol Toxicol* **26**: 333–369, 1986.
18. Gonzalez FJ, Molecular Genetics of the P450-superfamily. *Pharmacol Ther* **45**: 1–38, 1990.
19. Morrison VM, Burnett AK, Forrester LM, Wolf CR and Craft JA. The contribution of specific cytochrome P450s in the metabolism of 7,12-dimethylbenz[*a*]anthracene in rat and human liver microsomal membranes. Submitted.
20. White RE and Coon MJ, Oxygen activation by cytochrome P450. *Annu Rev Biochem* **49**: 315–356, 1980.
21. Waxman DJ and Walsh C, Cytochrome P450 isoenzyme 1 from phenobarbital induced rat liver; purification, characterisation and interactions with metyrapone and cytochrome b_5. *Biochemistry* **22**: 4846–4855, 1983.
22. Elshourbagy NA and Guzelian PS, Separation, purification and characterization of a novel form of hepatic cytochrome P450 from rats treated with pregnenalone-16α-carbonitrile. *J Biol Chem* **255**: 1279–1285, 1980.
23. Scheutz EG, Wrighton SA, Safe SH and Guzelian PS, Regulation of cytochrome P450 by phenobarbital and phenobarbital-like inducers in adult rat hepatocytes in monolayer culture and *in vivo*. *Biochemistry* **25**: 1124–1133, 1986.
24. Gibson GC, Orton TC and Tamburini PP, Cytochrome P450 induction by clofibrate. Purification and properties of hepatic cytochrome P450 relatively specific for 12 and 11-hydroxylation of dodecanoic acid (lauric acid). *Biochem J* **203**: 161–168, 1982.
25. Thomas PE, Bandiera S, Maines SL, Ryan DE and Levin W, Regulation of cytochrome P450j, a high affinity *N*-nitrosodimethylamine demethylase, in rat hepatic microsomes. *Biochemistry* **26**: 2280–2289, 1987.
26. Johansson I and Ingelman-Sunberg M, Benzene metabolism by ethanol-, acetone- and benzene-inducible cytochrome P450(IIE1) in rat and rabbit microsomes. *Cancer Res* **48**: 5387–5390, 1988.
27. Terelius Y and Ingelman-Sundberg M, Metabolism of *n*-pentane by ethanol-inducible cytochrome P450 in liver microsomes and reconstituted membranes. *Eur J Biochem* **161**: 303–308, 1986.
28. Wolf CR, Seilman S, Oesch F, Mayer RT and Burke DM, Multiple forms of cytochrome P450 related to forms marginally induced by phenobarbital. Differences in structure and in the metabolism of alkylresorufins. *Biochem J* **240**: 27–33, 1986.
29. Wolf CR, Moll E, Friedburg T, Oesch F, Buchmann A, Kuhlmann WP and Kunz HW, Characterization, localization and regulation of a novel phenobarbital inducible form of cytochrome P450 compared with three further forms of cytochrome P450, NADPH cytochrome P-450-reductase, glutathione transferase and microsomal epoxide hydrolase. *Carcinogenesis* **5**: 993–1001, 1984.
30. Wolf CR and Oesch F, Isolation of a high spin form of cytochrome P450 induced by 3-methylcholanthrene. *Biochem Biophys Res Commun* **111**: 504–511, 1983.
31. Adams DJ, Seilman S, Amelziad Z, Oesch F and Wolf CR, Identification of human cytochromes P450 analogous to forms induced by phenobarbital and 3-methylcholanthrene in the rat. *Biochem J* **232**: 869–876, 1985.
32. Bullied NJ and Craft JA, Effects of metyrapone and norharmane on microsomal monooxygenase and

epoxide hydrolase activities. *Biochem Pharmacol* **33**: 1451–1457, 1984.
33. Bradford MM, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principal of protein dye binding. *Anal Biochem* **72**: 248–254, 1976.
34. Omura T and Sato R, The carbon monoxide binding pigment of liver microsomes: I Evidence for its haemoprotein nature. *J Biol Chem* **239**: 2370–2378, 1964.
35. Nash T, The colorimetric estimation of formaldehyde by means of the Hansch reaction. *Biochem J* **55**: 416–421, 1953.
36. Laemmli UK, Cleavage of structural proteins during the assembly of head of bacteriophage T4. *Nature (London)* **227**: 680–685, 1970.
37. Towbin H, Staehelin T and Gordon J, Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**: 4350–4354, 1979.
38. Domin BA, Serabjit-Singh CJ and Philpot RM, Quantitation of rabbit cytochrome P-450, form 2 in microsomal preparations bound to nitrocellulose paper using a modified peroxidase immunostaining procedure. *Anal Biochem* **136**: 390–396, 1984.
39. Meehan RR, Gosden JR, Rout D, Hastie ND, Friedberg T, Adesnik M, Buckland R, van Heyningen V, Fletcher J, Spurr NK, Sweeny J and Wolf CR, Human cytochrome P450 PB-1: A multigene family involved in mephenytoin and steroid oxidations that maps to chromosome 10. *Am J Hum Genet* **42**: 26–37, 1988.
40. Hanniu M, Ryan DE, Iida S, Leiber CS, Levin W and Shirley JE, NH_4 terminal sequence analysis of four rat hepatic microsomal P450. *Arch Biochem Biophys* **235**: 304–311, 1984.
41. Bandiera S, Ryan DE, Levin W and Thomas PE, Evidence for a family of immunochemically related isoenzymes cytochrome P450 purified from untreated rats. *Arch Biochem Biophys* **240**: 478–482, 1985.
42. Cristou M, Mitchell MJ, Javinovich MC, Wilson NM and Jefcoate CR, Selective potent restriction of cytochrome P450b but not P450e-dependent 7,12-dimethylbenz(*a*)anthracene metabolism by the microsomal environment. *Arch Biochem Biophys* **270**: 162–172, 1989.
43. Sharma R, Lake BG, Foster J and Gibson GG, Microsomal cytochrome P452 induction and peroxisome proliferation by hypolipidaemic agents in rat liver. *Biochem Pharmacol* **37**: 1193–1201, 1988.
44. Craft JA, Effects of pyrazole on nitrosodimethylamine demethylase and other microsomal xenobiotic metabolising enzymes. *Biochem Pharmacol* **34**: 1507–1513, 1985.
45. Ortiz de Montellano PR and Correia MA, Suicidal destruction of cytochrome P450 during oxidative drug metabolism. *Annu Rev Pharmacol Toxicol* **23**: 481–503, 1983.
46. Meehan RR, Forrester LM, Stevenson K, Hastie ND, Buchman A, Kunz HW and Wolf CR, Regulation of phenobarbital-inducible cytochrome P450s in rat and mouse liver following dexamethasone administration and hypophysectomy. *Biochem J* **254**: 789–797, 1988.
47. Yamazoe Y, Shimada M, Murayama N and Kato R, Suppression of levels of phenobarbital-inducible rat liver cytochrome P450 by pituitary hormones. *J Biol Chem* **262**: 7423–7428, 1987.
48. Simmons DL, McQuiddy P and Kaspar CB, Induction of the hepatic mixed-function oxidase system by synthetic glucocorticoids. Transcriptional and post-transcriptional regulation. *J Biol Chem* **262**: 326–332, 1987.