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STUDIES ON 5,5-DIPHENYLHYDANTOIN IRREVERSIBLE BINDING TO RAT LIVER MICROSOMAL PROTEINS

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Abstract—5,5-Diphenylhydantoin irreversibly binds to rat liver microsomes and the process requires NADPH and O_2. Protein binding was significantly enhanced when experiments were carried out with liver microsomal preparations from β-naphthoflavone and 3-methylcholanthrene pretreated animals whereas pretreatment with phenobarbital significantly reduced it. Carbon monoxide, β-diethylaminoethyl-diphenylpropylacetate and glutathione inhibited drug covalent binding to microsomal proteins. In contrast, enhanced drug binding was observed when trichloropropene oxide and cyclohexene oxide, two epoxide hydrolase inhibitors, were added to the incubation mixture. 5,5-Diphenylhydantoin *in vitro* metabolism was quantitatively determined by gas liquid chromatography with selected ion monitoring. A good correlation seems to exist between drug covalent binding and the microsomal process of 5,5-diphenylhydantoin hydroxylation to 5-(4-hydroxyphenyl)-5-phenylhydantoin. The results presented support a previous hypothesis on the intermediacy of arene oxides in the biotransformation of this drug.

5,5-Diphenylhydantoin (DPH) is a drug mainly used in the treatment of convulsive disorders [1, 2]. The biotransformation of DPH has been the object of extensive studies both in laboratory animals and man [3]. DPH is reportedly metabolized following two main pathways, one involving the hydantoin moiety of the molecule with formation of diphenylhydantoic acid and α-aminodiphenylacetic acid, the other occurring at the aromatic rings with formation of several hydroxylated products such as 5-(3,4-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin (DHPH), 5-(3-hydroxyphenyl)-5-phenylhydantoin, 5-(4-hydroxyphenyl)-5-phenylhydantoin (HPPH), 5-(3,4-dihydroxyphenyl)-5-phenylhydantoin (DHP) and 5-(3-methoxy-4-hydroxyphenyl)-5-phenylhydantoin (MOPH) [4–9].

The identification of DHPH led to the hypothesis that an arene oxide intermediate, namely 5-(phenyl-3,4-oxide)-5-phenylhydantoin, might be formed in DPH metabolism. It is known that arene oxides represent highly electrophilic species able to bind irreversibly to cellular molecules and macromolecules. Interest in the mechanisms of formation of these metabolic intermediates and in their chemical reactivity towards cell constituents prompted us to investigate the *in vitro* covalent binding and hydroxylation of this drug.

MATERIALS AND METHODS

Chemicals. DPH, HPPH and 5-(4-methylphenyl)-5-phenylhydantoin (MPPH) were obtained from Aldrich (Beerse, Belgium). DHP, as reference material, was extracted with chloroform at pH 4.5 from the urine of DPH treated rats and first purified by preparative thin layer chromatography (TLC). Further purification was achieved by high performance liquid chromatography (HPLC) using a reversed-phase column (0.25 m × 2.6 mm) packed with 10 μm (average particle diameter) LiChrosorb RP-18 (Merck, Darmstadt, West Germany). The column was eluted with a mixture of acetonitrile and acetate buffer 0.02 M, pH 4.5 (40:60, v/v). [4-^{14}C]DPH (sp. act. 54.5 mCi/mmole) was obtained from the Radiochemical Centre (Amersham, U.K.). The radiochemical purity of labelled DPH was checked by HPLC as described above. The compound was found to be more than 99% pure.

Phenobarbital (PB) was obtained from Merck (Darmstadt, West Germany); 3-methylcholanthrene (3-MC) from Sigma (St. Louis, MO); β-naphthoflavone (BNF), cyclohexene oxide (CyO) and 1,2-epoxy-3,3,3-trichloropropene (TCPO) from Aldrich; β-diethylaminoethyl-diphenyl-propylacetate (SKF-525A) from Smith, Kline and French Laboratories (Philadelphia, PA). The following reagents were used: glucose-6-phosphate, glucose-6-phosphate dehydrogenase, nicotinamide adenine dinucleotide phosphate (NADP) and reduced glutathione (GSH), Boehringer (Mannheim, West Germany); trideuteromethyl iodide, CEA (Gif-sur-Yvette, France); tetrahexylammonium hydrogen sulphate, Hässle (Goteborg, Sweden); and Tri-Deuter-8®, Pierce (Rockford, IL). All other chemicals and solvents were of the purest grade commercially available.

Animals. Male CD-COBS rats (200–220 g, body wt) from Charles River Italy (Calco, Como, Italy) were used. Five groups, each consisting of ten animals, were pretreated respectively with 0.9% saline (0.5 ml, twice daily for three days), PB (40 mg/kg, i.p. in 0.5 ml of 0.9% saline, twice daily for three days), corn oil (0.5 ml, once daily for three days),

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BNF (60 mg/kg, i.p. in 0.5 ml of corn oil, once daily for two days) and 3-MC (40 mg/kg, i.p. in 0.5 ml of corn oil, once daily for three days). Animals were fasted for 24 hr before killing.

Preparation of microsomes. Untreated animals and the groups of ten pretreated rats were used for the preparation of separate microsomal batches. The animals were killed by decapitation and the livers were excised, pooled and washed with 0.05 M phosphate buffer (pH 7.4). The livers were minced and homogenized at 0° in 4 vol. of cold 0.05 M phosphate buffer (pH 7.4) using a Potter–Elvehjem homogenizer with a Teflon pestle. Microsomes were prepared according to Kato and Takayanaghi [10]. The protein concentration was determined by the method of Lowry *et al.* [11] with bovine serum albumin fraction V as the standard. Cytochrome P-450 content was measured as described by Omura and Sato [12].

Covalent binding of [4-^{14}C]*DPH to microsomal proteins.* The incubation mixture in a final volume of 2 ml contained an NADPH regenerating system (2.5 μmole of $NADP^+$, 50 μmole of glucose-6-phosphate and two units of glucose-6-phosphate dehydrogenase), 0.05 M phosphate buffer (pH 7.4), 5 mM magnesium chloride, 0.15 M potassium chloride and 10 mg of microsomal protein. The different inhibitors used, and GSH, were added to the incubation medium at a concentration of 1 mM. After 5 min of preincubation at 37° the reaction was started by the addition of 10 μl of an ethanol solution of labelled DPH to obtain a final substrate concentration of 0.1 mM. The labelled DPH used in these experiments was diluted with the cold drug to obtain a specific activity of 2 mCi/mmole.

Incubation was at 37° for 2 hr in subdued light. The reaction was stopped by the addition of 5 ml of ethanol. The samples were centrifuged, the supernatant was discarded and the precipitate was extracted three times with 5 ml vol. of the following solvent sequence: 70% ethanol, three times with ethanol, boiling ethanol, benzene/ethanol (1:4, v/v), twice with acetone/chloroform (4:1, v/v), acetone/ethanol (5:1, v/v), diethyl ether/ethanol (5:1, v/v), twice with ethyl acetate/ethanol (5:1, v/v), ethanol and ethanol/water (2:3, v/v). All the extraction supernatants were tested for radioactivity. After the last cycle of extraction no further radioactivity could be removed from the proteins.

This exhaustive extraction thus removed the original DPH, as well as all metabolites reversibly bound to proteins. Protein loss was very low during the procedure. The precipitate was dissolved in 0.5 ml of Soluene®-350, Packard (Downers Grove, IL,) and transferred into a counting vial containing 10 ml of a PPO-POPOP solution in toluene. Radioactivity was measured in a Nuclear Chicago Isocap 300 liquid scintillation counter. The values obtained were corrected for quenching by the external standardization method.

Quantitative determination of HPPH in microsomal preparations. In parallel to covalent binding studies, experiments were carried out to investigate DPH *in vitro* hydroxylation. Microsomes were incubated with cold DPH (0.1 mM) under the same conditions as described above.

HPPH and DHP were quantitatively evaluated according to Arboix and Pantarotto [13]. The procedure consists of a salt–solvent pair method where DPH and some of its metabolites are converted to their per-trideuteromethylated derivatives and assayed by gas liquid chromatography (GLC) with selected ion monitoring. After 2 hr incubation, the microsomal mixtures were transferred to hermetically sealable glass tubes bearing a Teflon disc in the inner part of the cap and 500 ng of MPPH as internal standard were added. The samples were extracted with 5 ml of 0.5 M trideuteromethyl iodide methylene chloride solution after addition of 100 μl of 10 N NaOH and 50 μl of 0.1 M tetrahexylammonium hydrogen sulphate solution in 0.1 N NaOH. Extractions were carried out for 2 hr by shaking at 60° in subdued light. The organic phase (4.5 ml) was transferred to conical glass tubes and evaporated to dryness under a gentle stream of nitrogen in a water bath at 35°. The residue was resuspended in 50 μl of methylene chloride and the tetrahexylammonium salt was precipitated by addition of 2 ml of n-hexane. The tubes were capped and centrifuged for 5 min at 4000 *g*. The supernatant fraction (1.8 ml) was transferred to conical glass tubes and evaporated to dryness under a nitrogen stream at 35°. The residue was dissolved in Tri-Deuter-8® and a 1–5 μl portion of this solution was injected for gas chromatography with selected ion monitoring. Tri-Deuter-8® was used in order to achieve complete derivatization of any DHP metabolite formed.

Selected ion monitoring was carried out on a LKB mass spectrometer, model 2091 B, equipped with a gas chromatograph and a computer system, model 2130, for data acquisition and calculation, which was used under the following conditions: oven temperature, 250°; injection port heater temperature, 270°; carrier gas (helium) flow rate, 30 ml/min; energy of the ionization beam, 70 eV; ion source temperature, 290°; accelerating voltage, 3.5 kV; trap current, 60 μA.

The gas chromatographic column was a glass tube, 2 m long and 4 mm i.d., packed with 100–120 mesh Gas-Chrom Q coated with 3% OV 17. For determinations the instrument was focused on the ions at *m/e* 286, 300, 319 and 352, corresponding to the molecular ions in the spectra of the per-trideuteromethylated derivatives of DPH, MPPH, HPPH and DHP respectively. Interference from endogenous substrates was never observed.

This method can be considered precise as shown by the low percentage of coefficient of variation (% CV) calculated as S.D./mean × 100 (see Table 1).

Table 1. Precision of the gas chromatographic-selected ion monitoring procedure for the determination of DPH, HPPH and DHP in rat liver microsomal preparations

Compound	Concentration (μg/ml) 0.02	0.05	0.25	1
DPH	4.2	3.8	4.6	5.4
HPPH	5.0	6.1	1.8	7.0
DHP	6.3	3.6	7.2	3.9

Values reported represent % CV.
Each figure is the mean ± S.E. of five determinations.

RESULTS

Table 2 shows the results of studies on [4-^{14}C]DPH covalent binding to microsomal proteins in three groups of control rats. Binding was observed only when the drug was incubated in the presence of NADPH, this process being catalysed by an enzymatic activity. With boiled microsomes or in the absence of a NADPH regenerating system [4-^{14}C]DPH covalent binding to proteins did not occur. Furthermore, animal pretreatment with corn oil resulted in slight increase in drug binding compared to that observed when microsomal preparations from saline treated or untreated rats were used.

Table 3 reports the effect of animal pretreatment with known inducers of the different cytochrome P-450 forms, such as PB, BNF and 3-MC, on the *in vitro* irreversible binding of [4-^{14}C]DPH to proteins. Following PB pretreatment the amount of drug metabolites bound to microsomes was significantly lower than in preparations from saline treated controls. However, an increase was observed when microsomes from BNF and especially 3-MC pretreated rats were used. The content of cytochrome P-450 in the different liver microsomal preparations is given in Table 4.

Table 5 illustrates the effect on [4-^{14}C]DPH binding to microsomes when incubations are carried out in the presence of: (i) inhibitors of microsomal mixed function monooxygenases such as 95% CO and SKF-525A; (ii) uncompetitive inhibitors of epoxide hydrolase such as CyO and TCPO; (iii) GSH, a compound known as a scavenger of reactive species. Carbon monoxide and SKF-525A both significantly inhibited irreversible [4-^{14}C]DPH protein binding. A similar effect was observed in experiments where GSH was added to the incubation mixture. Conversely, CyO and TCPO were found to enhance drug covalent binding.

The *in vitro* metabolism of DPH was also quantitatively evaluated by gas chromatography with selected ion monitoring. Figure 1 shows a typical recording obtained from the extract of a microsomal preparation after incubation with DPH. Three peaks are present, corresponding to DPH, MPPH and HPPH; DHP was not detectable probably because it fell below the limit of sensitivity of our procedure (20 ng/sample). 5-(3-4-Dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin(DHPH), if present in the incubation mixture, undergoes chemical degradation under our analytical conditions and, by losing a water molecule, is converted to the fully aromatic and stable HPPH. Therefore the HPPH concentrations determined represent the sum of HPPH itself and DHPH.

Table 6 shows the results of these experiments. DPH was metabolized to a limited extent by rat liver microsomes, the only detectable metabolite (HPPH) corresponding to about 2% of the amount of the drug incubated. Significantly higher HPPH concentrations were found after DPH incubation with microsomes from BNF and 3-MC pretreated rats. Pretreatment with PB did not result in any appreciable change in the amount of HPPH present in the medium with respect to the concentrations found in the microsomal preparations from untreated, saline treated and corn oil treated animals.

DISCUSSION

A large number of exogenous compounds are known to be transformed, *in vitro* and *in vivo*, into chemically reactive intermediates able to bind irreversibly to cellular macromolecules such as proteins and nucleic acids. These 'metabolic activation processes' are in general catalysed by enzymes present in the cell endoplasmic reticulum and, to a lesser extent, also in the nuclear membrane. The study of how a chemical binds covalently to cellular macromolecules may offer useful information for a better characterization of the metabolic intermediate(s) and enzymatic system(s) involved in these interactions.

In the present paper a covalent interaction between [4-^{14}C]DPH and proteins of the endoplasmic reticulum has been demonstrated. We have shown that drug covalent binding is mediated by an enzymatic process; NADPH is required for metabolic activation and SKF-525A and CO significantly reduced the extent of [4-^{14}C]DPH binding by inhibiting the activity of microsomal mixed function monooxygenases. This enzymatic activity was found to be cytochrome P-448 dependent; [4-^{14}C]DPH binding to microsomes was in fact increased when incubations were carried out with preparations from BNF and 3-MC pretreated animals. Nevertheless, since the measurement of cytochrome P-448 in our experimental conditions relies on determination of the light absorption maximum at 448 nm of the reduced CO-complex, possible involvement of other, differently inducible cytochrome P-450 forms cannot be excluded [14]. More thorough study of the types of enzymes catalysing DPH metabolic activation to reactive intermediates should therefore use other techniques, such as electrophoresis, which we are at present setting up in our laboratory.

The chemical species involved in this interaction is probably an electrophilic arene oxide since GSH, added to the incubation medium, reduced the extent of [4-^{14}C]DPH covalent binding to proteins whereas CyO and TCPO significantly enhanced it by inhibiting the inactivating epoxide hydrolase activity. For a correct interpretation of these covalent binding results, a parallel evaluation of the metabolic pathway thought to be implicated in this interaction was also carried out.

From the known metabolism of DPH, summarized in Scheme 1, we can reasonably assume that arene oxide metabolites represent intermediates in drug hydroxylation to phenolic, dihydrodiol and catechol derivatives. It is thus clear that measuring HPPH and, if possible, DHP levels in the incubation media was an interesting way of possibly correlating covalent binding studies with quantitative information on the *in vitro* metabolism of this drug. DPH was found to be only poorly metabolized by rat liver microsomes, but our results show a good relationship between the pattern of DPH hydroxylation to HPPH and the extent of drug covalent binding. The two processes are in fact catalysed by a similar enzymatic system.

Furthermore, if we consider the percentage of DPH and metabolites irreversibly bound to microsomes and the percentage of the drug converted to HPPH, the arene oxide intermediate shows

Table 2. Irreversible protein binding of [4-^{14}C]DPH (0.1 mM) catalysed by liver microsomes (M) from control rats (untreated animals and animals pretreated with either saline or corn oil) in the presence of a NADPH regenerating system after 120 min of incubation*

Treatment	Sample	[4-^{14}C]DPH and metabolites irreversibly bound to 1 mg of microsomal protein (nmole ± S.E.)	Drug and metabolites irreversibly bound as % of drug concentration incubated
Untreated	Boiled M + NADPH + [4-^{14}C]DPH	0.016 ± 0.002	0.08
	M − NADPH + [4-^{14}C]DPH	0.015 ± 0.001	0.07
	M + NADPH + [4-^{14}C]DPH	0.163 ± 0.005†	0.81
Saline	Boiled M + NADPH + [4-^{14}C]DPH	0.015 ± 0.001	0.07
	M − NADPH + [4-^{14}]DPH	0.023 ± 0.001	0.11
	M + NADPH + [4-^{14}C]DPH	0.161 ± 0.008†	0.80
Corn oil	Boiled M + NADPH + [4-^{14}C]DPH	0.016 ± 0.001	0.08
	M − NADPH + [4-^{14}C]DPH	0.019 ± 0.001	0.09
	M + NADPH + [4-^{14}C]DPH	0.200 ± 0.011†	1.00

* Each figure is the mean ± S.E. of five determinations.
† $P < 0.01$, compared with the respective control values (boiled M + NADH + [4-^{14}C]DPH and M − NADPH + [4-^{14}C]DPH).

Table 3. Effect of rat pretreatment with PB, BNF or 3-MC on the irreversible protein binding of [4-^{14}C]DPH (0.1 mM) catalysed by liver microsomes (M) in the presence of a NADPH regenerating system after 120 min of incubation*

Treatment	Sample	[4-^{14}C]DPH and metabolites irreversibly bound to 1 mg of microsomal protein (nmole ± S.E.)	Per cent difference from control condition taken as 100%	Drug and metabolites irreversibly bound as % of drug concentration incubated
Saline	M + NADPH + [4-^{14}C]DPH	0.161 ± 0.008	100	0.80
PB in saline	Boiled M + NADPH + [4-^{14}C]DPH	0.018 ± 0.001		0.09
	M − NADPH + [4-^{14}C]DPH	0.019 ± 0.001		0.09
	M + NADPH + [4-^{14}C]DPH	0.111 ± 0.006†	69‡	0.55
Corn oil	M + NADPH + [4-^{14}C]DPH	0.200 ± 0.011	100	1.00
BNF in corn oil	Boiled M + NADPH + [4-^{14}C]DPH	0.016 ± 0.001		0.08
	M − NADPH + [4-^{14}C]DPH	0.020 ± 0.001		0.10
	M + NADPH + [4-^{14}C]DPH	0.262 ± 0.004†	131§	1.31
3-MC in corn oil	Boiled M + NADPH + [4-^{14}C]DPH	0.016 ± 0.001		0.08
	M − NADPH + [4-^{14}C]DPH	0.028 ± 0.002		0.14
	M + NADPH + [4-^{14}C]DPH	0.314 ± 0.010†	157§	1.57

* Each figure is the mean ± S.E. of five determinations.
† $P < 0.01$, compared with the respective control values (boiled M + NADPH + [4-^{14}C]DPH and M − NADPH + [4-^{14}C]DPH).
‡ $P < 0.01$, lower than the saline pretreated group covalent binding value.
§ $P < 0.01$, higher than the corn oil pretreated group covalent binding value.

Table 4. Microsomal cytochrome P-450/448 content in the liver of control rats and rats pretreated with PB, BNF and 3-MC*

Treatment	nmole/mg protein ± S.E.
Untreated	0.69 ± 0.04†
Saline	0.67 ± 0.07†
Corn oil	0.70 ± 0.01†
PB	1.19 ± 0.15†
BNF	1.04 ± 0.18‡
3-MC	1.26 ± 0.11‡

* Each figure is the mean ± S.E. of three determinations.
† Values calculated on the basis of the difference in absorbance at 480 and 450 nm of the reduced CO-complex of these microsomal preparations.
‡ Values calculated on the basis of the difference in absorbance at 480 and 448 nm of the reduced CO-complex of these microsomal preparations.

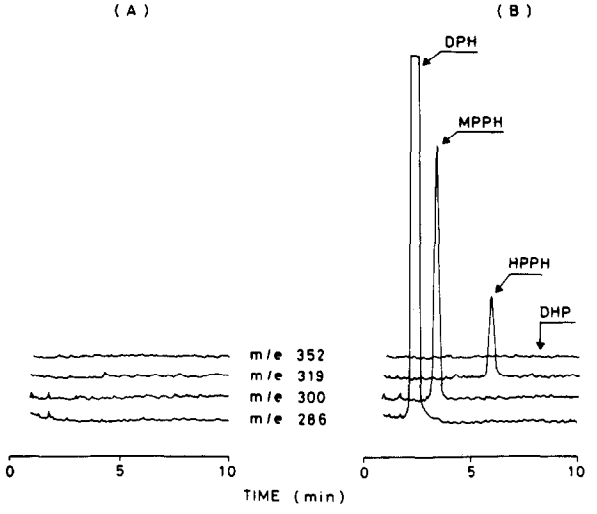


Fig. 1. Selected ion monitorings from extracts of rat liver microsomal preparations. (A) Monitoring of an extract from microsomes incubated for 120 min without DPH. (B) Monitoring of an extract from microsomes incubated for 120 min with DPH.

extremely high chemical reactivity towards the nucleophilic sites of proteins, these values being comparable (see Table 7).

Hydroxylation and irreversible binding seem to correlate well with the degree of drug mutagenicity. *In vitro*, using Ames' test, we found that both DPH and HPPH are weak frame-shift mutagens [15]. A slight but significant increase in the number of revertants per plate was observed only when the drug and its hydroxy-metabolite were incubated with TA 98 and TA 1538 *Salmonella* strains in the presence of an activating system consisting of the supernatant of the 9000 *g* (S-9) fraction from the liver of rats pretreated with BNF, 3-MC and Arochlor 1254. Without S-9 or using preparations from control animals and animals pretreated with PB, no mutagenic effect could be detected.

Table 5. Effect of GSH and inhibitors of drug metabolizing enzymes on the irreversible protein binding of [4-^{14}C]DPH (0.1 mM) catalysed by liver microsomes (M) from untreated rats in the presence of a NADPH regenerating system after 120 min of incubation*

Sample	[4-^{14}C]DPH and metabolites irreversibly bound to 1 mg of microsomal protein (nmole ± S.E.)	Per cent difference from control condition taken as 100%	Drug and metabolites irreversibly bound as % of drug concentration incubated
M + NADPH + [4-^{14}C]DPH	0.163 ± 0.005	100	0.81
M + NADPH + [4-^{14}C]DPH + GSH (1 mM)	0.119 ± 0.009†	73	0.59
M + NADPH + [4-^{14}C]DPH + CO (95%)	0.047 ± 0.006†	29	0.23
M + NADPH + [4-^{14}C]DPH + SKF-525A (1 mM)	0.053 ± 0.005†	32	0.26
M + NADPH + [4-^{14}C]DPH + CyO (1 mM)	0.204 ± 0.006‡	125	1.02
M + NADPH + [4-^{14}C]DPH + TCPO (1 mM)	0.249 ± 0.008‡	153	1.24

* Each figure is the mean ± S.E. of five determinations.
† $P < 0.01$, lower than the control value (M + NADPH + [4-^{14}C]DPH).
‡ $P < 0.01$, higher than the control value (M + NADPH + [4-^{14}C]DPH).

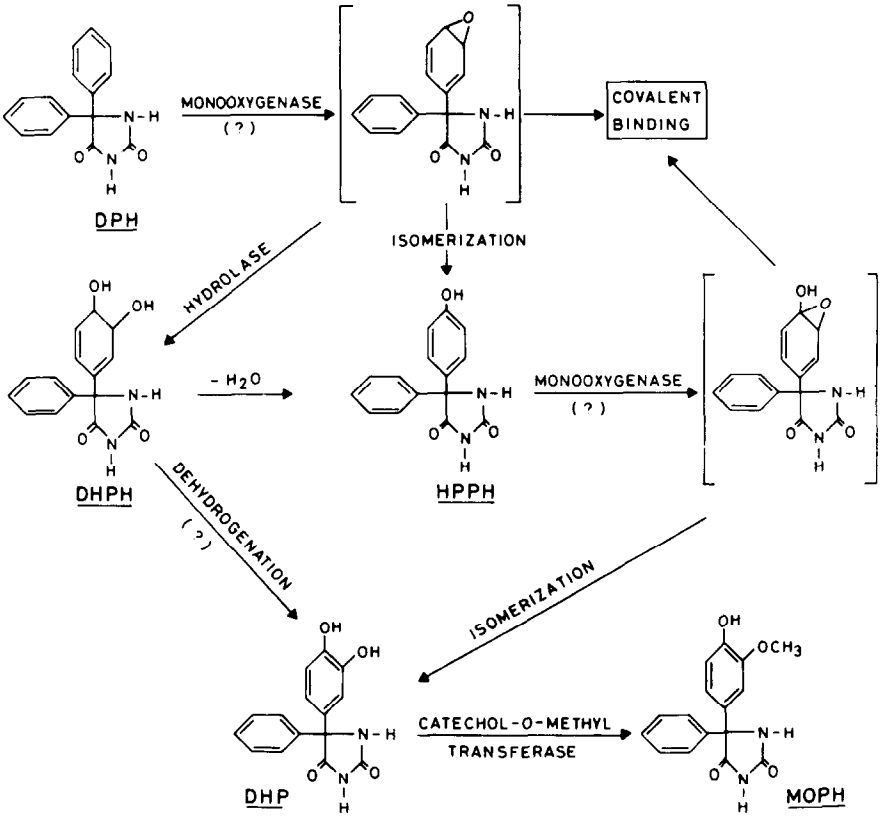
Table 6. Hydroxylation of DPH (0.1 mM) catalysed by liver microsomes from control rats and rats pretreated with different cytochrome P-450/448 inducers in the presence of a NADPH regenerating system after 120 min of incubation*

Treatment	Amount of metabolites produced (μg) Per cent of the DPH concentration incubated HPPH	DHP
Untreated	0.73 ± 0.11	1.44	<0.02
Saline	0.68 ± 0.03	1.35	<0.02
PB in saline	0.58 ± 0.13	1.14	<0.02
Corn oil	0.74 ± 0.09	1.46	<0.02
BNF in corn oil	1.14 ± 0.10†	2.25	<0.02
3-MC in corn oil	1.59 ± 0.21†	3.15	<0.02

* Each figure is the mean ± S.E. of five determinations.
† $P < 0.01$, higher than the HPPH concentration determined after DPH incubation with microsomes from corn oil pretreated animals.

Table 7. Comparison of the irreversible binding of [4-^{14}C]DPH and metabolites to rat liver microsomal proteins and the process of drug hydroxylation to HPPH

Treatment	(A) DPH and metabolites irreversibly bound as % of drug concentration incubated	(B) HPH as % of DPH concentration incubated	B/A
Untreated	0.81	1.44	1.77
Saline	0.80	1.35	1.68
PB in saline	0.55	1.14	2.07
Corn oil	1.00	1.46	1.46
BNF in corn oil	1.31	2.25	1.71
3-MC in corn oil	1.57	3.15	2.00



Scheme 1. Known and postulated DPH *in vivo* metabolism through formation of chemically reactive intermediates.

In the light of these observations it would now be interesting to establish the importance of DPH reactive metabolites in determining toxic effects such as skin reactions, immunological disorders, liver damage, teratogenicity and even carcinogenicity reported in patients under repeated treatment with this drug [16, 17].

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