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EFFECT OF RIFAMPICIN ON THE MOUSE HEPATIC MIXED-FUNCTION OXIDASE SYSTEM

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Abstract—The properties of the mouse hepatic microsomal mixed-function oxidase system were compared following induction by rifampicin, β-naphthoflavone (BNF), phenobarbitone (PB) and pregnenolone 16α-carbonitrile (PCN). Close similarities were observed between rifampicin and PCN with regard to the profile of mixed-function oxidase activities induced. Testosterone 6β-hydroxylation was especially affected by both compounds which increased its activity disproportionately relative to PB and BNF. The responses of other mixed-function oxidase activities to *in vitro* additions of α-naphthoflavone, metyrapone, SKF 525A and sodium fluoride, and the spectral characteristics of the reduced cytochrome P-450–CO complex were also comparable after pretreatment with rifampicin and PCN, but different from those observed after PB or BNF. At the doses used, rifampicin was a less potent inducer of the mixed-function oxidase system than PCN and differences in potency could underlie the ability of PCN but not rifampicin to increase relative liver weight and microsomal protein content. However, it seems less likely that differences in potency could explain the dissimilarities in the reduced ethylisocyanide difference spectrum induced by rifampicin and PCN or the observation that, of the two microsomal polypeptide bands of 52–57000 mol.wt induced by rifampicin and PCN, only one appeared common to both inducers.

Rifampicin (rifampin), an antibiotic commonly used in the treatment of tuberculosis [1], is a known inducer of the hepatic mixed-function oxidase system [2]. Prolonged administration of the drug in man has been shown to produce proliferation of hepatic smooth endoplasmic reticulum [3] and to increase urinary D-glucaric acid excretion [4, 5], the metabolism of other drugs [4–8], and the cytochrome P-450 content of isolated liver subfractions [9]. In guinea pigs [10] and mice [11, 12] pretreatment with rifampicin has been shown to increase hepatic microsomal enzyme activities and cytochrome P-450 content. However, in rats the same activities were not affected by pretreatment with rifampicin [9, 11].

Different types of purified cytochrome P-450 catalyse different mixed-function oxidations [13] and the responses of corresponding microsomal enzyme activities to certain inhibitors, activators and enzyme inducing agents also differ [14–19]. In order to identify the inducing characteristics of rifampicin, we have compared its effects on mouse hepatic microsomal systems with those of the widely studied enzyme inducing agents β-naphthoflavone, phenobarbitone and pregnenolone 16α-carbonitrile [17, 18, 20]. Further characterisation was made by using the inhibitors α-naphthoflavone (ANF), *SKF 525A, metyrapone and NaF and by comparing the results of SDS-polyacrylamide gel electrophoresis in microsomes from mice pretreated with the four enzyme inducers.

MATERIALS AND METHODS

Chemicals. Bovine serum albumin, Coomassie brilliant blue R, cytochrome *c*, glucose 6-phosphate, glucose 6-phosphate dehydrogenase, *N*,*N*′methylene-bisacrylamide, paracetamol, testosterone, *N*,*N*,*N*′,*N*′-tetramethylethylenediamine and Tris were purchased from Sigma (London), Poole, Dorset; acetanilide, acrylamide, Hepes, semicarbazide, sodium dodecyl sulphate and sodium fluoride from Hopkin and Williams, Romford, Essex; benzo[α]pyrene, metyrapone, α-naphthoflavone and β-naphthoflavone from Aldrich Chemicals Co. Ltd., Gillingham, Dorset; and ammonium persulphate and phenobarbitone sodium from British Drug Houses, Poole, Dorset. $NADP^+$ was bought from International Enzymes Ltd., Windsor, Berks, corn oil from Renown Products (Penge) Ltd., Belvedere, Kent, and carbon monoxide from Cambrian Gases, Croydon.

[4-^{14}C]Testosterone (sp. act. 51 m Ci/mmole) was obtained from the Radiochemical Centre, Amersham, Bucks. Its radiochemical purity was > 98% on thin layer chromatography in dichloromethane: acetone (90 : 50). Using unlabelled testosterone, the isotope was diluted to 0.417 mCi/mmole before use.

Rifampicin was kindly provided by Ciba Laboratories, Horsham, W. Sussex, ethylmorphine hydrochloride by May and Baker, Dagenham, Essex,

* Abbreviations and trivial names. ANF: α-naphthoflavone (7,8-benzoflavone); BNF: β-naphthoflavone (5,6-benzoflavone); Hepes: *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulphonic acid; Metyrapone: 2-methyl-1,2-di-3-pyridyl-1-propane; PB: phenobarbitone sodium; PCN: pregnenolone 16α-carbonitrile (16α-cyano-3β-hydroxy-5-pregnen-20-one); SDS: sodium dodecyl sulphate; SKF 525A: 2-diethylaminoethyl-2,2-diphenylvalerate; testosterone: 17β-hydroxy-4-androsten-3-one.

6β-, 7α-, and 16α-hydroxytestosterones by the Medical Research Council Steroid Reference Collection, pregnenolone 16α-carbonitrile by G. D. Searle & Co., Chicago, Illinois, U.S.A., SKF 525 A by Smith, Kline and French, Welwyn Garden City, Herts, and ethylisocyanide by Dr G. Sweeney, MacMaster University, Toronto, Canada.

Animals and pretreatment regimens. Male C57Bl6 inbred mice (11 weeks old, 18–24 g) were obtained from Bantin and Kingman, Hull, Yorkshire. They were housed four or less per cage on commercially available dried clay bedding and fed on Labsure C.R.M. Diet (Rank-Hovis-MacDougal, Poole, Dorset) and tap water *ad libitum*. These conditions were maintained for at least two weeks before pretreatment began.

All inducing agents were given by intraperitoneal injection in the appropriate vehicle (10 ml/kg body weight). The regimens used were: β-naphthoflavone (80 mg/kg in corn oil): one dose, 48 hr before death; phenobarbitone (80 mg/kg in saline), pregnenolone 16α-carbonitrile (30 mg/kg in corn oil) or rifampicin (40 mg/kg in HCl, pH3): one dose on three consecutive days with the final injection 24 hr before death.

Tissue preparation. Mice were killed by cervical dislocation between 8 am and 9 am and livers (at least four per pool) were excised into ice-cold KCl–Hepes (1.15 % KCl containing 10 mM Hepes buffer, pH 7.6). Washed microsomal suspensions were prepared as previously described [21] and their protein content was determined [22] before making the appropriate dilution for use in the enzyme assays.

Biochemical analyses. Cytochrome P-450 was determined in microsomal suspensions containing 2 mg protein/ml 0.1 M Hepes buffer, pH 7.6 by the method of Omura and Sato [23]. Ethylisocyanide-induced difference spectra were determined according to Imai and Sato [24] by addition of 5 μl of a 2% solution of ethylisocyanide in methanol to a dithionite-reduced microsomal suspension containing 2 mg protein/ml 0.5 M Hepes buffer, pH 7.8. All spectra were recorded on a Pye Unicam SP 800 double-beam spectrophotometer using the front cell compartment. Peak heights were measured from the appropriate baseline after adjustment to a standard absorbance at 490–500 nm. Positions of maximal absorbance were measured from recordings made using chart expansion.

NADPH-cytochrome *c* reductase activities were measured at 30° on a Pye Unicam SP 800 dual beam spectrophotometer by the method of Peters and Fouts [25]. Cuvettes contained 200 μmoles Hepes buffer, pH 8.1, 417 μmoles KCl, 4.2 μmoles KCN, 0.2 μmoles cytochrome *c*, 100 μg microsomal protein and water to a final volume of 2.5 ml. The reaction was initiated by the addition to the sample cuvette of 0.2 ml of a premixed NADPH generating system containing 6.25 μmoles glucose 6-phosphate, 6.25 μmoles $MgCl_2$, 1.25 μmoles $NADP^+$ and 2 units glucose 6-phosphate dehydrogenase. An extinction coefficient of 19,100 mol^{-1} was used in the calculation of the amounts of cytochrome *c* reduced.

Minor modifications of the methods of Bend *et al.* [26], Shimazu [27] and Wattenberg *et al.* [28] were used in the assays for ethylmorphine *N*-demethylase, acetanilide, 4-hydroxylase and benzo[α]pyrene hydroxylase activities, respectively. Incubations contained 200 μmoles Hepes buffer (pH 7.6 for ethylmorphine, pH 7.8 for acetanilide and pH 7.4 for benzpyrene), 5 μmoles glucose-6-phosphate, 10 μmoles $MgCl_2$, 1 μmole NADP, 1 unit glucose 6-phosphate dehydrogenase, microsomal protein (3 mg for ethylmorphine and acetanilide, 50 μg for benzo[α]pyrene) and water to a final volume of 2 ml (including substrate). Semicarbazide (10 μmoles) or bovine serum albumin (2mg) were also included in the incubations for ethylmorphine demethylase and benzo[α]pyrene hydroxylase, respectively. When present, modifiers of enzyme activity were added in acetonitrile (10 μl) for α-naphthoflavone (0.2 μmole) or water for metyrapone (1 μmole), SKF 525 A (0.2 μmole) and NaF (0.2 mmole). After 5 min preincubation at 37°, reactions were initiated by the addition of 18 μmoles ethylmorphine, 16 μmoles acetanilide or 100 nmoles benzo[α]pyrene (in 50 μl acetone), and proceeded for 15, 15 and 10 min respectively.

The hydroxylation of testosterone was measured using minor modifications of the method of Orton and Philpot [29]. Incubations contained 200 μmoles Hepes buffer (pH 7.6), 5 μmoles glucose 6-phosphate, 10 μmoles $MgCl_2$, 1 μmol $NADP^+$, 1 unit glucose 6-phosphate dehydrogenase, 1 mg microsomal protein and water to 2.0 ml (including substrate) After 5 min preincubation at 37°, the reaction was initiated by addition of 800 nmoles (740,000 d.p.m.) [4-^{14}C]testosterone in 20 μl methanol and proceeded for 10 min. Testosterone and its metabolites were extracted into dichloromethane and, after evaporation to dryness, an aliquot of this extract, redissolved in methanol, was applied to a pre-activated (110° for 60 min) silica gel HF_{254} precoated thin layer chromatography plate (Merck, Darmstadt, Germany) as a 50 mm band, together with the appropriate standards. The plate was developed in dichloromethane/acetone (90 : 50) and metabolites co-chromatographing with and subsequently referred to as testosterone (R_f~0.70), 6β-hydroxytestosterone (R_f~0.50), 7α-hydroxytestosterone (R_f~0.30) and 16α-hydroxytestosterone (R_f~0.21) were visualised under ultraviolet light and scraped off into scintillation vials. The samples were counted in the presence of 1 ml H_2O and 10 ml Liquid Scintillation Cocktail T (Hopkins and Williams, Romford, Essex) in a Packard Liquid Scintillation Spectrometer, Model 3320 (Packard Instrument Co., Chicago, Illinois, USA).

Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. Microsomes for electrophoresis were diluted to a concentration of 1 mg protein/ml in 0.0625 M Tris buffer (pH 6.8) containing 15% glycerol and 2.3% (w/v) SDS. Microsomal samples (40 μg protein) and a mixture of reference standards containing horse heart cytochrome *c* (subunit mol. wt 12,500), ovalbumin (43,000), catalase (58,000) and bovine serum albumin (68,000) were separately loaded into 9 mm wide wells in a stacking gel (30 × 140 × 1.5 mm) prepared from 3% acrylamide and overlaid on a vertical gradient gel (100 × 140 × 1.5 mm) prepared from 7% and 12% acrylamide solutions with a standard dual-chamber

gradient mixer. Other conditions employed in the preparation of the gels have been described by Laemmli [30]. The gels were run at 140 V for 3 to 4 hr at room temperature, fixed in 50% (w/v) trichloracetic acid for 1 hr, stained overnight with Coomassie Brilliant Blue R (0.2% (w/v) in methanol/glacial acetic acid/water, 50 : 7 : 50) and destained in methanol/glacial acetic acid/water (9 : 1 : 10).

Calculation and statistical evaluation of results. Ethylmorphine *N*-demethylase and acetanilide 4-hydroxylase specific activities were calculated as nmoles product (formaldehyde and paracetamol, respectively) per mg microsomal protein per min. Benzo[α]pyrene hydroxylase specific activities (per mg microsomal protein per min) were calculated by measurement of the fluorescence (400 nm excitation, 514 nm emission) of its phenolic metabolites as Relative Fluorescent (RF) units. A standard solution of quinine sulphate (3 μg/ml 0.1 M H_2SO_4) had a RF of 200 units when its fluorescence was measured at 350 nm excitation, 450 nm emission.

Quantitation of the hydroxylated metabolites of testosterone was made by expressing the counts co-chromatographing with the appropriate standard as a percentage of the total applied to the chromatography plate. Calculations of the amounts of testosterone metabolised or hydroxytestosterones produced (nmoles/mg microsomal protein/min) were made using the appropriate dilutions and by assuming that 1 μmole[^{14}C]hydroxytestosterone was derived from 1 μmole of [^{14}C]testosterone substrate. Allowances were made for the counts appearing in each band in the blanks i.e. those incubations in which [4-^{14}C]testosterone was added after termination of the incubation.

Molecular weights of polypeptide components separated by polyacrylamide gel electrophoresis were determined as described by Laemmli [30] from a standard curve of distance migrated vs $\log_{10}$ mol. wt. The values presented are apparent mol. wts pertaining to the conditions employed.

All values were considered significantly different when the probability of their chance occurrence was less than 5%. The statistical tests applied are given in the appropriate figure legends.

RESULTS

Comparative effects of enzyme inducing agents on factors associated with the mixed-function oxidase system of mouse liver. The slight increases in relative liver weight and microsomal protein content noted after rifampicin pretreatment (Table 1) contrasted with the greater and statistically significant increases noted after phenobarbitone (PB) or pregnenolone 16α-carbonitrile (PCN). β-Naphthoflavone (BNF) affected neither of these parameters significantly. All four compounds increased NADPH-cytochrome *c* reductase activity (25–89%) and cytochrome P-450 content (50–170%), but only PB pretreatment gave a reduced cytochrome P-450–CO difference spectrum with an absorption maximum at the same wavelength as that found in control preparations (Table 1). The corresponding peak position showed a hypsochromic shift of 1 nm following rifampicin or PCN pretreatment and of 2 nm following BNF (Table 1). The peak position of the ethylisocyanide-induced absorption maximum around 455 nm also appeared at lower wavelengths following BNF (3 nm hypsochromic shift), PCN (3 nm shift) or rifampicin (1 nm shift), but was unchanged by PB pretreatment (Table 1). Relative to control values, PB decreased the 455/430 nm absorbance ratio of the reduced ethylisocyanide difference spectrum (measured at pH 7.8) while rifampicin, PCN or BNF pretreatment increased this ratio, albeit by different amounts.

Effects on mixed function oxygenation. Pretreatment with the four enzyme inducing agents affected the *in vitro* mixed-function oxygenation of the model substrates ethylmorphine, acetanilide and benzo[α]pyrene (Fig. 1) and the endogenous substrate testosterone (Fig. 2) to a variable extent. Relative to control activities, rifampicin more than doubled the rate of ethylmorphine demethylation and benzo[α]pyrene hydroxylation, but decreased acetanilide hydroxylase activity (Fig. 1). PCN produced a qualitatively similar induction profile, but the

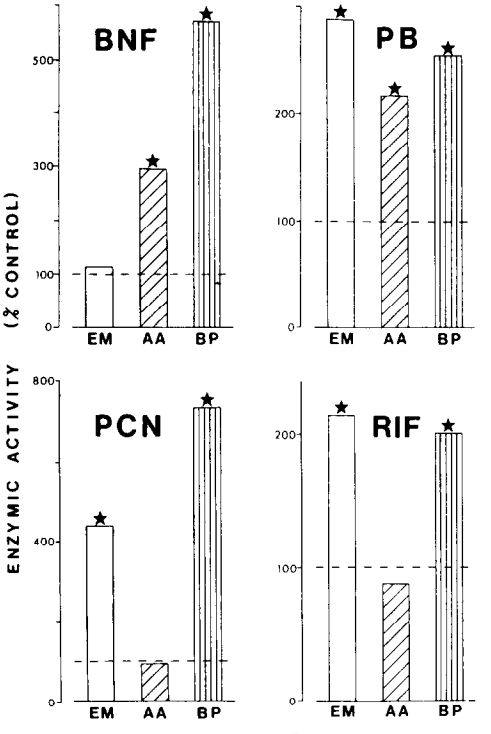

Fig. 1. Effect of pretreatment with β-naphthoflavone, phenobarbitone, pregnenolone 16α-carbonitrile and rifampicin on mixed-function oxidase activities in mouse liver microsomes. Each value is the mean of four separate determinations (associated S.E. < 10%) and is presented as a percentage of the corresponding activity in control animals pretreated with vehicle alone. Enzyme activities were determined as described in Methods and in control animals were, for ethylmorphine *N*-demethylation (EM, □): 3.54 ± 0.30 nmoles formaldehyde produced/mg microsomal protein/min; for acetanilide hydroxylation (AA, ▨): 2.65 ± 0.07 nmoles paracetamol produced/mg microsomal protein/min; for benzo[α]pyrene hydroxylation (BP, ▥): 3.09 ± 0.33 RF units produced/mg microsomal protein/min (mean ± S.E., n = 16). *Significantly different from the corresponding control ($P < 0.05$; Student's t-test).

Table 1. Effects of pretreatment with rifampicin, β-naphthoflavone, phenobarbitone and pregnenolone 16α-carbonitrile on various parameters associated with hepatic drug metabolism in C57 Bl 6 mice

	Relative liver weight	Microsomal protein	NADPH-cytochrome *c* reductase	Cytochrome P-450		Ethyl isocyanide difference spectrum	
				Content	Absorbance maximum	Peak ratio	Absorbance maximum
Pretreatment	g/100g body wt	mg/g liver	nmoles cyt. *c*. reduced/ mg mic. prot./min	nmoles/mg mic. prot.	nm	$A_{455-430}$	nm
HCl	5.37 ± 0.07	26.9 ± 0.6	112 ± 3	0.68 ± 0.03	450	0.83	455
Rifampicin	5.58 ± 0.05	27.9 ± 0.3	171 ± 6*	1.02 ± 0.03*	449–450	0.89	454
Corn oil	5.45 ± 0.05	25.8 ± 0.8	110 ± 7	0.44 ± 0.03	450	(0.83)	(455)
β-Naphthoflavone	5.43 ± 0.05	26.5 ± 0.6	136 ± 4*	0.83 ± 0.04*	448	1.48	452
Saline	5.12 ± 0.12	28.1 ± 0.5	140 ± 3	0.58 ± 0.02	450	(0.83)	(455)
Phenobarbitone	5.89 ± 0.07*	36.4 ± 2.3*	274 ± 10*	1.26 ± 0.12*	450	0.74	455
Corn oil	4.83 ± 0.07	23.2 ± 0.8	150 ± 3	0.57 ± 0.02	450	(0.83)	(455)
Pregnenolone 16α-carbonitrile	5.82 ± 0.09*	29.0 ± 0.7*	283 ± 15*	1.54 ± 0.01*	449–450	1.15	452

Pretreatment regimens and biochemical determinations were described under Methods. Values relating to ethylisocyanide difference spectra are the average of two determinations; only one set of control (no inducer) data was obtained and other control values, in parentheses, show this data repeated for comparative purposes. Cytochrome P-450 absorbance maxima show the mean or range of four determinations. All other results are the mean ± S.E. of four determinations.

* Significantly different from the appropriate control ($P < 0.05$; Student's *t*-test).

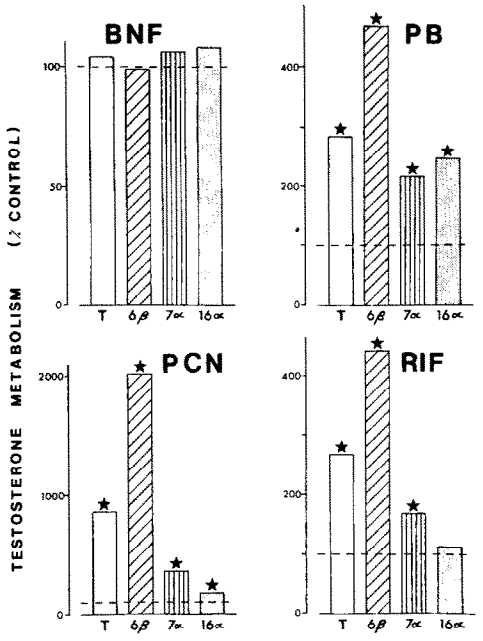

Fig. 2. Effects of pretreatment with β-naphthoflavone, phenobarbitone, pregnenolone 16α-carbonitrile and rifampicin on testosterone metabolism in mouse liver microsomes. Each value is the mean of four separate determinations (associated S.E. < 10%) and is presented as a percentage of the corresponding activity in control animals pretreated with vehicle alone. The parameters determined (as described under Methods) with their associated control activities (nmoles/mg microsomal protein/min; mean ± S.E.; n = 16), were: disappearance of testosterone (T, □; 5.04 ± 0.23); production of 6β-hydroxytestosterone (6β, ▨; 1.74 ± 0.14); production of 7α-hydroxytestosterone (7α, ▥; 0.69 ± 0.03; production of 16α-hydroxytestosterone (16α, ▧; 0.84 ± 0.05). *Significantly different from the corresponding control ($P < 0.05$; Student's t-test).

increases in ethylmorphine N-demethylase activity (four-fold) and benzo[α]pyrene hydroxylase activity (seven-fold) were greater than those observed using rifampicin. BNF substantially increased the microsomal hydroxylations of benzo[α]pyrene (six-fold) and acetanilide (three-fold), but had no effect on ethylmorphine demethylation. In contrast, PB pretreatment increased all three enzyme activities two- to three-fold relative to the controls.

Testosterone catabolism was not significantly affected by BNF, but was considerably increased following pretreatment with rifampicin, PB or PCN (Fig. 2). This effect was largely achieved by substantial increases (four- to twenty-fold) in the activity of testosterone 6β-hydroxylase. Rifampicin and PCN induced this enzyme activity relative to those of the remaining mixed-function oxidases by greater amounts than PB. In addition, rifampicin and PCN induced testosterone 7α-hydroxylation more than 16α-hydroxylation, while PB pretreatment affected both activities similarly.

Effects of mixed-function oxidase inhibitors and activators. In microsomal preparations from control mice (pretreated with vehicle alone), additions of 0.1 mM α-naphthoflavone (ANF) inhibited ethylmorphine N-demethylase (by 26–42%) and acetanilide 4-hydroxylase (by 65–76%), but increased benzo[α]pyrene hydroxylase activities (by 59–187%) (Fig. 3). The inhibition of ethylmorphine demethylase and acetanilide hydroxylase activities was augmented by BNF pretreatment, decreased by PB pretreatment, but unaffected by pretreatment with rifampicin or PCN. In contrast, the activation of benzo[α]pyrene activity by ANF was enhanced following rifampicin or PCN pretreatment, was unaffected by PB but was transformed into an inhibition following BNF pretreatment.

The extent of the inhibition observed using 0.1 mM SKF 525A with control microsomal preparations was greatest for ethylmorphine demethylation (60–71%),

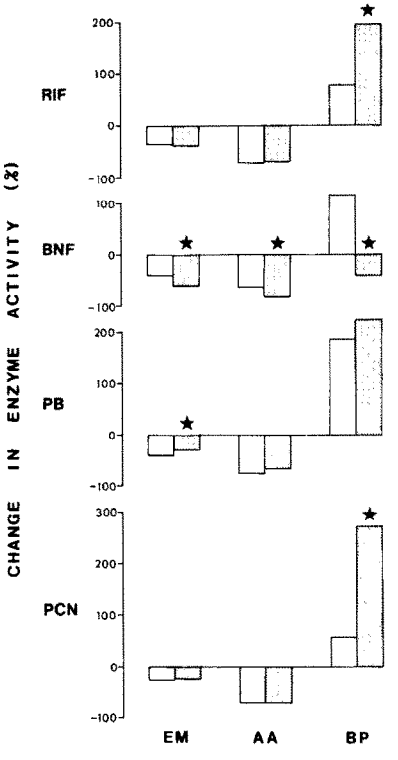

Fig. 3. Effect of α-naphthoflavone (0.1 mM) on mixed-function oxidase activities in liver microsomes from mice pretreated with β-naphthoflavone (BNF), phenobarbitone (PB), pregnenolone 16α-carbonitrile (PCN) or rifampicin (RIF). Activities measured in the presence of α-naphthoflavone using preparations from animals pretreated with vehicle alone (□) or enzyme inducing agent (▩) are presented as a percentage change relative to the activity obtained in the presence of acetonitrile (solvent for α-naphthoflavone) alone. Each value represents the mean (associated S.E. < 15%) of four determinations made as described under Methods for ethylmorphine N-demethylation (EM), acetanilide hydroxylation (AA) and benzo[α]pyrene hydroxylation (BP). Specific enzyme activities in preparations containing no α-naphthoflavone can be derived from the data in Fig. 1. Acetonitrile affected these activities only marginally. All values are significantly different from those in the corresponding incubations containing acetonitrile but no α-naphthoflavone ($P < 0.05$; Student's paired t-test). *Significant difference between the effect of α-naphthoflavone in microsomes from mice pretreated with inducer or the appropriate vehicle alone ($P < 0.05$; Mann-Whitney 'U' test).

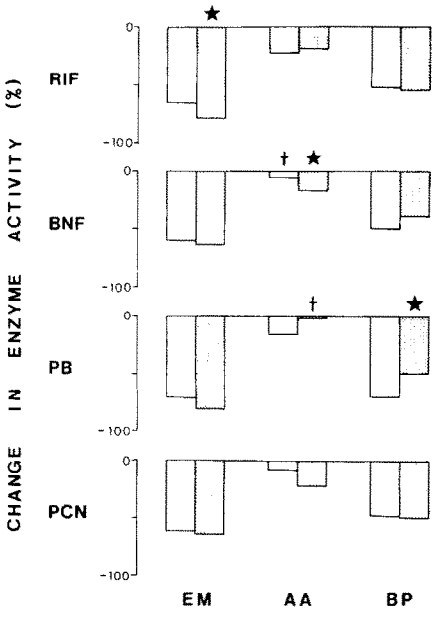

Fig. 4. Effects of SKF 525A (0.1 mM) on mixed-function oxidase activities in liver microsomes from mice pretreated with β-naphthoflavone (BNF), phenobarbitone (PB), pregnenolone 16α-carbonitrile (PCN) or rifampicin (RIF). The percentage change of the mean of each activity (associated S.E. < 15%; $n = 4$) is presented relative to the mean obtained in the absence of the modifier used. Other details have been given in Fig. 3. *Significant difference between the effect of SKF 525A in microsomes from mice pretreated with inducer or the appropriate vehicle alone ($P < 0.05$; Mann-Whitney 'U' test). †Unless so indicated, values were significantly different from the corresponding activity obtained in the absence of SKF 525A ($P < 0.05$; Student's paired t-test).

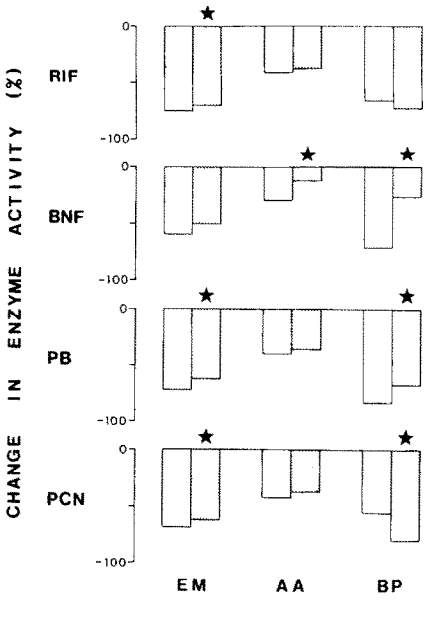

Fig. 5. Effects of metyrapone (0.5 mM) on mixed-function oxidase activities in liver microsomes from mice pretreated with β-naphthoflavone (BNF), phenobarbitone (PB), pregnenolone 16α-carbonitrile (PCN) or rifampicin (RIF). The legend to Fig. 4 applies. All values are significantly different from those in the corresponding incubations containing no metyrapone ($P < 0.05$, Student's paired t-test). *Significant difference between the effect of metyrapone in microsomes from mice pretreated with inducer or the appropriate vehicle alone ($P < 0.05$; Mann-Whitney 'U' test).

less for benzo[α]pyrene hydroxylation (47–70%) and least for acetanilide hydroxylation (5–22%) (Fig. 4). Following pretreatment with any of the four enzyme inducing agents, the pattern of inhibition of the three enzyme activities relative to each other was unchanged. However, SKF 525A showed significantly greater inhibition of ethylmorphine demethylase activity after rifampicin pretreatment and of acetanilide hydroxylase activity after BNF pretreatment, whereas it was a less effective inhibitor of benzo[α]pyrene hydroxylase activity after PB pretreatment.

Metyrapone inhibited ethylmorphine demethylase and benzpyrene hydroxylase activities more than acetanilide hydroxylase activity in preparations from control mice (Fig. 5). Following pretreatment with all the inducers, the inhibition of ethylmorphine demethylase and acetanilide hydroxylase activities by metyrapone was less marked. Benzo[α]pyrene hydroxylase activity showed a differential response to metyrapone inhibition following pretreatment. After rifampicin and PCN the inhibition was enhanced, but after BNF and PB inhibition was less marked.

In control microsomal preparations, NaF inhibited ethylmorphine demethylase and benzo[α]pyrene hydroxylase activities (15–59%), but increased acetanilide hydroxylation 21–37% (Fig. 6). NaF also

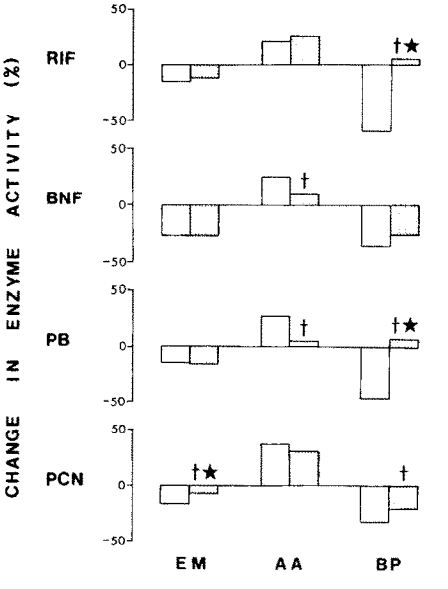

Fig. 6. Effects of NaF (100 mM) on mixed-function oxidase activities in liver microsomes from mice pretreated with β-naphthoflavone (BNF), phenobarbitone (PB), pregnenolone 16α-carbonitrile (PCN) or rifampicin (RIF). The legend to Fig. 4 applies. *Significant difference between the effect of NaF in microsomes from mice pretreated with inducer or the appropriate vehicle alone ($P < 0.05$; Mann-Whitney 'U' test). †Unless so indicated, values are significantly different from the corresponding activity obtained in the absence of NaF ($P < 0.05$; Student's paired t-test).

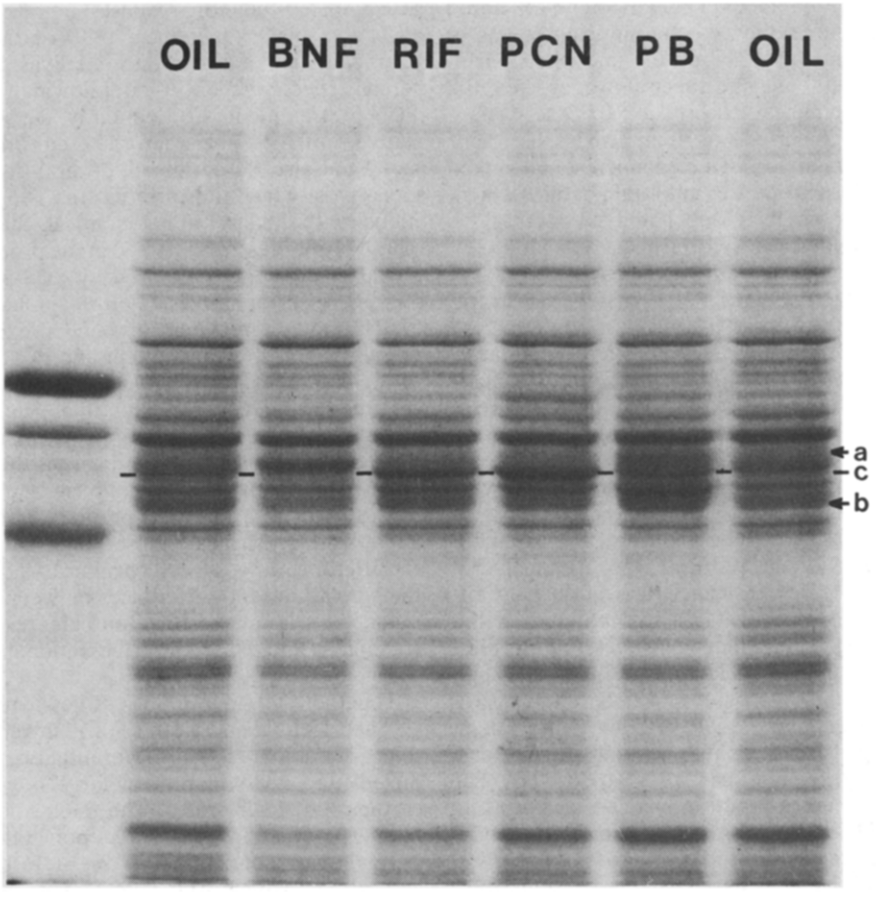

Fig. 7. SDS-Polyacrylamide gel electrophoresis of liver microsomal fractions isolated from mice pretreated with corn oil (OIL), β-naphthoflavone (BNF), phenobarbitone (PB), pregnenolone 16α-carbonitrile (PCN) or rifampicin (RIF). The methods involved in the preparation, running and staining of gels were described under Methods. The left-hand track contained the standards previously described (Methods). The markings 'a' and 'b' denote the position of components with mol. wts of 54,000 and 46,000, respectively, while 'c' marks the position of the 52,000 mol. wt polypeptide band referred to in the text.

stimulated acetanilide hydroxylase activity after pretreatment with rifampicin or PCN, but not after PB or BNF. Similarly, NaF had no significant effect on benzo[α]pyrene hydroxylase activity after pretreatment with rifampicin, PCN or PB, nor on ethylmorphine demethylase activity after PCN.

SDS-Polyacrylamide gel electrophoresis. The electrophoretic profiles of microsomal fractions isolated from mice pretreated with corn oil, BNF, PB, PCN or rifampicin are shown in Fig. 7. Electrophoretograms of HCl (vehicle for rifampicin) or saline-pretreated preparations are not shown since they were comparable with those obtained using corn oil pretreatment.

Since equal amounts of protein were applied to each track, changes in the relative intensity of different bands can be considered to reflect comparable changes in the proportions of the integral polypeptide components. Thus, following induction, there were changes in the relative proportions of various polypeptide components, especially in the mol. wt range 46,000 (marked 'b') to 54,000 (marked 'a'). Particularly of note was the polypeptide band of mol. wt 52,000 (marked 'c') which was substantially induced only following pretreatment with rifampicin and PCN. In contrast, BNF induced a band of mol. wt > 52,000 (above 'c') which was not apparent after rifampicin or PCN. Although less obvious on the photograph than on the original gel, two comparable high mol. wt bands were induced by PB. The profile of the polypeptide components in the 46,000–52,000 mol. wt range (i.e. between 'b' and 'c') was also modified differently depending on the inducer used. Relative to control preparations, rifampicin pretreatment appeared to have little effect on the intensity of these polypeptide bands, but, in contrast, their intensity was generally decreased by BNF pretreatment. PCN induced some increases in intensity, but these were less marked than those noted following PB pretreatment.

DISCUSSION

Despite widespread observations that rifampicin is an inducer of the hepatic microsomal mixed-function oxidase system both in man [2–9] and in experimental animals [10–12], the characteristics associated with enzyme induction by the drug are poorly

defined. Recently, Heubel and Netter [12] noted several differences between the inducing effects of rifampicin, 3-methylcholanthrene and phenobarbitone on the metabolic and spectral properties of the mouse microsomal mixed function oxidase system. By comparing the effects of rifampicin, BNF, PB or PCN pretreatment on selected microsomal mixed function oxidase properties in a strain of mice which, unlike those used by Heubel and Netter [12], is responsive to polycyclic hydrocarbon induction [32], we have more clearly defined the characteristics of rifampicin induction and the types of P-450 substrates that may be most sensitive to its inducing effects.

Rifampicin induction showed several qualitative similarities with that following PCN pretreatment, and marked differences from the effects of PB or BNF. Thus, both rifampicin and PCN failed to induce acetanilide hydroxylase activity, but markedly increased testosterone 6β-hydroxylase activity by an amount which was relatively greater than that of other P-450 substrates. The slight shift of the absorbance maximum of the reduced cytochrome P-450–CO complex to lower wavelengths following rifampicin pretreatment was also comparable to that noted in PCN-pretreated mice (Table 1) or rats [33]. While in agreement with Heubel and Netter [12] on the extent of this hypsochromic shift following rifampicin pretreatment, we have been able to demonstrate that it is different from that obtained following polycyclic hydrocarbon pretreatment. The responses of mixed-function oxidase activities to ANF and NaF following rifampicin pretreatment were also more similar to those produced by PCN than PB or, especially, BNF. Comparable similarities were noted using SKF 525A and metyrapone, but the inhibiting effects of these compounds in the present study differed from those obtained by other workers in rats [15, 16, 34, 35]. Thus, in agreement with the results of Mull *et al.* using metyrapone in mice [36], we could detect no augmented effect of SKF 525A or metyrapone on enzyme activities induced by PB, although a reduced efficacy against activities induced by polycyclic hydrocarbons was observed. The reasons for these species differences in the effects of SKF 525A and metyrapone are presently unclear, but may reflect an ability to interact with cytochromes P-450 induced by PB to a dissimilar extent in rats and mice.

Despite the qualitative similarities between the inducing properties of rifampicin and PCN, the latter was a more effective inducer. In the absence of absolute differences between inducing agents, such characteristics have been associated with enzyme induction mediated by common mechanisms whose expression is dependent on relative potency [37]. The failure of rifampicin, but ability of PCN, to increase relative liver weight and microsomal protein content in the present study could therefore be explained by differences in potency, particularly since previous reports on the effects of rifampicin on these parameters suggest that dose may be important [11, 12]. However, other differences between the inducing characteristics of rifampicin and PCN are less easily explained on this basis. Thus, it is less likely that differences in potency could underlie the failure of high doses of rifampicin to affect mixed-function oxidases in rats [11] where PCN is a powerful inducer [18], or the dissimilar characteristics of the reduced ethylisocyanide difference spectra we observed following rifampicin or PCN pretreatment. Consequently, despite the qualitative similarities between the effects of rifampicin and PCN already discussed, several distinct differences exist which indicate that the two compounds induce with similar, but not identical, characteristics. These properties are reflected in the profile of microsomal components separated by SDS-polyacrylamide gel electrophoresis. For example, while the 52,000 mol. wt polypeptide band was only preferentially induced by rifampicin and PCN, the extent of the induction achieved was greater following pretreatment with PCN than with rifampicin. Moreover, there were other differences in the relative proportions of the polypeptide bands in the 46,000–52,000 mol. wt range by which rifampicin and PCN could be differentiated. If, as our preliminary results using haem staining suggest, these bands can be related to different forms of cytochrome P-450, then both the similarities and differences between the characteristics of the mixed-function oxidases induced by rifampicin and PCN could be rationalised on this basis.

Together with the findings of Pessayre and Mazel [11] and Heubel and Netter [12], our results suggest that rifampicin may preferentially induce the mixed-function oxidation of large polycyclic cytochrome P-450 substrates. The implications of these findings in relation to the large, polycyclic structural characteristics of rifampicin merit further investigation, but apparently endorse Conney's original concept of structure as an important determinant of inducing characteristics [38]. The considerable induction of testosterone 6β-hydroxylation observed in rifampicin- and PCN-pretreated mice reflects comparable effects on steroid hydroxylases in other laboratory animals [10, 39, 40]. In man also, rifampicin affects the hydroxylation of ethinyloestradiol [31], mestranol [41] and cortisol [42]. Together, these observations suggest that steroid hydroxylations may be particularly susceptible to induction by rifampicin and emphasise the necessity for selecting appropriate dosages of glucocorticoids and oral contraceptives during rifampicin therapy in man.

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