A Fluorimetric Study of the Hydroxylation of Biphenyl *in vitro* by Liver Preparations of Various Species

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1. A study has been made of the enzymic hydroxylation of biphenyl by liver microsomal preparations from 11 species of animals, by using a fluorescence method for the micro-estimation of the hydroxylation products, 2- and 4-hydroxybiphenyl. 2. Livers from all species examined produced 4-hydroxybiphenyl, but only those from mice, hamsters, cats, coypus and frogs produced 2-hydroxybiphenyl as well. 3. Adult rat and rabbit livers produced only the 4-isomer, but livers from the young of these species also produced the 2-isomer. 4. The properties and requirements of the 4-hydroxylating enzyme of rabbit liver were studied. 5. The results are discussed and it is suggested that the 2- and 4-hydroxylating enzymes are different.

Rabbit-liver preparations hydroxylate biphenyl to 2- and 4-hydroxybiphenyl (Mitoma, Posner, Reitz & Udenfriend, 1956). These phenols are highly fluorescent and the quantitative aspects of these hydroxylations can be investigated fluorimetrically, as in the 7-hydroxylation of coumarin (Creaven, Parke & Williams, 1965). 2- and 4-Hydroxybiphenyl in the same preparation can be estimated fluorimetrically because the 2-isomer shows excited-state ionization, whereas the 4-isomer does not (Bridges, Creaven & Williams, 1965). In the present paper this method of estimation, the properties of the microsomal enzyme systems involved in the hydroxylations and the variation in the production of the two isomers by liver preparations from various species are described. Our results suggest that two enzymes may be involved, one hydroxylating the 2- and the other the 4-position of biphenyl. These enzymes appear to be different from coumarin 7-hydroxylase (Creaven *et al.* 1965). Some of the results described below have been briefly reported (Creaven, Parke & Williams, 1962).

EXPERIMENTAL

Materials. Biphenyl, m.p. 70°, 2-hydroxybiphenyl, m.p. 56·5°, 4-hydroxybiphenyl, m.p. 166·5°, and 4,4′-dihydroxybiphenyl, m.p. 277–279° (decomp.), were purified as described in the preceding paper (Bridges *et al.* 1965), and succinic acid (British Drug Houses Ltd., Poole, Dorset) was purified by recrystallization from water. Nicotinamide, m.p. 131°, the sodium salts of $NADH_2$ and $NADPH_2$, disodium glucose 6-phosphate (all from British Drug Houses Ltd.), NADP and Tween 80 (polyoxyethylene sorbitan mono-oleate) (both from Koch–Light Laboratories Ltd., Colnbrook, Bucks.) were used as purchased.

Methods. The maintenance of animals, preparation and fractionation of liver homogenates and conditions of incubation were as described by Creaven *et al.* (1965). The substrate solution was prepared by dissolving biphenyl (185mg.) in Tween 80 (2g.) and diluting to 100ml. with aq. 1·15% (w/v) KCl. On standing, this solution deposited crystals of biphenyl, which were redissolved by warming before use.

In the standard procedure each incubation mixture contained 1ml. of liver preparation (≡250mg. of liver and containing 10μmoles of nicotinamide), 6μmoles of biphenyl, 10mg. of Tween 80, 0·25μmole of NADP and 1ml. of 0·05M-tris–HCl buffer, pH 8·6, in a final volume of 2·7ml.

Fluorescence measurements. These were done in an Aminco–Bowman spectrophotofluorimeter with 1cm.^{2} quartz cuvettes and 150w Osram xenon arc lamp as light source. All the wavelengths of excitation and fluorescence quoted in this paper are instrumental values and are not corrected.

Determination of 4-*hydroxybiphenyl alone.* This method is used only in the absence of 2-hydroxybiphenyl. The hydroxylation is stopped by the addition of 2N-HCl (0·5ml.) to the incubation mixture, which is then shaken mechanically with *n*-heptane (10ml.) for 5min. in 20ml. ground-glass stoppered tubes. The tubes are centrifuged at 2000rev./min. in an MSE Major head no. 6885 centrifuge for 15min. and 2ml. of the heptane layer in each tube is shaken for 5min. in similar tubes with 0·1N-NaOH (10ml.) made up in all-glass distilled water. The mixture is then centrifuged as before for 10min. The fluorescence intensity of the lower alkaline layer (3–4ml.) is determined at 400mμ with excitation at 311mμ. Known amounts of 4-hydroxybiphenyl are run through the same procedure and used as fluorimetric standards. The recovery of 4-hydroxybiphenyl (30μg.) from tissue homogenates (3ml.) by a single heptane extraction is 81±3%. By this procedure the fluorimetric determination of 4-hydroxybiphenyl has an accuracy of 100±3%.

Determination of 2- *and* 4-*hydroxybiphenyl in the same solution.* At pH 13, a mixture of 2- and 4-hydroxybiphenyl shows a single fluorescence peak since the fluorescence

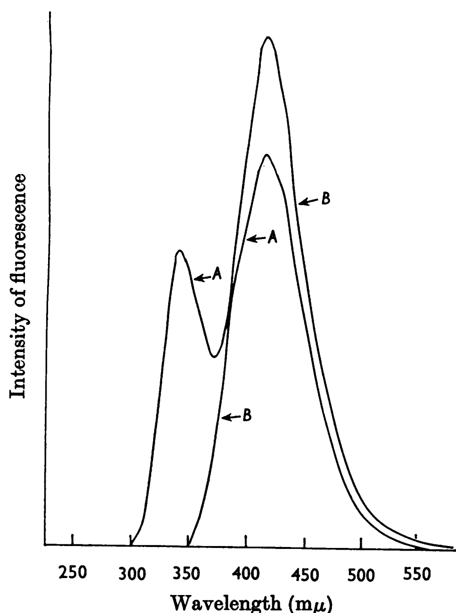

Fig. 1. Tracing of the fluorescence of a mixture of 2- and 4-hydroxybiphenyl (2-isomer, 3 μg./ml.; 4-isomer, 5 μg./ml. in water; see the text): *A*, at pH 6; *B*, at pH 13.

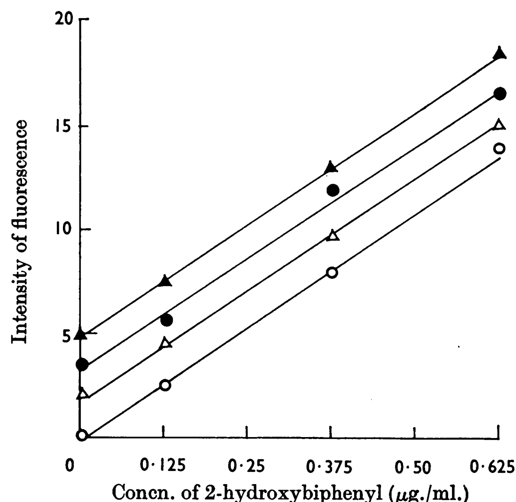

Fig. 2. Fluorescence intensity ($\lambda_{exc.}$ 295 mμ; $\lambda_{fl.}$ 415 mμ) of 2-hydroxybiphenyl at pH 5·5 in the presence of different amounts of 4-hydroxybiphenyl: ○, none; △, 0·18 μg./ml.; ●, 0·36 μg./ml.; ▲, 0·48 μg./ml.

maxima of the anions of these phenols are close together. 2-Hydroxybiphenyl shows excited-state ionization but its 4-isomer does not (Bridges *et al.* 1965). In the range pH 2–9, the 2-isomer absorbs light in the un-ionized form but emits the fluorescence of the anion ($\lambda_{fl.}$ 415 mμ; $\lambda_{exc.}$ 295 mμ), whereas the 4-isomer absorbs in the un-ionized form and emits the fluorescence of the un-ionized form ($\lambda_{fl.}$ 338 mμ; $\lambda_{exc.}$ 275 mμ). [In the preceding paper (Bridges *et al.* 1965) the excitation now reported at 275 mμ was recorded at 288 mμ; the difference is due to changing the xenon arc lamp of the fluorimeter.] The instrumental tracings of the fluorescence curves of a mixture of 2- and 4-hydroxybiphenyl at pH 6 and 13 are shown in Fig. 1.

The incubation mixtures are extracted with *n*-heptane and the extracted phenols transferred to 0·1 N-NaOH as described in the preceding section. After centrifuging, 2 ml. of the NaOH layer is pipetted into a cuvette and brought to pH 5·5 with 0·5 ml. of 0·5 N-succinic acid. Fluorescence intensity is then determined at two wavelengths: (*a*) at 338 mμ with $\lambda_{exc.}$ 275 mμ [$\lambda_{exc.}$ recorded at 288 mμ by Bridges *et al.* (1965); see above] and (*b*) at 415 mμ with $\lambda_{exc.}$ 295 mμ. From reading (*a*) 4-hydroxybiphenyl can be determined directly since the 2-isomer does not interfere at this wavelength. From reading (*b*) 2-hydroxybiphenyl can be determined after making a correction for the contribution of the 4-isomer (see Fig. 2) at this wavelength, which is appreciable at low concentrations of 2-hydroxybiphenyl. For this correction three standard solutions are needed: (i) 4-hydroxybiphenyl (30 μg./ml. in aq. 5% ethanol); (ii) a mixture of 2-isomer (6 μg./ml.) and 4-isomer (30 μg./ml.); (iii) 2-hydroxybiphenyl (6 μg./ml.). These standard solutions (1 ml.) are added separately to incubation mixtures that had been incubated without substrate. The substrate solution (0·5 ml.) is now added and the mixture extracted and estimated as above. At 338 mμ, standard (i) and (ii) should give the same reading and either can be used as a standard for 4-hydroxybiphenyl. At 415 mμ the reading for standard (i) should be the same as the difference between the readings for standard (ii) and (iii) at this wavelength. Let the amount of 4-hydroxybiphenyl in the unknown be x, which can be determined at 338 mμ, and the amount of 4-hydroxybiphenyl in standard (i) be y. If the reading of standard (i) at 415 mμ is A, then the contribution of 4-hydroxybiphenyl in the unknown at 415 mμ is Ax/y. If the reading of the unknown at 415 mμ is B, then the corrected reading C for 2-hydroxybiphenyl is $B-(Ax/y)$. The calculations can be made by using standards (ii) and (iii) only, but the use of three standards is a check that the operations have been carried out correctly.

2- and 4-Hydroxybiphenyl were stable at pH 5·5 and 13 at 25°, but when exposed to ultraviolet light for more than 10 min. the fluorescence at pH 13, particularly of the 2-isomer, diminished (Bridges *et al.* 1965). Spectrofluorimeter readings were therefore taken immediately on exposure to the light source.

Identification of the products of hydroxylation in vitro. A mixture of biphenyl (240 μmoles), NADP (10 μmoles), 10000 *g* supernatant of liver homogenate (40 ml.) and 0·05 M-tris–HCl buffer, pH 8·6 (40 ml.), was incubated with shaking in 20 equal portions (25 ml. beakers) at 37° for 45 min. in air. The contents were pooled and extracted with ether (3 × 300 ml.). The extract was evaporated to 2 ml. and chromatographed on a column (2·3 cm. × 25 cm.) of alumina (Spence grade H, partially deactivated with 10% acetic acid), and eluted with light petroleum (b.p. 40–60°), chloroform and finally methanol. Each eluate was evaporated to 0·5 ml. and samples were chromatographed on thin-layer chromatoplates (Desaga) of alumina (E. Merck,

Table 1. R_F *values and colour reactions of some hydroxybiphenyls*

The hydroxybiphenyls were chromatographed on thin-layer chromatoplates of alumina (Merck) in the following solvent systems: *A*, benzene–$CHCl_3$ (19:1, v/v); *B*, *n*-hexane–methanol (49:1, v/v); *C*, acetone–$CHCl_3$ (1:1, v/v). The compounds were detected by spraying with Gibbs reagent [ethanolic 0·1% (w/v) 2,6-dichlorobenzoquinone-4-chloroimine] and Brentamine Fast Red B salt [aq. 0·1% (w/v) stabilized diazotized 2-amino-5-nitroanisole] and by the fluorescence when examined under u.v. light (265 mμ; Chromatolite lamp).

	R_F			Colour reactions		
Compound	Solvent *A*	Solvent *B*	Solvent *C*	With Gibbs reagent	With Bentamine Fast Red B salt	Fluorescence
2-Hydroxybiphenyl	0·25	0·53	0·83	Blue	Orange	Bright blue
4-Hydroxybiphenyl	0·06	0·16	0·70	Buff	Red	q.*
4,4′-Dihydroxybiphenyl	0·00	0·00	0·41	Grey	Green	q.*

* Quenching of background fluorescence.

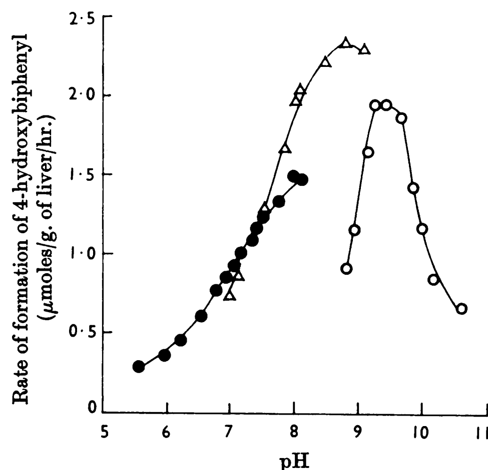

Fig. 3. Effect of buffer and pH on the rate of formation of 4-hydroxybiphenyl from biphenyl by the 10000*g* supernatant preparation of rabbit liver (see the text): ●, 0·02M-sodium phosphate buffer; △, 0·02M-tris–HCl buffer; ○, 0·02M-glycine–NaOH buffer.

Darmstadt, Germany) in solvents *A*, *B* and *C*. The R_F values and colour reactions of 2-hydroxy-, 4-hydroxy- and 4,4′-dihydroxy-biphenyl are given in Table 1.

RESULTS

Hydroxylation of biphenyl by adult rabbit liver

Biphenyl was hydroxylated by the 10000*g* supernatant of adult rabbit liver almost exclusively to 4-hydroxybiphenyl, the amounts of 2-hydroxybiphenyl formed being negligible. Rabbit-liver preparations could therefore be used for the study of the requirements of the system 4-hydroxylating biphenyl. Hydroxylation occurred more readily in tris–hydrochloric acid buffer than in phosphate or glycine–sodium hydroxide buffer. The optimum pH of the reaction also differed slightly in these buffers: in tris–hydrochloric acid buffer, which was used in most experiments, the optimum pH was 8·6–8·8 (see Fig. 3). The reaction requires oxygen, for in an atmosphere of nitrogen hydroxylation hardly occurred. In pure oxygen hydroxylation was slightly greater than in air (Table 2). The optimum temperature of the reaction is 35–40°. The percentage hydroxylation was maximal at a substrate concentration of 1 μmole/3 ml. of incubation mixture, but the maximum yield of 4-hydroxybiphenyl was obtained with 6 μmoles/3 ml. (Fig. 4). The rate of production of 4-hydroxybiphenyl was constant for the first 30 min. of incubation (Fig. 5) and an incubation period of 15 min.

Table 2. *Effect of incubation atmosphere on the hydroxylation of biphenyl*

The 10000*g* supernatant (1 ml.) from liver homogenates of adult Chinchilla doe rabbits was incubated with 6·0 μmoles of biphenyl and 10 mg. of Tween 80, 1·0 ml. of 0·05M tris–HCl buffer, pH 8·6, 0·25 μmole of NADP and 6·0 μmoles of glucose 6-phosphate, in a total volume of 3·0 ml. of 1·15% KCl, for 15 min. in air at 37°. The results are the means (S.E.M. = 0·05) of two to four experiments.

Animal no.	Atmosphere	Yield of 4-hydroxybiphenyl (μmoles/g. of liver/hr.)
1	Air	2·3
	O_2	2·5
	N_2	0·1
2	Air	1·75
	O_2	1·95
	N_2	0·00
3	Air	2·6
	O_2	2·9

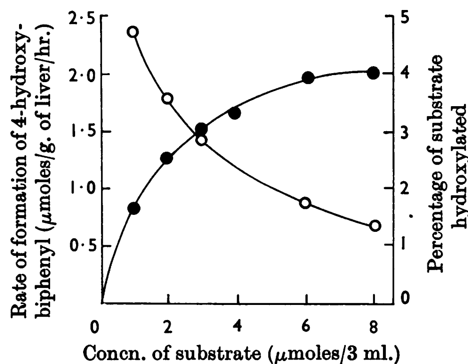

Fig. 4. Effect of substrate concentration on the rate of formation of 4-hydroxybiphenyl from biphenyl by the 10000*g* supernatant preparation of rabbit liver (see the text). ●, Rate of formation of 4-hydroxybiphenyl; ○, percentage of substrate hydroxylated.

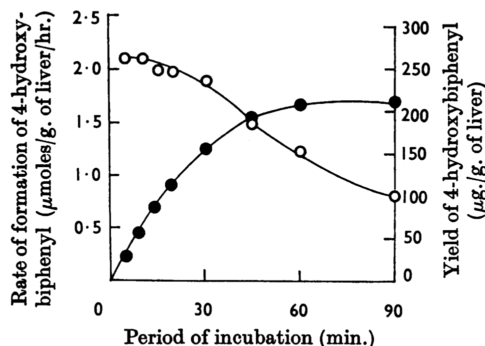

Fig. 5. Variation in the rate of formation of 4-hydroxybiphenyl from biphenyl by the 10000*g* supernatant preparation of rabbit liver with time of incubation. ○, Rate of formation of 4-hydroxybiphenyl; ●, yield of 4-hydroxybiphenyl.

was therefore adopted in most experiments. In the presence of an excess of soluble fraction, the rate of production of 4-hydroxybiphenyl was linearly related to the amount of microsomal fraction used, i.e. to the enzyme concentration.

Cofactors required for optimum activity. These were found to be similar to those required in the 7-hydroxylation of coumarin (Creaven *et al.* 1965). The addition of NADP (0·25μmole/3ml.) caused a threefold increase in hydroxylation (Table 3). There was also increased hydroxylation if nicotinamide was added during the preparation of the supernatant, but the effects of NADP and nicotinamide are not additive and the effect of both is, in fact, less than that of NADP alone. The addition of glucose 6-phosphate (6μmoles/3 ml.) alone or in

Table 3. *Effects of nicotinamide, NADP and glucose 6-phosphate on the hydroxylation of biphenyl*

The 10000*g* supernatant (1 ml.) from the liver of adult Chinchilla doe rabbits was incubated with 6·0μmoles of biphenyl and 10mg. of Tween 80, 1·0ml. of 0·05M-tris–HCl buffer, pH 8·6, and various cofactors, in a total volume of 3·0ml. of 1·15% KCl, at 37° in air for 15min. Fresh tissue was used 2hr. after slaughter of the animal and aged tissue was kept for 18 days at −12° before incubation. Nicotinamide (10μmoles) was added during the preparation of the 10000*g* supernatant; NADP and glucose 6-phosphate were added just before incubation. The results are the means (S.E.M. = 0·05) of two experiments.

Additions			Yield of 4-hydroxybiphenyl (μmoles/g. of liver/hr.)	
Nicotinamide (μmoles)	NADP (μmole)	Glucose 6-phosphate (μmoles)	Fresh liver	Aged liver
0	0	0	1·04	0·25
0	0	6	1·36	0·34
0	0·25	0	3·04*	2·06
0	0·25	6	2·86	2·06
10	0	0	2·34	0·94 (0·35)†
10	0	6	2·32	1·10
10	0·25	0	2·74	1·34
10	0·25	6	2·54	1·60

* 0·50 and 0·75μmole of NADP give 3·22 and 3·31μmoles of 4-hydroxybiphenyl/g. of liver/hr. respectively in this experiment.

† No nicotinamide was added during preparation of the 10000*g* supernatant, but 10μmoles were added just before incubation.

combination with the other cofactors has little effect on hydroxylation.

If the 10000*g* supernatant is kept at −12° for 18 days, it shows negligible hydroxylating activity. However, if nicotinamide (40μmoles/g. of liver) is added before storage some 40% of the original activity is retained, though if the nicotinamide is added at the end of the storage period hydroxylating activity is not restored. The addition of NADP (0·25μmole/3 ml.) to the stored supernatant restores 70% of the original activity, but if the NADP is added to the stored supernatant to which nicotinamide was added before storage the restoration of hydroxylating activity is no greater than when NADP is used alone. NADP was therefore added as a routine to incubation mixtures in amounts of 0·25μmole/3 ml. Larger quantities of NADP produced little increase in hydroxylation, although NADP has no tendency to inhibit (Table 3).

The microsomal fraction (105000*g* for 1hr.) and supernatant were separated by centrifugation. Neither fraction alone hydroxylated biphenyl. However, the addition of $NADPH_2$ to the microsomal fraction completely restored the hydroxy-

Table 4. *Subcellular distribution of biphenyl-hydroxylating activity and the effects of reduced nicotinamide nucleotides*

Subcellular fractions equivalent to 0·25g. of liver/ml. were prepared from the liver of a Chinchilla doe rabbit, 10μmoles of nicotinamide/ml. being added during the preparation. Portions (1·0ml.) of the tissue fractions were incubated with 1·0ml. of 0·05M-tris–HCl buffer, pH 8·6, 6·0μmoles of biphenyl and 10mg. of Tween 80, 6μmoles of glucose 6-phosphate, 2·0μmoles of ATP and 10μmoles of $MgCl_2$, in a total volume of 3·0ml. of 1·15% KCl, at 37° for 15min. in air. The results are the means (S.E.M. = 0·05) of two experiments.

Subcellular fraction	$NADPH_2$ added (μmoles)	$NADH_2$ added (μmoles)	Yield of 4-hydroxy-biphenyl (μmoles/g. of liver/hr.)
10000g Supernatant	0	0	1·04
Microsomal fraction	0	0	0·00
Soluble fraction	0	0	0·00
Microsomal fraction + soluble fraction	0	0	1·10
Microsomal fraction	1	0	0·86
	2	0	1·12
	3	0	1·15
	4	0	1·19
	5	0	1·27
	0	1	0·07
	0	3	0·12

Table 5. *Effects of inhibitors on the hydroxylation of biphenyl*

The 10000g supernatant (1 ml.) from liver homogenates of adult Chinchilla doe rabbits was incubated with 6μmoles of biphenyl and 10mg. of Tween 80, 0·25μmole of NADP, 6·0μmoles of glucose 6-phosphate and 1·0ml. of 0·05M-tris–HCl buffer, pH 8·6, in a final volume of 3·4ml. of 1·15% KCl, for 15min. at 37° in air. The results are the means of two experiments.

Inhibitor	Inhibition (%)
p-Chloromercuribenzoate (0·15mM)	42
SKF 525A (0·4mM)	30
2,4-Dichlorophenol (0·85mM)	36
$\alpha\alpha'$-Bipyridyl (1·4mM)	44
Ascorbic acid (1·0mM)	19
Potassium cyanide (1·0mM)	4

lating activity of the washed (three times) microsomal fraction, but $NADH_2$ was less effective (Table 4).

Effects of inhibitors. The results (Table 5) are substantially the same as found for coumarin 7-hydroxylase (Creaven *et al.* 1965), except that $\alpha\alpha'$-bipyridyl inhibits the 4-hydroxylation of biphenyl whereas it has no effect on coumarin 7-hydroxylase. The inhibition by $\alpha\alpha'$-bipyridyl was abolished by equimolar ferrous sulphate.

Hydroxylation of biphenyl by livers of different species

Adult rat. Rat-liver preparations hydroxylated biphenyl almost entirely in the 4-position. The amounts of 2-hydroxybiphenyl produced were too small to be estimated, but it was detected fluorimetrically on chromatograms. The properties of the rat-liver enzyme were similar to those of the rabbit except that there was no inhibition by $\alpha\alpha'$-bipyridyl.

Young rats. These were 1–3 months old (60–140g. body wt.) and liver preparations from these produced measurable amounts of 2-hydroxybiphenyl (0·15–0·4μmole/g./hr.) (Table 6).

Young rabbits. These were 3–6 months old (1·0–1·5kg. body wt.) and they also produced 2-hydroxybiphenyl (Table 6).

Other species. The results with liver preparations from other species are shown in Table 6.

Metabolism of 4-hydroxybiphenyl by guinea-pig liver

The yield of 4-hydroxybiphenyl with guinea-pig liver preparations was low. However, on acid hydrolysis of the incubation mixture, appreciable amounts of the phenol were obtained (Table 6). In this case it appeared that hydroxylation occurred but the product at the end of the incubation was in a bound form. On incubating 1ml. of the guinea-pig liver preparation with 30μg. of 4-hydroxybiphenyl, the amounts of the phenol recoverable in 5, 10, 15 and 20 min. were 30, 14, 11 and 7% respectively. (Recoveries after incubation with rat-liver preparation were 97, 83, 73 and 67% respectively.) After the 20 min. incubation, the mixture was heated under reflux in 3N-hydrochloric acid for 20 min., whereby 100% of the 4-hydroxybiphenyl was recovered. It is probable that 4-hydroxybiphenyl is conjugated, possibly with glucuronic acid, in the guinea-pig liver preparation, as was the case with 7-hydroxycoumarin (Creaven *et al.* 1965).

DISCUSSION

The metabolites of biphenyl in urine in the rabbit have been shown to be 4-hydroxybiphenyl and its conjugates (Block & Cornish, 1959), whereas in the rat they were 4-hydroxy-, 4,4'-dihydroxy- and 3,4-dihydroxy-biphenyl and their conjugates and biphenylmercapturic acid (West, Lawson, Miller & Mathura, 1956). In both species, 4-hydroxybiphenyl was the major hydroxylated product, but 2-hydroxybiphenyl was not found. Mitoma *et al.* (1956)

Table 6. *Hydroxylation of biphenyl* in vitro *by liver preparations from various species*

The liver preparations were 10000*g* supernatants of liver homogenates containing additives and incubated as described in the text for the standard procedure. The animals used were adult except where otherwise indicated: M, male; F, female; Y, young (see the text). Where appropriate, ranges are given in parentheses; — indicates less than 0·1 μmole/g./hr.

Species and strain	Sex and no. of animals	Hydroxylation (μmoles/g. of liver/hr.) In 4-position	In 2-position
Rabbit			
English	M 2	2·65, 3·0	—
	F 6	3·15 (2·70–3·95)	—
	YM 2	2·7, 3·7	0·11, 0·14
Chinchilla	F 5	2·1 (1·65–2·75)	—
New Zealand	M 1	1·7	—
Rat			
Wistar albino	M 24	1·4 (1·24–1·63)	—
	F 4	1·5 (1·03–2·25)	—
	YM 11	2·5 (2·04–3·1)	0·32 (0·14–0·53)
Black hooded	YM 2	1·36, 1·40	0·12, 0·16
Mouse			
A_2G	M 12	5·7 (4·56–6·57)*	2·2 (2·15–2·25)*
I.C.I.	M 4	7·1†	0·37†
	YM 6	4·1†	0·63†
Hamster	M 6	3·8 (3·45–4·0)*	1·8 (1·8–1·8)*
	F 7	1·7 (1·1–2·3)*	0·6 (0·4–0·94)*
Cat	M 2	0·8, 1·0	0·27, 0·17
Coypu (*Myocastor coypus*)	F 2	5·8, 6·4	0·25, 0·27
Guinea pig	M 2	1·35, 1·4‡	—
Hen (Light Sussex)	F 4	1·7 (1·4–1·85)	—
Frog (*Rana temporaria*)	M & F 23	1·15 (1·05–1·35)*§	0·15 (0·1–0·2)*§
		0·75 (0·65–0·80)*	ca. 0·1
Trout (*Salmo fario*)	M & F 6	0·22 (0·21–0·23)*	—
Fox	M 1	3·0	—

* Two or more livers were pooled for these estimations.
† All livers pooled.
‡ After acid hydrolysis (see the text).
§ Values for the whole homogenate (see the text).

reported that liver preparations from New Zealand White rabbits converted biphenyl into 2- and 4-hydroxybiphenyl, the 2-isomer being formed in larger amounts than the 4-isomer. With adult New Zealand White, Chinchilla and English rabbits, we found that liver preparations formed 4-hydroxybiphenyl and practically no 2-isomer, and these findings are in agreement with the findings on the whole animals.

The rabbit-liver enzyme system that 4-hydroxylates biphenyl is similar to that which 7-hydroxylates coumarin (Creaven *et al.* 1965), but there is evidence that they may not be the same (Table 7). Further, coumarin-7-hydroxylase activity does not occur in rat-liver preparations that actively 4-hydroxylate biphenyl. Table 8 summarizes the occurrence of the coumarin hydroxylase and biphenyl hydroxylase in various species.

Species differences. Liver preparations from all 11 species examined (Table 6) are able to convert biphenyl into 4-hydroxybiphenyl. However, the extent of this hydroxylation varies with species, being poor in cat and trout (less than 1 μmole/g. of liver/hr.) and high in mouse and coypu (about 6–7 μmoles/g./hr.). The ability to form 2-hydroxybiphenyl, however, is almost absent from the livers of adult rabbits, rats, guinea pigs, hens, trout and one fox, whereas the livers of mice (two strains), hamsters, cats, coypus and frogs form measurable amounts of this isomer. Mouse and hamster livers are active in 2-hydroxylating biphenyl, and the A_2G strain mice are much more active than the I.C.I. strain in this respect, although both are about the same with respect to 4-hydroxylating activity. In the rabbit and rat, 2-hydroxylating activity is present in young animals (Table 6), but in adult

Table 7. *Properties of rabbit-liver biphenyl hydroxylase and coumarin hydroxylase*

	Biphenyl 4-hydroxylase	Coumarin 7-hydroxylase*
Buffer giving optimum activity	Tris	Phosphate
Optimum pH in above buffer	8·6	7·4
Optimum substrate concentration (M)	2·14	0·356
Action of αα′-bipyridyl	Inhibits	None

* Creaven *et al.* (1965).

Table 8. *Occurrence of coumarin hydroxylase and biphenyl hydroxylases in the livers of various species*

Adult animals were used unless otherwise stated.

Species	Coumarin 7-hydroxylase*	Biphenyl 4-hydroxylase	Biphenyl 2-hydroxylase
Rabbit			
Adult	+	+	−
Young	+	+	+
Rat			
Adult	−	+	−
Young	−	+	+
Guinea pig	+	+	−
Cat	+	+	+
Coypu	+	+	+
Hamster	+	+	+
Mouse	−	+	+
Hen	+	+	−
Frog	+	+	+

* Creaven *et al.* (1965).

animals it is barely detectable. These findings suggest that the 2- and 4-hydroxylating systems are different. There does not appear to be a sex difference in rats and rabbits, but in the hamster the livers from the male are much more active than those from the female in terms of both 2- and 4-hydroxylation.

Brodie & Maickel (1962) suggested that amphibia and fish are unable to metabolize foreign compounds, but Table 6 shows that the frog and the trout can hydroxylate biphenyl, although not as effectively as some of the mammalian species. With frog liver, the whole homogenate gave a higher rate of hydroxylation than the 10000 *g* supernatant, which is the reverse of the finding with mammalian species. A similar pattern was observed in the 7-hydroxylation of coumarin by frog liver (P. J. Creaven, D. V. Parke & R. T. Williams, unpublished work). The frog-liver hydroxylating system is, however, located in frog microsomes and is $NADPH_2$-dependent. It is possible that the deposit at 10000 *g* of frog-liver homogenates contains an activator, although the deposit itself has no hydroxylating activity.

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