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THE STIMULATION OF HYDROXYLATION BY CARCINOGENIC AND NON-CARCINOGENIC COMPOUNDS

P. J. CREAVEN and D. V. PARKE

Department of Biochemistry, St. Mary's Hospital Medical School, London, W.2., England

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Abstract—Pretreatment of young rats and mice with certain drugs and polycyclic hydrocarbons stimulates the hydroxylation of biphenyl by liver microsomal preparations. Phenobarbitone, nikethamide and meprobamate, all increase the 4-hydroxylation of biphenyl but have little effect on the 2-hydroxylation. In contrast, the carcinogenic polycyclic hydrocarbons, 3,4-benzpyrene, 20-methylcholanthrene, and 1, 2, 5, 6-dibenzanthracene preferentially stimulate the 2-hydroxylation of biphenyl. Non-carcinogenic polycyclic hydrocarbons such as 1, 2, 6, 7-dibenzpyrene and 2, 3, 6, 7-dibenzanthracene stimulate neither mode of hydroxylation.

Biphenyl is metabolized *in vivo* into 4-hydroxybiphenyl (20 per cent of the dose in rats, 25 per cent in mice) and 2-hydroxybiphenyl (2 per cent in rats, 5·5 per cent in mice). Pretreatment with phenobarbitone produces moderate stimulation of the 4-hydroxylation in rats, but not mice: 2-hydroxylation is unaffected. Pretreatment with benzpyrene produces stimulation of 2-hydroxylation in both species, but does not affect the 4-hydroxylation.

It is suggested that a correlation may exist between the carcinogenicity of polycyclic hydrocarbons and their induction of the hepatic microsomal enzyme responsible for the 2-hydroxylation of biphenyl.

THE RATE of biological oxidation of drugs and other foreign compounds may be increased, both *in vivo* and *in vitro*, by pretreatment of the animals with a variety of compounds.[1, 2] This increase has been attributed to an induction of the microsomal enzyme systems responsible for the metabolism of foreign compounds.[3] Some of the most potent compounds showing this effect are carcinogenic polycyclic hydrocarbons but the relationship, if any, between the inductive effect and carcinogenesis has remained obscure[4] as many non-carcinogenic compounds produce similar stimulation of the microsomal enzymes.[1, 2, 5] We have studied the effect of a number of carcinogenic and non-carcinogenic substances on the rate and pattern of the biological hydroxylation of biphenyl and have shown that the two classes of compound produce different inductive effects. Pretreatment with the carcinogenic polycyclic hydrocarbons stimulates the hydroxylation of biphenyl in the *ortho* position, while non-carcinogenic compounds stimulate *para* hydroxylation. Part of this work has been published previously in a preliminary form.[6]

MATERIALS AND METHODS

Pretreatment of animals

The polycyclic hydrocarbons were administered as a single intraperitoneal dose to

weanling Wistar albino rats (1·0 mg in 0·2 ml arachis oil) and weanling albino mice (I.C.I. strain) (0·5–1·0 mg in 0·1–0·2 ml arachis oil) 24 hr before the experiments. The drugs were administered by repeated injection intraperitoneally or subcutaneously. Control animals received the same dose of arachis oil.

In vitro *experiments*

The pretreated animals were killed, the livers quickly removed and homogenized, and 10,000 *g* supernatant preparations (microsomes + soluble fraction) were prepared.[7] The biphenyl-2- and 4-hydroxylase activities of the liver preparations were determined as previously described.[8]

In vivo *experiments*

Biphenyl (2·0% w/v solution in arachis oil) at a dose level of 100 mg/kg was administered orally and intraperitoneally to pretreated rats, and intraperitoneally to pretreated mice. The urines of rats, and of groups of five mice, were collected for 48 hr, and refluxed with an equal volume of conc. HCl for 3 hr to hydrolyse the conjugated phenols. The 2- and 4-hydroxybiphenyl present in the hydrolysed urines were determined spectrofluorimetrically as previously described.[8]

Identification of biphenyl metabolites

The quantitative spectrofluorimetric determinations of 2- and 4-hydroxybiphenyl were confirmed by separation, identification and semi-quantitative estimation of these two phenols on thin-layer chromatoplates of alumina as previously described.[8]

RESULTS

Hydroxylation in vitro

The effects on hepatic microsomal hydroxylation of pretreating rats with a series of polycyclic hydrocarbons and the drugs phenobarbitone, meprobamate and nikethamide are shown in Tables 1 and 2. The drugs increase the 4-hydroxylation of biphenyl without having any effect on 2-hydroxylation. Of the hydrocarbons, 20-methylcholanthrene and 3, 4-benzpyrene stimulate only 2-hydroxylation; 1:2:5:6-dibenzanthracene, 1:2:3:4-, 1:2:4:5:-, 3:4:9:10- and 3:4:8:9-dibenzpyrenes and 22-methylcholanthrene stimulate both 2- and 4-hydroxylation, but mostly the former. 1:2:6:7-Dibenzpyrene and 2:3:6:7-dibenzanthracene show no stimulation of either mode of hydroxylation. Administration of ethionine (50 mg intraperitoneally to three rats 1 hr before administration of benzpyrene) removes the stimulatory effect of the latter on biphenyl 2-hydroxylation.

When the ratio of 4-hydroxylation/2-hydroxylation is considered, it is seen that on pretreatment with 20-methylcholanthrene or 3, 4-benzpyrene, which stimulate only 2-hydroxylation, the normal value of 5 is reduced to 2. The polycyclic hydrocarbons which stimulate both 2- and 4-hydroxylation show values ranging from 2 to 5. The drugs meprobamate, nikethamide and phenobarbitone increase the ratio to values of 9–13.

In mice a similar pattern is seen (see Table 3). Benzpyrene increases only the 2-hydroxylation of biphenyl and lowers the ratio from 6·5 to 2, whereas phenobarbitone increases 4-hydroxylation and raises the ratio to 16.

TABLE 1. EFFECT OF PRETREATMENT ON THE HYDROXLATION OF BIPHENYL BY RAT LIVER

Pretreatment	Yield of		No. of animals	Percentage stimulation of		Ratio 4-Hydroxylation / 2-Hydroxylation
	4-Hydroxybiphenyl (μmole/g liver/hr)	2-Hydroxybiphenyl (μmole/g liver/hr)		4-Hydroxylation	2-Hydroxylation	
Control	1·55 ± 0·1	0·30 ± 0·04	8	—	—	5·2
20-Methyl cholanthrene	1·50 ± 0·1	0·75 ± 0·05	8	0	150	2·0
1, 2, 5, 6-Dibenzanthracene	3·4 ± 0·1	1·6 ± 0·04	12	120	440	2·1
3, 4, 9, 10-Dibenzpyrene	2·4 ± 0·1	1·1 ± 0·01	12	55	265	2·2
3, 4, 8, 9-Dibenzpyrene	2·75 ± 0·05	1·0 ± 0·02	12	75	230	2·75
22-Methyl cholanthrene	4·2 ± 0·25	1·5 ± 0·05	6	170	400	2·8
1, 2, 3, 4-Dibenzpyrene	2·2 ± 0·1	0·62 ± 0·02	12	40	105	3·5
1, 2, 4, 5-Dibenzpyrene	1·8 ± 0·05	0·51 ± 0·06	3	15	70	3·6
1, 2, 6, 7-Dibenzpyrene	1·35 ± 0·05	0·32 ± 0·03	3	0	0	4·4
2, 3, 6, 7-Dibenzanthracene (pentacene)	1·4 ± 0·3	0·26 ± 0·04	3	0	0	5·5
Meprobamate	2·5 ± 0·25	0·29 ± 0·08	5	60	0	8·8
Nikethamide	3·4 ± 0·1	0·31 ± 0·02	7	120	0	10·7

Weanling male rats (50–70 g bodyweight) were pretreated with a single intraperitoneal injection (1·0 mg) of the various polycyclic hydrocarbons 24 hr before experiment, or with meprobamate (10 mg) daily for 5 days subcutaneously, or nikethamide (2·5 mg) daily for 5 days intraperitoneally, the last dose being administered 24 hr prior to experiment. Repeated pretreatment with 20-methylcholanthrene (1 mg/day for 3 days) gave results identical with those obtained after pretreatment with a single dose. Mean values are given ± the standard error of the mean.

TABLE 2. EFFECT OF PRETREATMENT WITH BENZPYRENE AND PHENOBARITONE ON BIPHENYL HYBROXYLATION BY RAT LIVER

Pretreatment	Yield of		No. of animals	Percentage stimulation of		Ratio 4-Hydroxylation / 2-Hydroxylation
	4-Hydroxybiphenyl (μmole/g liver/hr)	2-Hydroxybiphenyl (μmole/g liver/hr)		4-Hydroxylation	2-Hydroxylation	
Control	2·8 ± 0·2	0·23 ± 0·01	3	—	—	12·0
3, 4-Benzpyrene	2·9 ± 0·1	1·26 ± 0·07	5	0	450	2·3
Phenobarbitone	4·9 ± 0·2	0·36 ± 0·02	4	75	55	13·6

Weanling female rats (50–70 g bodyweight) were pretreated with a single intraperitoneal injection of 3, 4-benzpyrene (1·0 mg) and three consecutive daily injections of phenobarbitone (3 × 1·0 mg). Mean values are given ± the standard error of the mean.

TABLE 3. EFFECT OF PRETREATMENT WITH BENZPYRENE AND PHENOBARBITONE ON BIPHENYL HYDROXYLATION BY MOUSE LIVER

Pretreatment	Yield of		Percentage stimulation of		Ratio 4-Hydroxylation / 2-Hydroxylation
	4-Hydroxybiphenyl	2-Hydroxybiphenyl	4-Hydroxylation	2-Hydroxylation	
Control	4·1	0·63	—	—	6·5
Benzpyrene	3·7	1·68	−10	165	2·2
Phenobarbitone	11·9	0·75	190	20	15·9

The values given each represent the pooled livers of six weanling mice.

Weanling mice (15–20 g bodyweight) were pretreated with a single intraperitoneal injection of 3, 4-benzpyrene (1·0 mg) or with three consecutive daily injections of phenobarbitone (3 × 0·3 mg) the last being administered 24 hr prior to experiments.

TABLE 4. EFFECT OF PRETREATMEAT ON THE HYDROXYLATION OF BIPHENYL BY RATS *in vivo*

Pretreatment	Route of administration of biphenyl	Excretion in urine of		Percentage stimulation of		Ratio 4-Hydroxylation / 2-Hydroxylation
		4-Hydroxybiphenyl (% dose)	2-Hydroxybiphenyl (% dose)	4-Hydroxylation	2-Hydroxylation	
Control	oral	17·5 (16·5–18)	1·9 (1·7–2·0)	—	—	9·2
Benzpyrene	oral	16·5 (16–17)	3·3 (3·2–3·5)	0	70	5·0
Phenobarbitone	oral	26 (23–29)	2·0 (1·6–2·4)	50	0	13
Control	i.p.	20 (19–20)	1·9 (1·4–2·4)	—	—	10·5
Benzpyrene	i.p.	15 (14·5–15)	2·2 (1·9–2·4)	−25	15	6·8
Phenobarbitone	i.p.	26 (25–27·5)	2·0 (1·8–2·2)	30	0	13

Weanling female rats (70–75 g bodyweight) were pretreated with a single dose of benzpyrene or phenobarbitone (1 mg) 24 hr before administration of biphenyl (100 mg/kg). Urines were collected for 48 hr after administration of the biphenyl. Each value is the mean for 3 animals with the limiting values in parentheses.

TABLE 5. EFFECT OF PRETREATMEET ON THE HYDROXYLATION OF BIPHENYL BY MICE *in vivo*

Pretreatment	Excretion in urine of		Percentage stimulation of		Ratio 4-Hydroxylation / 2-Hydroxylation
	4-Hydroxybiphenyl (% dose)	2-Hydroxybiphenyl (% dose)	4-Hydroxylation	2-Hydroxylation	
Controls	25·0	5·4	—	—	
	25·6	5·6			4·6
Benzpyrene	15·5	11·9	−40	100	
	13·5	10·4			1·3
Phenobarbitone	19·2	3·5	−20	−30	5·1
	20·0	4·0			

Weanling female mice (20–25 g bodyweight) in groups of five were pretreated with a single dose of benzpyrene or phenobarbitone (0·5 mg) 24 hr before intraperitoneal administration of biphenyl (100 mg/kg). Urines were collected for 48 hr after administration of biphenyl.

Hydroxylation in vivo

After oral or intraperitoneal administration of biphenyl to normal rats the urine contains 18–20 per cent of the dose as 4-hydroxybiphenyl and its conjugates, and 2 per cent as 2-hydroxybiphenyl and conjugates. This is considerably less 4-hydroxybiphenyl than was found by West, *et al.*,[9] who isolated nearly 50 per cent of a dose of biphenyl from rat urine as this metabolite, and is more in accordance with the findings of Stroud[10] that in rabbits 25 per cent of the dose is excreted in the urine as 4-hydroxybiphenyl. However, in our experiments only a single dose (7–7·5 mg) was administered, whereas West *et al.*,[9] fed a total of 15 g of biphenyl, probably over a period of several weeks. The disparity in results could therefore be due to an induction of biphenyl-4-hydroxylation by biphenyl itself, or to an enterohepatic circulation of the compound and its metabolites, resulting in a prolonged urinary excretion.

After intraperitoneal administration of biphenyl to mice the urinary excretion of hydroxylated products amounts to 25 per cent of the dose as 4-hydroxybiphenyl and 5·5 per cent as 2-hydroxybiphenyl. The 4-/2- ratios for the *in vivo* experiments agree with those obtained for the *in vitro* experiments in mice and female rats.

Pretreatment of rats with phenobarbitone produces a moderate stimulation of the 4-hydroxylation of biphenyl and raises the ratio of 4-hydroxylation/2-hydroxylation from about 10 to 13. In mice no similar stimulation was observed with phenobarbitone, on the contrary, a slight inhibition of both 2- and 4-hydroxylation was observed, with no significant change in the ratio. This is in direct contrast to the *in vitro* experiments and may have been due to the fact that in the *in vivo* experiments only a single dose was used.

Pretreatment of rats with benzpyrene produces stimulation of biphenyl-2-hydroxylation only, and although the ratio of 4-/2-hydroxylation is lowered from the normal value of 10 to about 6 this is not so marked as in the *in vitro* experiments where the ratio falls to about 2·5. Pretreatment of mice with benzpyrene results in a marked stimulation of 2-hydroxylation, with some inhibition of 4-hydroxylation, and a lowering of the ratio from 4·6 to 1·3. This shows close correlation to the *in vitro* experiments in which the ratio drops from 6·5 to 2·2.

The *in vivo* experiments therefore largely confirm the findings *in vitro* that pretreatment with phenobarbitone stimulates biphenyl-4-hydroxylase, whereas benzpyrene stimulates the 2-hydroxylase.

DISCUSSION

The metabolism of foreign compounds by the microsomal enzymes of the liver may be stimulated both *in vivo* and *in vitro* by pretreatment with many different compounds, including steroids, drugs, pesticides and polycyclic hydrocarbons. The mechanisms of stimulation by these different classes of compound are not identical however, for whereas drugs and pesticides produce a non-specific induction of microsomal enzymes and cause a marked proliferation of the smooth-surfaced endoplasmic reticulum of the liver (SER) the polycyclic hydrocarbons appear to effect a more specific induction of the enzymes which metabolize foreign compounds and do not produce any marked increase in the liver SER.[11, 12]

Biphenyl is largely metabolized by hydroxylation at the 4-position, and in some species, particularly young animals, also at the 2-position. In the mouse 2-hydroxylation of biphenyl occurs in both adult and young animals, but in the rat is appreciable

only in immature animals. These two modes of hydroxylation have been shown to be effected by different microsomal enzyme systems.[8] Using these two enzymic reactions as a model we have now shown that compounds which produce induction of microsomal enzymes do not stimulate these two reactions to the same extent. Pretreatment with drugs (phenobarbitone, nikethamide, or meprobamate) stimulates predominantly the 4-hydroxylation of biphenyl in both the rat and the mouse. In contrast, pretreatment with polycyclic hydrocarbons known to be potent carcinogens (3,4-benzpyrene, 20-methylcholanthrene, and 1,2,5,6-dibenzanthracene) preferentially stimulates the 2-hydroxylation of biphenyl. Polycyclic hydrocarbons known to be non-carcinogenic (1,2,6,7-dibenzpyrene and 2,3,6,7-dibenzanthracene) do not appear to stimulate either mode of hydroxylation, and those hydrocarbons with weak or doubtful carcinogenic activity (1,2,3,4- and 1,2,4,5-dibenzpyrene) fall into an intermediate category and produce moderate stimulation, predominantly of the 2-hydroxylation.

There thus appears to be a positive correlation between the carcinogenicity of the polycyclic hydrocarbons we have studied and the induction of hepatic microsomal enzymes responsible for the hydroxylation of biphenyl, in particular, biphenyl-2-hydroxylase. It is therefore tempting to speculate that the carcinogenicity of polycyclic hydrocarbons may in some way be associated with their induction of the enzymes which metabolize foreign compounds, particularly those concerned in the ortho- hydroxylation of aromatic compounds. The ingestion of carcinogenic polycyclic hydrocarbons could thus result in quantitative changes in the metabolic fate of other foreign compounds by the preferential formation of ortho- hydroxylated metabolites. In the case of carcinogenic polycyclic amines this would result in an increased production of ortho-aminophenol metabolites, which are the active carcinogens. An increase in the metabolism of β-naphthylamine into the active carcinogens 2-naphthylhydroxylamine and 2-amino-1-naphthol by rats after pretreatment with 1,2,5,6-dibenzanthracene has been reported.[13] It has further been suggested that polycyclic hydrocarbons could result in a similar increase of carcinogenic ortho-aminophenol metabolites of tryptophan.[13]

If this correlation between the induction of *ortho-* hydroxylation and carcinogenicity is confirmed, determination of the stimulation of biphenyl-2-hydroxylase could provide a rapid *in vitro* screening technique for potential carcinogens.

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