Table 1. *Nuclear-magnetic-resonance spectra of hydrogen atoms in the isolated lactones*

The chemical shifts are expressed in p.p.m. from internal 2-methylpropan-2-ol.

Position of protons	Chemical shift (p.p.m.)	
	Lactone (<i>V. sativa</i>)	Lactone (<i>L. tingitani</i>)
3	1.42	1.75
5	2.36	0.88
6	—	2.18

The spectrum of the compound from *V. sativa* shows two bands, the more complex one, at higher field, is the AB portion of an ABX spectrum which has been further split by a single proton. The lower field band is the AB part of an ABX spectrum. The position and multiplicities of these bands in the observed spectrum are those expected of the 3- and 5-protons of the proposed formula. The 2- and 4-proton signals are masked by the band arising from the few per cent of water in the commercial D₂O used as solvent.

The most prominent feature of the spectrum of the compound from *L. tingitani* is the triplet at a shift of 2.18 p.p.m. This has been assigned to the 6-protons in formula (II): the triplet splitting, and the small upfield shift of this band compared with the corresponding band in the spectrum of the compound from *V. sativa*, is in agreement with the introduction of a —CH₂— grouping between the C-4 and C-5 of formula (I). Two other complex bands were observed in the spectrum of this compound and their positions and multiplicities are suitable for the 3- and 5-protons of formula (II). Again the methine protons are masked by the solvent line.

These spectra are in agreement with the chemical evidence and indicate that the compounds isolated from *V. sativa* and *L. tingitani* are the lactones derived respectively from γ -hydroxyarginine and γ -hydroxyhomoarginine.

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The Biochemistry of Aromatic Amines

10. ENZYMIC *N*-HYDROXYLATION OF ARYLAMINES AND CONVERSION OF ARYLHYDROXYLAMINES INTO *o*-AMINOPHENOLS*

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Since the finding that one of the routes of metabolism of 2-acetamidofluorene in the rat is by *N*-hydroxylation and subsequent conjugation with glucuronic acid (Cramer, Miller & Miller, 1960) it has been shown that 2-naphthylamine (Troll & Nelson, 1961), 4-acetamidobiphenyl (Miller, Wyatt, Miller & Hartmann, 1961*b*), 4-aminostilbene (Miller & Miller, 1962) and benzidine (Troll, Belman & Rinde, 1963) are also excreted in urine as *N*-hydroxy metabolites by a variety of species. This reaction is likely to play an important part in the carcinogenic properties of the arylamines since the *N*-hydroxy derivatives of 2-acetamidofluorene (Miller, Miller & Hartmann, 1961*a*), 4-acetamidobiphenyl (Miller *et al.* 1961*b*), 4-acetamidostilbene (Anderson, Enomoto, Miller & Miller, 1963) and

2-naphthylhydroxylamine (Boyland, Dukes & Grover, 1963) are more potent carcinogens than the parent compounds under certain experimental conditions.

The *N*-oxidation of several arylamines by rat-liver microsomes and NADPH has been demonstrated by the estimation of the hydroxylamine and nitroso derivatives (Uehleke, 1961, 1963; Kampffmeyer & Kiese, 1963). The formation of 2-(*N*-hydroxyacetamido)fluorene from 2-acetamidofluorene by rabbit-liver microsomes has been detected and estimated (Irving, 1962*a*).

The present paper describes the enzyme systems in rabbit liver which are concerned with the *N*-hydroxylation of acetamidoaryl compounds, with special reference to 4-acetamidobiphenyl, and the further reactions of the metabolic *N*-hydroxyacetamidoaryl compounds.

* Part 9: Boyland, Manson & Nery (1963).

EXPERIMENTAL

Materials. NADH as the disodium salt was obtained from Sigma Chemical Co., St Louis, Mo., U.S.A. NAD^+ , NADP^+ (sodium salt), glucose 6-phosphate (sodium salt) and glucose 6-phosphate dehydrogenase (D-glucose 6-phosphate-NADP⁺ oxidoreductase, EC 1.1.1.49) as a suspension in 3.3 M-ammonium sulphate, were obtained from C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany. *N*-Acetylbenzidine was prepared by the method of Bilow & Baur (1925).

Washed microsome suspensions. Fresh rabbit liver was homogenized in 4 vol. of KCl (1.15%, w/v) in an Ato-Mix blender (Measuring and Scientific Equipment Ltd.) for 1 min., and the homogenate was centrifuged in an Angle 50 centrifuge (Measuring and Scientific Equipment Ltd.) at 1480 g_{av} for 20 min. The supernatant phase was centrifuged at 105400 g_{av} in a Spinco model L preparative ultracentrifuge with rotor no. 40 for 1 hr. The supernatant fraction (soluble liver fraction) was removed and two of the loosely packed microsomal pellets were tipped into a clean Spinco tube, leaving behind the bulk of the glycogen. The microsomes were suspended in the KCl solution (12.5 ml.) and again centrifuged at 105400 g_{av} for 1 hr. The supernatant phase was discarded and the microsomal pellets were again suspended in 1.15% (w/v) KCl (12.5 ml.) and again centrifuged at 105400 g_{av} for 1 hr. The supernatant fraction was discarded, the pellets were covered with 1.15% (w/v) KCl (1 ml.) and stored at -5° . All operations were carried out at approx. 5° . Immediately before use the microsomal pellets were thawed and homogenized in 2 M-NaOH with a Potter & Elvehjem (1936) type homogenizer with a Teflon pestle. The suspension of microsomes was diluted to contain the microsomes from 1 g. of liver/ml. and samples of this suspension were added to the incubation flasks.

Identification of metabolites by thin-layer chromatography. For experiments on the reactions catalysed by microsomes complete incubation mixtures contained: rabbit-liver microsomes from 500 mg. of liver; NaH_2PO_4 - Na_2HPO_4 buffer, pH 7.4 (500 μ moles of phosphate); nicotinamide (400 μ moles); glucose 6-phosphate (20 μ moles); NADP^+ (1 μ mole); NADH (2 μ moles); glucose 6-phosphate dehydrogenase (25 μ g.); KF (1 m-mole); in a total vol. of 10 ml. When soluble liver fraction was studied the incubation mixtures contained NADH (2 μ moles), phosphate buffer (500 μ moles of phosphate), pH 7.4, and soluble liver fraction (2 ml.) in a total volume of 10 ml. The substrates (2 μ moles) were added in 0.2 ml. of 2-methoxyethanol (methylCellosolve). The mixtures were incubated in air in a metabolic shaker (H. Mickle, Gomshall, Surrey) at 37° and after 1 hr. anaesthetic ether (14 ml.) (Duncan Flockhart and Co. Ltd.) was added. After shaking, a sample of the ether (10 ml.) was removed and evaporated to dryness in a water bath at 50° . The residue was dissolved in acetone (0.1 ml.) and applied to thin-layer chromatograms. Thin-layer chromatography was carried out on glass plates, 20 cm. $\times$ 20 cm. or 20 cm. $\times$ 10 cm., spread with silica gel G (Merck). A layer 0.25 mm. thick was convenient for general use and the plates were dried at 80° for 1–2 hr. Samples were applied as spots (approx. 5 mm. diam.) 1 cm. from the edge of the silica gel and the chromatograms developed until the solvent front had travelled 14 cm. from the point of application. The tanks were lined with Whatman no. 3 MM chromatography paper saturated with the solvent

system. The routine solvent systems used were light petroleum (b.p. 40 – 60°)-acetone (7:3, v/v) and chloroform-ethyl acetate-acetic acid (6:3:1, by vol.), except when 4-acetamido-4'-aminobiphenyl (*N*-acetylbenzidine) was the substrate and in these experiments the solvent system was light petroleum (b.p. 40 – 60°)-acetone (1:1, v/v). The plates were dried at room temperature and sprayed with one of the following reagents: (a) Folin and Ciocalteu's reagent (British Drug Houses Ltd.) followed by Na_2CO_3 (10%, w/v); (b) ammoniacal 2% (w/v) AgNO_3 ; (c) *p*-dimethylaminobenzaldehyde (0.5%, w/v, in ethanol containing 1 ml. of conc. HCl /100 ml.); (d) *p*-dimethylaminocinnamaldehyde (1%, w/v, in 50%, v/v, ethanolic 3 N-HCl) (Harley-Mason & Archer, 1958); (e) 2,6-dichloroquinonechloroimide (0.5%, w/v, in ethanol) followed by Na_2CO_3 (10%, w/v) (Gibbs, 1927); (f) sodium aminoprusside (0.5%, w/v; this reagent reacts with arylhydroxylamines to form coloured complexes having the general formula $[\text{Fe}(\text{CN})_5\text{ArNHOH}]^{3-}$; (Boyland & Nery, 1964); (g) $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (5%, w/v, in 95%, v/v, ethanolic 0.1 N-HCl) (Stadtman & Barker, 1950).

Estimation of 4-(*N*-hydroxyacetamido)biphenyl (I) formed from 4-acetamidobiphenyl (II) by liver microsomes. The spectrophotometric method for the estimation of 4-(*N*-hydroxyacetamido)biphenyl (I) was based on the observation that hydroxamic acids react with ferric salts to form coloured complexes (Lossen, 1892). Complete reaction mixtures as described in the qualitative experiments were used, with the exceptions that microsomes from 1 g. of liver were present and the phosphate buffer was replaced with 0.1 M-glycine-NaOH buffer, pH 8.6. Incubations were in air for 30 min. At the end of the incubation period the reaction mixtures were shaken with anaesthetic ether (12 ml.) and a sample of ether (10 ml.) was removed and evaporated to dryness on a water bath at 50° . The concentrated ether extracts from experimental incubations, and zero-time controls which contained all the constituents, were dissolved in 0.005% anhydrous FeCl_3 in A.R. methanol (2.5 ml.) (Bayer & Reuther, 1956) and the extinctions at 530 m μ read on a Unicam SP. 500 spectrophotometer. Under these conditions an extinction reading of 0.1 represented 0.17 ± 0.02 (11) μ mole of 4-(*N*-hydroxyacetamido)biphenyl (I) in the reaction mixture, expressed as the mean of 11 determinations $\pm$ s.d.

Protein was estimated by a modification of the biuret method (Fincham, 1954).

RESULTS

Chromatographic properties of *N*-hydroxyacetamidoaryl compounds. The following properties are exhibited by the four *N*-hydroxyacetamidoaryl compounds listed in Table 1. (a) All the compounds examined on thin-layer chromatograms appeared as approximately circular spots with the exception of the *N*-hydroxyacetamidoaryl compounds, the spots of which had sharply defined leading edges and indistinct trailing edges. The R_F values of other compounds were reproducible when amounts varying from 0.05 to 1 μ mole were applied, but the distance travelled by the leading edge of the *N*-hydroxyacetamidoaryl compounds varied, with-

in certain limits, according to the amount of material applied. When amounts of 4-(*N*-hydroxyacetamido)biphenyl (I) varying from 0.05 to 0.25 μ mole were applied to silica-gel plates 0.25 mm. thick, the leading edge of the greatest amounts applied travelled the greatest distance. This property might be utilized for the quantitative estimation of these compounds. (b) When chromatograms were sprayed with the Folin and Ciocalteu's reagent, metabolites other than the *N*-hydroxyacetamidoaryl compounds were immediately visible although the colours were intensified by spraying with sodium carbonate (10%). *N*-Hydroxyacetamidoaryl compounds, however, were not visible until the sodium carbonate had also been applied or after standing for approx. 30 min. (c) When sprayed with ammoniacal 2% silver nitrate the *N*-hydroxyacetamidoaryl compounds developed a grey colour in about 10 min. (d) When sprayed with the ferric chloride reagent *N*-hydroxyacetamidoaryl compounds gave a characteristic brownish-purple colour immediately but none of the other metabolites reacted in this way.

Metabolism of 4-acetamidobiphenyl (II) by rabbit-liver microsomes. Rabbit-liver microsomes were incubated with various substrates in the reaction mixtures described in the Experimental section,

potassium fluoride being omitted in some experiments. After extraction with ether and application of the ether solution to thin-layer chromatograms, the metabolites were identified by comparison of their chromatographic properties and colour reactions with those of authentic specimens (Table 1).

Hydroxylation in the 4'-position. 4-Acetamido-4'-hydroxybiphenyl (III) and 4-amino-4'-hydroxybiphenyl (VIII) were detected as metabolites of 4-acetamidobiphenyl (II) in the absence of potassium fluoride, but in its presence the deacetylated metabolite (VIII) was absent except for traces formed after incubation for 2 hr.

Hydroxylation in the 3-position. No 4-acetamido-3-hydroxybiphenyl (IV) was detected from 4-acetamidobiphenyl (II) in the presence of potassium fluoride, but 4-amino-3-hydroxybiphenyl (V) was formed in its absence. This probably arose from hydroxylation of the 4-aminobiphenyl (VII), which is formed under these conditions.

N-Hydroxylation. 4-(*N*-Hydroxyacetamido)biphenyl (I) was detected as a metabolite of 4-acetamidobiphenyl (II) both in the absence and presence of potassium fluoride. In the absence of potassium fluoride the further metabolism of this derivative (I) gave rise to 4-(hydroxyamino)bi-

Table 1. *Thin-layer chromatography of arylamine metabolites*

Thin-layer chromatography was on glass plates spread with a 0.25 mm. layer of silica gel G. Solvent systems: 1, light petroleum (b.p. 40–60°)–acetone (7:3, v/v); 2, chloroform–ethyl acetate–acetic acid (6:3:1, by vol.). Colours reported were visible immediately after spraying unless indicated otherwise. Spray reagents: (a) Folin and Ciocalteu's reagent followed by Na₂CO₃ (10%, w/v); (b) ammoniacal 2% (w/v) AgNO₃; (c) *p*-dimethylaminobenzaldehyde (0.5%, w/v, in ethanol containing 1 ml. of conc. HCl/100 ml.); (d) *p*-dimethylaminocinnamaldehyde (1%, w/v, in 50%, v/v, ethanolic 3*N*-HCl); (e) 2,6-dichloroquinonechloroimide (0.5%, w/v, in ethanol) followed by Na₂CO₃ (10%, w/v); (f) sodium amminopruesside (0.5%, w/v); (g) FeCl₃·6H₂O (5%, w/v, in 95%, v/v, ethanolic 0.1*N*-HCl).

Compound	<i>R_F</i>		Colours with spray reagents						
	Solvent 1	Solvent 2	(a)	(b)	(c)	(d)	(e)	(f)	(g)
4-Aminobiphenyl	0.45	0.41	Grey	None	Yellow	Red	Brown	None	None
4-Acetamido-4'-hydroxybiphenyl	0.12	0.40	Grey	Brown*	None	None	None	None	None
4-Amino-4'-hydroxybiphenyl	0.21	0.17	Green	Green	Orange	Mauve	None	None	None
4-Acetamido-3-hydroxybiphenyl	0.32	0.52	Grey	Brown*	None	None	Blue	None	None
4-Amino-3-hydroxybiphenyl	0.33	0.17	Grey	Black	Orange	Mauve	Blue	Yellow	Grey
4-(<i>N</i> -Hydroxyacetamido)-biphenyl	0.10–0.35	0.25–0.50	Grey	Black*	Pink	Pink	None	None	Purple
4-(Hydroxyamino)biphenyl	0.39	†	Grey	Black	Yellow	Red	Green	Mauve*	None
2-Aminofluorene	0.45	0.26	Blue	Brown*	Yellow	Red	None	None	None
2-Acetamido-7-hydroxyfluorene	0.11	0.44	Grey	Black*	None	None	Blue	None	Grey
2-Amino-7-hydroxyfluorene	0.19	0.09	Green	Green	Orange	Mauve	Blue	Mauve*	None
2-Acetamido-1-hydroxyfluorene	0.34	0.52	Grey	Brown*	None	None	Blue	None	Grey
2-Amino-1-hydroxyfluorene	0.37	0.10	Grey	Black	Orange	Blue	Blue	Blue	None
2-(<i>N</i> -Hydroxyacetamido)fluorene	0.10–0.30	0.25–0.50	Grey	Black*	Pink	Pink	None	None	Purple
2-(Hydroxyamino)fluorene	0.41	†	Grey	Black	Yellow	Red	Yellow	Mauve*	None
2-(<i>N</i> -Hydroxyacetamido)-naphthalene	0.10–0.30	0.25–0.48	Grey	Black*	Pink	Pink	None	None	Purple
2-Acetamido-1-hydroxy-naphthalene	0.36	0.51	Mauve	Mauve	Mauve	Mauve	Mauve	Pink	Mauve
<i>N</i> -Hydroxyacetanilide	0.10–0.30	0.20–0.45	Grey	Black*	Pink	Pink	None	None	Purple
2-Hydroxyacetanilide	0.29	0.49	Grey	Black*	None	None	Blue	None	None

* Visible after 10 min.

† Decomposed.

phenyl (VI), 4-aminobiphenyl (VII) and 4-amino-3-hydroxybiphenyl (V). The *N*-oxidation of the arylamine (VII) to the *N*-hydroxy metabolite (VI) was shown to be a reversible reaction. The reduction (VI) $\rightarrow$ (VII), however, may be partly a chemical reaction, since, when the microsomes were heated at 100° for 5 min. before incubation, trace amounts of the arylamine (VII) could still be

detected. This is in contrast with the other reactions studied. With fresh microsomes, reaction mixtures with 4-(hydroxyamino)biphenyl (VI) added as substrate also contained 4-amino-3-hydroxybiphenyl (V), but this could have arisen from the *o*-hydroxylation of the arylamine (VII) which is also formed in this system. These results are summarized in Table 2 and Scheme 1.

Scheme 1. Reactions which have been demonstrated by thin-layer chromatography in the metabolism of 4-acetamidobiphenyl by rabbit-liver fractions. $\longrightarrow$, Catalysed by microsomes and unaffected by KF; $-\cdot-\cdot-$, inhibited by KF; $\cdots\cdots\cdots$, catalysed by soluble fraction.

Table 2. Metabolism of 4-acetamidobiphenyl by rabbit-liver microsomes

Reaction mixtures and incubation conditions were as described in the Experimental section with KF omitted as indicated. The metabolites were identified on thin-layer chromatograms by comparison of their R_F values and colour reactions with those of authentic specimens as shown in Table 1. Both 4-acetamidobiphenyl and 4-aminobiphenyl were hydroxylated in the 4'-position in the presence of KF but these metabolites are not included in the Table.

Substrate	KF	Metabolites identified
4-Acetamidobiphenyl	Absent	4-(<i>N</i> -Hydroxyacetamido)biphenyl, 4-(hydroxyamino)biphenyl, 4-aminobiphenyl, 4-amino-3-hydroxybiphenyl
	Present	4-(<i>N</i> -Hydroxyacetamido)biphenyl
4-(<i>N</i> -Hydroxyacetamido)biphenyl	Absent	4-(Hydroxyamino)biphenyl, 4-aminobiphenyl, 4-amino-3-hydroxybiphenyl
	Present	None
4-(Hydroxyamino)biphenyl	Absent	4-Aminobiphenyl, 4-amino-3-hydroxybiphenyl
	Present	4-Aminobiphenyl, 4-amino-3-hydroxybiphenyl
4-Aminobiphenyl	Absent	4-(Hydroxyamino)biphenyl, 4-amino-3-hydroxybiphenyl
	Present	4-(Hydroxyamino)biphenyl, 4-amino-3-hydroxybiphenyl

Conditions affecting the formation of 4-(*N*-hydroxyacetamido)biphenyl (I) from 4-acetamidobiphenyl (II). In qualitative experiments in which 4-(*N*-hydroxyacetamido)biphenyl (I) was detected on thin-layer chromatograms, the reaction mixtures were buffered with sodium phosphate, pH 7.4, but quantitative experiments with the complete reaction mixtures indicated that the maximum amount of 4-(*N*-hydroxyacetamido)biphenyl (I) was formed at pH values of 8 or higher. Determinations with 0.1M-tris, $\text{Na}_2\text{H}_2\text{P}_2\text{O}_7$ - $\text{K}_4\text{P}_2\text{O}_7$ or glycine-sodium hydroxide buffers at various pH values showed that the greatest amounts were obtained with glycine-sodium hydroxide buffer, pH 8.6.

With this buffer system and microsomes from 1 g. of liver the amount of 4-(*N*-hydroxyacetamido)biphenyl (I) formed was proportional to the time of incubation up to 30 min., but this rate could not be maintained for 45 min. With 30 min. incubation periods the amount of the *N*-hydroxy metabolite (I) formed was proportional to the concentration of microsomes up to the amount obtained from 1 g. of liver in 10 ml. of reaction mixture.

The cofactor requirements to obtain the maximum amount of 4-(*N*-hydroxyacetamido)biphenyl (I) from 4-acetamidobiphenyl (II) were studied with 0.1M-glycine-sodium hydroxide buffer, pH 8.6, 30 min. incubation periods and microsomes from 1 g. of liver (27 mg. of protein) (Table 3). This shows that NADPH is essential for the reaction, since if either NADP^+ or the system which reduces it to NADPH (glucose 6-phosphate and glucose 6-phosphate dehydrogenase) is omitted from the incubation mixture no reaction takes place. The addition of NADPH to the reaction mixture has no effect on the amount of 4-(*N*-hydroxyacetamido)biphenyl formed and hence the role of NADH is not understood, since this cofactor is ineffective itself but causes an increase in the amount of 4-(*N*-hydroxyacetamido)biphenyl (I) formed in the presence of NADPH. The *N*-hydroxylation does not occur under anaerobic conditions, showing that oxygen is also required, and the enzymic nature of the reaction is demonstrated by the fact that 4-(*N*-hydroxyacetamido)biphenyl (I) is not formed if the microsomes are heated at 100° for 5 min. before incubation.

The enhancement caused by potassium fluoride is attributed to protection of the 4-(*N*-hydroxyacetamido)biphenyl (I) from deacetylation, and the highest values for the *N*-hydroxy metabolite (I) were obtained by the addition of 1 m-mole of potassium fluoride (final concn. 0.1M). With the complete reaction mixture and 30 min. incubation period 0.17 μmole of 4-(*N*-hydroxyacetamido)biphenyl (I) was formed; therefore this amount was added to a complete incubation mixture (with the

exception of 4-acetamidobiphenyl, II) to determine the amount that could be recovered. Without incubation 98 % was recovered but after 30 min. of incubation recovery values were 70 % in the presence and 27 % in the absence of 1 m-mole of potassium fluoride. By adding 2 m-moles of potassium fluoride the recovery of added 4-(*N*-hydroxyacetamido)biphenyl (I) could be increased to 88 %, but this concentration of potassium fluoride decreased the amount of 4-(*N*-hydroxyacetamido)biphenyl (I) formed from 4-acetamidobiphenyl (II). The values reported in Table 3 have not been corrected for recovery and represent the amount of 4-(*N*-hydroxyacetamido)biphenyl (I) present after incubation for 30 min., and correspond to the formation of 0.013 $\mu\text{mole/mg.}$ of protein/hr. However, it is unlikely that this represents the true rate of *N*-hydroxylation since the overall reaction is complicated by the fact that the substrate is also metabolized by other routes and the product is further metabolized by this system.

N-Hydroxylation of *N*-acetylarylamines, other than 4-acetamidobiphenyl (II), by rabbit-liver microsomes. (a) 2-Acetamidofluorene. Thin-layer chromatograms of reaction mixtures containing rabbit-liver microsomes, the cofactors described in the Experimental section and 2-acetamidofluorene as substrate showed a metabolic pattern similar to that described for 4-acetamidobiphenyl (II). The metabolites were identified by comparison with authentic specimens, and the chromatographic properties and colour reactions used for identification are shown in Table 1. In the presence of potassium fluoride, 2-acetamido-7-hydroxyfluorene and 2-(*N*-hydroxy-

Table 3. Factors effecting the formation of 4-(*N*-hydroxyacetamido)biphenyl from 4-acetamidobiphenyl by rabbit-liver microsomes

Complete reaction mixture contained: rabbit-liver microsomes from 1 g. of liver; glycine-NaOH buffer, pH 8.6 (1 m-mole); 4-acetamidobiphenyl (2 μmoles); nicotinamide (400 μmoles); NADP^+ (1 μmole); NADH (2 μmoles); glucose 6-phosphate dehydrogenase (25 $\mu\text{g.}$); KF (1 m-mole); in total vol. 10 ml. Incubations were in air for 30 min. at 37°.

Constituent omitted from complete reaction mixture	4-(<i>N</i> -Hydroxyacetamido)-biphenyl (μmole)
NADP^+	0
Glucose 6-phosphate + glucose 6-phosphate dehydrogenase	0
Air	0
NADH	0.12
NADH replaced by NADPH (2 μmoles)	0.11
KF	0.08
Nil	0.17
Nil*	0

* Microsomes heated at 100° for 5 min. before incubation.

Table 4. *Isomerization of N-hydroxyacetamidoaryl compounds by soluble fraction of rabbit liver*

Reaction mixtures contained: substrate (2 μ moles); soluble liver fraction (2 ml.); NADH (2 μ moles); phosphate buffer (500 μ moles), pH 7.4; in total vol. 10 ml. Incubations were in air for 1 hr. at 37°.

Substrate	<i>o</i> -Hydroxy metabolite
<i>N</i> -Hydroxyacetanilide	<i>o</i> -Acetamidophenol
2-(<i>N</i> -Hydroxyacetamido)naphthalene	2-Acetamido-1-naphthol
4-(<i>N</i> -Hydroxyacetamido)biphenyl	4-Acetamido-3-hydroxybiphenyl
2-(<i>N</i> -Hydroxyacetamido)fluorene	2-Acetamido-1-hydroxyfluorene

acetamido)fluorene were the only metabolites identified, although chromatograms showed two other compounds which reacted with the Folin and Ciocalteu's reagent. These compounds were possibly other unidentified ring-hydroxylation products. When 2-(*N*-hydroxyacetamido)fluorene was added as substrate in the presence of potassium fluoride no new compounds were visible, but in the absence of potassium fluoride 2-amino-7-hydroxyfluorene, 2-amino-1-hydroxyfluorene, 2-(hydroxyamino)-fluorene and 2-aminofluorene were formed. 2-(Hydroxyamino)fluorene was reduced to 2-amino-fluorene and the addition of either of these compounds as substrate resulted in the appearance of the *o*-hydroxy metabolite (2-amino-1-hydroxy-fluorene) in an analogous manner to the metabolism of 4-acetamidobiphenyl (II).

(b) *N*-Acetylbenzidine. On incubation of 4-acetamido-4'-aminobiphenyl (*N*-acetylbenzidine) with microsomes under conditions which give maximum amounts of 4-(*N*-hydroxyacetamido)-biphenyl (II), one major metabolite is formed. The chromatographic behaviour and colour reactions of this metabolite indicate that it is 4'-amino-4-(*N*-hydroxyacetamido)biphenyl, since it has similar properties to the four *N*-hydroxyacetamidoaryl compounds listed in Table 1.

(c) 2-Acetamidonaphthalene and acetanilide. Although the corresponding *N*-hydroxy derivatives were available as reference compounds, attempts to identify the corresponding *N*-hydroxyacetamidoaryl compounds by using either 2-acetamidonaphthalene or acetanilide as substrates were not successful. The reaction mixtures used in these experiments were similar to those which gave maximum amounts of *N*-hydroxy metabolite from 4-acetamidobiphenyl (II).

Isomerization of N-hydroxyacetamidoaryl compounds by the soluble fraction of rat or rabbit liver. In initial experiments on the further metabolism of 4-(*N*-hydroxyacetamido)biphenyl (I) by rabbit-liver microsomes, soluble liver fraction was used as a source of glucose 6-phosphate dehydrogenase, and 4-acetamido-3-hydroxybiphenyl (IV) was detected on thin-layer chromatograms of the reaction mixtures. Further investigation showed that 4-(*N*-hydroxyacetamido)biphenyl (I) was also converted into 4-acetamido-3-hydroxybiphenyl

(IV) when microsomes were omitted from the reaction mixture. Preliminary experiments indicate that the factor in the soluble liver fraction which catalyses this reaction is an enzyme. The reaction proceeds in the presence of NAD⁺, NADH or NADPH, but only trace amounts could be detected without the addition of any of these co-factors and the activity is destroyed by heating at 100° for 5 min. It is possible that this is a general reaction for *N*-hydroxyacetamidoaryl compounds since all these compounds so far tested in this system have been isomerized to the corresponding *o*-hydroxy derivative (Table 4) by the soluble fraction of liver from rats or rabbits.

DISCUSSION

Studies on the metabolites of 4-aminobiphenyl (VII) and 4-acetamidobiphenyl (II) in urine have shown that hydroxylation takes place in the 3-position of the compounds in the dog (Bradshaw & Clayson, 1955), in the 4'-position of the compounds in the rabbit (Bradshaw, 1957) and on the nitrogen atom of the compounds in the rat and dog (Miller *et al.* 1961*b*), the metabolites being excreted as conjugates with sulphuric acid or glucosiduronic acid. In the presence of NADPH and oxygen, rabbit-liver microsomes catalysed all these oxidations. Scheme 1 shows reactions in which the products have been identified by thin-layer chromatography, but it is probable that further investigation would reveal additional reactions. A study of the conditions that affect the equilibrium of the reaction arylamine $\rightleftharpoons$ arylhydroxylamine and the formation of *o*-hydroxy metabolites from this system may help to explain the wide range in susceptibility to the carcinogenic amines.

The major metabolites in the urine of a rabbit dosed with 2-acetamidofluorene were 2-(*N*-hydroxyacetamido)fluorene and 2-acetamido-7-hydroxyfluorene (Irving, 1962*b*), and these were also formed by rabbit-liver microsomes (Irving, 1962*a*). In the present work the metabolism of 2-acetamidofluorene by rabbit-liver microsomes was analogous to that shown for 4-acetamidobiphenyl (II) in Scheme 1. In the absence of potassium fluoride, however, deacetylated metabolites [2-amino-

fluorene, 2-(hydroxyamino)fluorene, 2-amino-7-hydroxyfluorene and 2-amino-1-hydroxyfluorene] were also formed. The rabbit does not excrete 2-acetamido-1-hydroxyfluorene as a metabolite of 2-acetamidofluorene (Irving, 1962b), and this compound could not be detected in experiments with rabbit-liver microsomes in which deacetylation was inhibited by potassium fluoride. Therefore the 2-amino-1-hydroxyfluorene which is formed in the absence of potassium fluoride is unlikely to have arisen by deacetylation of 2-acetamido-1-hydroxyfluorene. This suggests that either deacetylation is necessary before *o*-hydroxylation can take place or that the 2-amino-1-hydroxyfluorene has been formed by rearrangement of 2-(hydroxyamino)fluorene.

Experiments with 2-acetamido[9-¹⁴C]fluorene have shown that in the rat 2-(*N*-hydroxyacetamido)fluorene gives rise to 2-acetamido-1-hydroxyfluorene (Miller & Miller, 1960). This rearrangement is catalysed by the soluble fraction of rat or rabbit liver. Although the soluble fraction of rat liver can reduce 2-(*N*-hydroxyacetamido)fluorene to 2-acetamidofluorene with NADH and NADPH (Lotlikar, Miller & Miller, 1963), it is unlikely that the 2-acetamido-1-hydroxyfluorene has arisen by reduction followed by hydroxylation since the hydroxylation of foreign compounds by soluble liver fractions has not been reported. The rearrangement is probably a general reaction for this type of compound because *N*-hydroxyacetanilide, 2-(*N*-hydroxyacetamido)naphthalene and 4-(*N*-hydroxyacetamido)biphenyl (I) are also converted into the corresponding *o*-hydroxy derivatives (Table 4). Preliminary experiments suggest this rearrangement is enzymic but the specificity for the type of substrate is not known. The rearrangement of *N*-hydroxyarylamines could not be followed because the soluble liver fractions rapidly reduce these compounds. It is possible that the glucosiduronic acid conjugates of aryl hydroxylamines are not changed by the enzyme, so that rapid conjugation of the *N*-hydroxy metabolites may prevent rearrangement and thus account for the fact that 1-acetamido-1-hydroxyfluorene is not excreted by the rabbit.

Benzidine is hydroxylated in the 3-position (Bradshaw & Clayson, 1955) and acetylated by the dog, rabbit, rat, mouse, guinea pig and man (Engelbertz & Babel, 1953; Clayson, 1959; Sciarini & Meigs, 1961), whereas Troll *et al.* (1963) have found a *N*-hydroxyacetamido compound in the urine of patients given benzidine. When rabbit-liver microsomes were incubated with 4-acetamido-4'-aminobiphenyl (*N*-acetylbenzidine) in reaction mixtures which support maximum *N*-hydroxylation of 4-acetamidobiphenyl (II), and include potassium fluoride, 4'-amino-4-(*N*-hydroxyacetami-

do)biphenyl could be detected. Thus three acetylarylamines, 4-acetamidobiphenyl (II), 2-acetamidofluorene and *N*-acetylbenzidine, were all converted into *N*-hydroxy metabolites by rabbit-liver microsomes. The failure to detect the corresponding metabolites from 2-acetamidonaphthalene and acetanilide may be because they were not formed or because they were formed and rapidly metabolized. Further metabolism could take place by reduction, rearrangement, deacetylation or reaction with microsomal constituents. Some chemical properties of arylhydroxylamines indicate possible reactions that may also take place in biological systems. 2-Naphthylhydroxylamine reacts with phosphoric acid, sulphuric acid, L-cysteine, *N*-acetyl-L-cysteine and glutathione to form 2-amino-1-naphthyl dihydrogen phosphate, 2-amino-1-naphthyl sulphuric acid, *S*-(2-amino-1-naphthyl)-L-cysteine, 2-amino-1-naphthylmercapturic acid and *S*-(2-amino-1-naphthyl)glutathione respectively (Boyland, Manson & Nery, 1960, 1962). There is evidence that this type of reaction also takes place *in vivo* between thiols and *N*-hydroxy metabolites of arylamines since aniline is converted into *o*-aminophenylmercapturic acid by rats and rabbits and 2-aminonaphthylmercapturic acid is a metabolite of 2-naphthylamine (Boyland, Manson & Nery, 1963). Further, some of the metabolites of 2-acetamidofluorene have the properties of mercapturic acids (Weisburger, Grantham & Weisburger, 1962).

SUMMARY

1. Rabbit-liver microsomes metabolized 4-acetamidobiphenyl by the following reactions: 4-acetamidobiphenyl $\rightarrow$ 4-(*N*-hydroxyacetamido)biphenyl $\rightarrow$ 4-(hydroxyamino)biphenyl $\rightleftharpoons$ 4-amino-biphenyl $\rightarrow$ 4-amino-3-hydroxybiphenyl. The initial *N*-hydroxylation required NADPH and oxygen but maximum amounts of 4-(*N*-hydroxyacetamido)biphenyl were obtained when NADH and potassium fluoride were also present. Potassium fluoride inhibits the deacetylation of 4-(*N*-hydroxyacetamido)biphenyl and thus reduces further metabolism.

2. *N*-Acetyl-*N*-hydroxy metabolites were also identified from 2-acetamidofluorene and *N*-acetylbenzidine, but not from 2-acetamidonaphthalene or acetanilide, under conditions which gave maximum amounts of 4-(*N*-hydroxyacetamido)biphenyl from 4-acetamidobiphenyl.

3. 4-(*N*-Hydroxyacetamido)biphenyl is converted into 4-acetamido-3-hydroxybiphenyl by the soluble fraction of rat or rabbit liver, and *N*-hydroxyacetanilide, 2-(*N*-hydroxyacetamido)naphthalene and 2-(*N*-hydroxyacetamido)fluorene are also isomerized to the corresponding *o*-hydroxy derivatives.

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Fatty Acid Synthesis in Cell-Free Preparations of Rat Liver containing Phosphorylating Mitochondria

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If rats are treated with thyroxine, fatty acid synthesis is depressed in cell-free fractions of their livers from which all the particles sedimenting at 10000*g* for 20 min., including the mitochondria, have been removed by centrifugation ('S₁₀ fractions') (Scaife & Migicovsky, 1957; Fletcher & Myant, 1960). Fletcher & Myant (1961*b*) have shown that the S₁₀ fraction from thyroxine-treated rats ('treated S₁₀ fractions') contains very little glycogen, and that synthesis of fatty acids can be partially restored by adding either glycogen or ATP *in vitro*. Since glycogen is the main source of

energy for the regeneration of ATP in S₁₀ fractions from untreated rats, Fletcher & Myant (1961*b*) suggested that the depression of fatty acid synthesis in the treated S₁₀ fractions was due indirectly to the fall in glycogen content of the liver brought about by thyroxine. They pointed out, however, that the energy for the regeneration of ATP in normally respiring liver cells is generally thought to be derived from oxidation in the mitochondria, and not from the breakdown of glycogen. It was therefore desirable to study the effect of thyroxine treatment on fatty acid synthesis in liver prepa-