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HYDROXYLATION OF ALKYL AND HALOGEN SUBSTITUTED ANILINES AND ACETANILIDES BY MICROSOMAL HYDROXYLASES

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Abstract—The hydroxylation of a variety of halo and alkyl substituted anilines and acetanilides with hepatic microsomal preparations was studied. 4 Chloro- and 4-fluoro-acetanilides and anilines were hydroxylated to the corresponding 4-hydroxy derivatives with loss of halogen. The 4-chloro derivatives were extremely poor substrates for the aryl hydroxylases. 3-Methyl-, 3-chloro-, and 3-fluoro-acetanilides were metabolized to the corresponding 4-hydroxy-3-substituted acetanilide. 4-Methylaniline, 4-methyl-acetanilide, and 4-ethylacetanilide were not substrates for aryl hydroxylation, but instead underwent side-chain oxidation to form, respectively, 4-hydroxymethylaniline, 4-hydroxymethylacetanilide, and 4-(1′-hydroxyethyl)acetanilide. The hydroxymethyl compounds were further oxidized to aldehydes.

THE DRUG-metabolizing enzymes of hepatic microsomes effect a variety of oxidative transformations,[1] among which are aromatic[2] and aliphatic hydroxylation.[3, 4] A number of alkyl- and halogen-substituted anilines and acetanilides have now been studied with microsomal preparations in order to ascertain the effect of these substituents on the orientation and extent of aromatic hydroxylation. Because of recent reports on the migration of halogen[5–7] and other substituents[8] during enzymatic hydroxylation, the metabolic products from these substituted anilines and acetanilides were also examined for instances of hydroxylation-induced migrations of substituents.

MATERIALS

4-Fluoroacetanilide, 3-fluoroacetanilide, 4-methylacetanilide, 3-methylacetanilide, and 4-ethylacetanilide were prepared by acetylation of the corresponding amine with acetic anhydride and sodium acetate in a mixture of acetone–water. Published procedures were followed for the preparation of 3-methyl-4-hydroxyacetanilide,[9] 3-hydroxy-4-methylacetanilide,[10] and 3-chloro-4-hydroxyacetanilide.[11] 3-Fluoroaniline and 4-ethylaniline were from Aldrich Chemical Co.; the remaining anilines, acetanilides, and intermediates were from Eastman Organic Chemicals.

METHODS

Microsomal hydroxylations. Rabbit liver microsomes were prepared and incubated with various acetanilides and anilines as described in Table 1.[12] Products were extracted

into ethyl acetate and purified by paper chromatography (Whatman No. 1; benzene–HOAc–H_2O, 2:2:1) and by TLC (Silica gel GF; benzene–methanol–HOAc, 90:16:8). The anilines were extracted and then acetylated with acetic anhydride and sodium acetate in a mixture of acetone–water prior to chromatography. Products were assayed by u.v. spectroscopy or, in the case of 4-hydroxyacetanilide, by colorimetry.[13] Phenols were detected by color reagents such as Folin-Ciocalteu, Gibbs, diazotized *p*-nitroaniline, and diazotized sulfanilic acid. Other metabolites were detected on paper or Silica gel GF with the aid of u.v. light. The bathochromic shift of the u.v. absorption spectrum of phenols on addition of base helped to differentiate between phenolic and nonphenolic products. Mass spectrometry and n.m.r. spectroscopy were used for further identification of products. The results are presented in Table 1.

TABLE 1. HYDROXYLATION OF ANILINES AND ACETANILIDES WITH HEPATIC HYDROXYLASES*

Substrate	Product	R_f	Conversion (μmole)
Aniline	4-Hydroxyaniline		0·80
4-Fluoroaniline	4-Hydroxyaniline		0·28
4-Chloroaniline†	4-Hydroxyaniline		trace
4-Methylaniline	4-Hydroxymethylaniline		
	4-Aminobenzaldehyde		
Acetanilide	4-Hydroxyacetanilide‡	0·51	1·30
4-Fluoroacetanilide	4-Hydroxyacetanilide‡	0·51	0·10
3-Fluoroacetanilide	3-Fluoro-4-hydroxyacetanilide	0·54	0·60
4-Chloroacetanilide†	4-Hydroxyacetanilide‡	0·51	trace
3-Chloroacetanilide	3-Chloro-4-hydroxyacetanilide‡	0·55	1·40
4-Methoxyacetanilide	4-Hydroxyacetanilide‡	0·51	0·65
4-Methylacetanilide	4-Hydroxymethylacetanilide‡	0·47	1·55
	4-Acetamidobenzaldehyde	0·60	0·20
3-Methylacetanilide	3-Methyl-4-hydroxyacetanilide‡	0·61	1·30
	5-Methyl-2-hydroxyacetanilide	0·85	0·20
	3-Hydroxymethylacetanilide	0·50	trace
4-Ethylacetanilide	4-(1′-Hydroxyethyl)acetanilide	0·53	1·25

* Incubations contain 3 ml of microsomal suspension corresponding to 750 mg liver, 1·5 ml; 0·5 M Tris buffer, pH 8·0; 5 μmole NADP; 2·5 μmole ATP, 25 μmole glucose 6-phosphate; 5 units glucose 6-phosphate dehydrogenase; and 5 μmole of substrate in a final volume of 5 ml. Conversion values are based on 5 μmole substrate incubated for 1 hr at 37°. R_f values are for Silica gel-GF, with benzene–methanol–HOAc (90:16:8).

† 25 μmole Substrate used; no detectable product was formed when 5 μmole substrate was used.

‡ R_f values identical to authentic compounds. The R_f value for 3-hydroxy-4-methylacetanilide is 0·68.

RESULTS

Both aniline and 4-fluoroaniline formed 4-hydroxyaniline as the major phenolic product in agreement with earlier reports.[2, 14] Five-fold higher substrate concentrations were required for 4-chloroaniline, which then gave only trace amounts of a phenolic product, 4-hydroxyaniline, identified by thin-layer and paper chromatography after prior acetylation. 4-Methylaniline formed 4-hydroxymethylaniline and *p*-aminobenzaldehyde (*cf.* 4-methylacetanilide).

Acetanilide,[2] 4-fluoroacetanilide, and 4-chloroacetanilide were all oxidized to 4-hydroxyacetanilide, which was identified by thin-layer and paper chromatography

and in addition by mass spectrometry (mol. wt. 151). Higher substrate concentrations were necessary to detect trace amounts of 4-hydroxyacetanilide formed from 4-chloroacetanilide. No 3-chloro-4-hydroxyacetanilide was detected from 4-chloroacetanilide (cf. Refs. 5–7) by paper or TLC.

3-Chloroacetanilide and 3-fluoroacetanilide were hydroxylated in the 4-position to form as major products either 3-chloro- or 3-fluoro-4-hydroxyacetanilide. The identity of the product from 3-chloroactanilide was confirmed by comparison of thin-layer and paper chromatographic properties, color reactions, mass and n.m.r. spectra with authentic 3-chloro-4-hydroxyacetanilide (Fig. 1). The identity of 3-fluoro-4-hydroxyacetanilide was confirmed by mass spectrometry (mol. wt. 169).

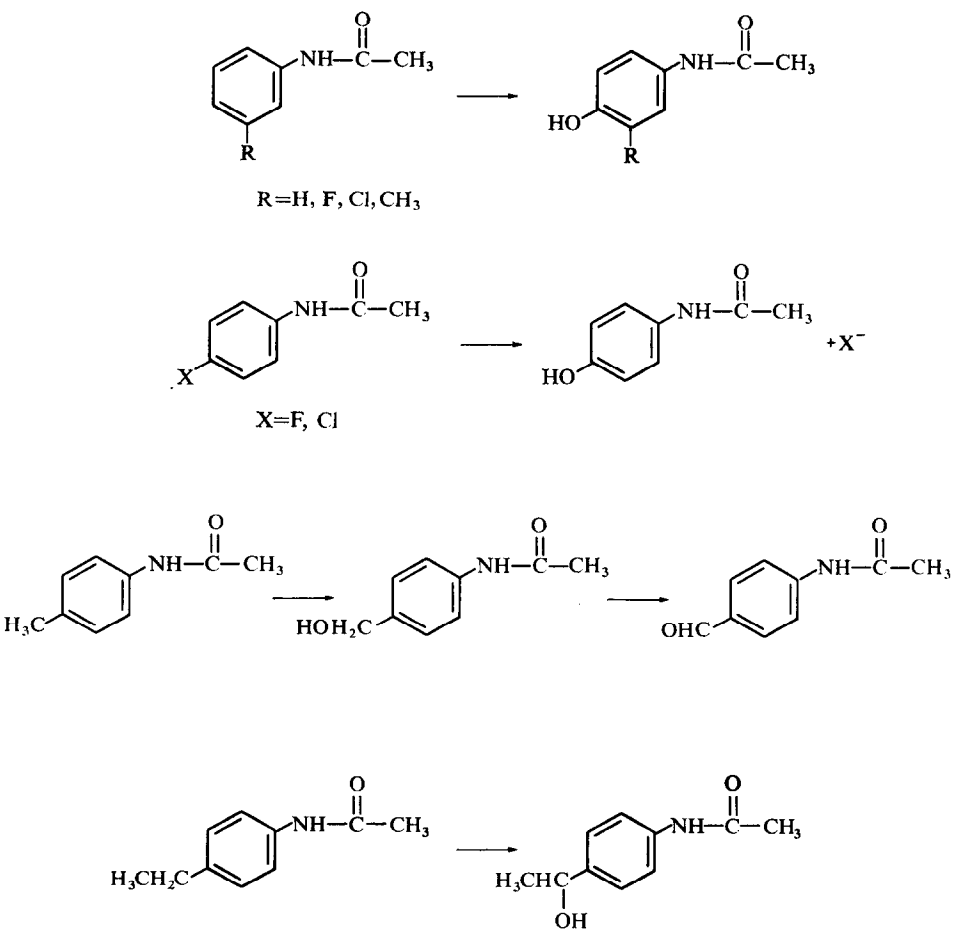

FIG. 1. Metabolism of substituted acetanilides.

Oxidation of 4-alkylacetanilides by liver microsomes led not to phenolic products but instead to oxidation of the side chain. 4-Methylacetanilide formed as a major product 4-hydroxymethylacetanilide (Fig. 1). The structure of this material was confirmed by mass spectrometry (mol. wt. 165) and by n.m.r. spectroscopy (one *N*-acetyl

group, 2·1 δ; one aryl-CH_2—O group, 4·5 δ; and 4-aromatic protons, A_2 B_2 pattern, 7·1 δ and 7·35 δ) and by comparison with an authentic sample prepared from 4-nitrobenzyl alcohol by reduction and acylation. A minor nonphenolic product formed by oxidation of 4-methylacetanilide had a u.v. maximum in methanol at 261 mμ. The material, on treatment with sodium borohydride, was reduced to 4-hydroxymethylacetanilide with a u.v. maximum at 244 mμ. The mass spectrum (mol. wt. 163) confirms the identity of the minor metabolite as 4-acetamidobenzaldehyde (Fig. 1). No 4-hydroxy-3-methylacetanilide or 3-hydroxy-4-methylacetanilide was detected chromatographically in extracts from the microsomal hydroxylation of 4-methylacetanilide. 4-Ethylacetanilide was converted to a nonphenolic product whose mass spectrum (mol. wt. 179, with an intense peak at 164, corresponding to loss of CH_3) indicated that it was 4-(1′-hydroxyethyl)acetanilide (Fig. 1).

By contrast, 3-methylacetanilide was hydroxylated to phenolic products. Only a trace of a nonphenolic metabolite was formed, which was judged to be 3-hydroxymethylacetanilide on the basis of chromatographic properties and color reactions. The major product from 3-methylacetanilide was shown to be 4-hydroxy-3-methylacetanilide (Fig. 1) by comparison with authentic material. A minor phenolic product was also formed and exhibited the high R_f values typical of 2-hydroxyacetanilides. The material gave phenolic color reactions with all reagents except Gibbs'.[15] Since a negative reaction of a phenol with Gibbs' reagent indicates a *para*-substituted phenol, this minor product is tenatively identified as 2-hydroxy-5-methylacetanilide.

DISCUSSION

The metabolism of aniline, acetanilide, and their halogen-containing congenors by microsomal preparations leads to 4-hydroxyanilines and acetanilides. The primary directing influence for aryl hydroxylation within this class of aromatic compounds thus appears to be derived from the acetamido or amino substituent.

Acetanilides with a 3-fluoro- or 3-chlorosubstituent remain excellent substrates for the aryl hydroxylases and form the corresponding 4-hydroxy derivative (Table 1, Fig. 1). The 4-fluoro or 4-chloro derivatives are, by contrast, poor substrates for aryl hydroxylation and form much smaller amounts of 4-hydroxyaniline or 4-hydroxyacetanilide with loss of the halogen. This effect is much more pronounced for the 4-chloro compounds where only at much higher substrate concentrations could trace amounts of phenolic products be detected.

The loss of fluorine on hydroxylation of 4-fluoroaniline and 4-fluoroacetanilide is not surprising in view of the report of Kaufman[16] on the enzymatic conversion of 4-fluorophenylalanine to tyrosine with (reductive) loss of fluoride ion. It was mildly surprising that with microsomes both 4-chloroaniline and 4-chloroacetanilide lost the halogen to form the 4-hydroxy derivative, since incubation of 4-chlorophenylalanine with phenylalaninehydroxylase leads via a hydroxylation-induced migration to 3-chlorotyrosine as the major product.[5] A minor product of the latter reaction is tyrosine, resulting from a loss of chloride ion. Both 4-chloroaniline and 4-chloroacetanilide were rigorously pure by the criteria of paper and TLC and melting point so that it is unlikely that the 4-hydroxy derivative was formed from trace amounts of aniline or acetanilide.

The inhibition of microsomal aryl hydroxylation by a halogen substituent is also observed with 6-fluoro-α-methyltryptamine (microsomes), 4-chloroamphetamine

(*in vivo*), and ethyl-2-(4′-chlorophenyl)-2-methylpropionate (Atromid) (microsomes). With none of these compounds were phenolic products detected,[17] whereas the related compounds, tryptamine[18] and 5-fluorotryptamine,[17] form 6-hydroxy derivatives with microsomes and the major pathway of metabolism (*in vivo*) for amphetamine is 4-hydroxylation.[19]

Thus, in many compounds, metabolism by aryl hydroxylation is inhibited when a halogen substituent is present at the position of usual oxygen substitution. Aryl hydroxylation is, however, unaffected or even enhanced by a halogen at the position ortho to oxygen substitution.

In the alkyl-substituted derivatives of acetanilide, the acetamido group still controls the course of oxidative metabolism. As with 3-chloro- and 3-fluoroacetanilide, microsomal preparations oxidize 3-methyl-acetanilide to a 4-hydroxy derivative. Small amounts of 2-hydroxy-5-methylacetanilide are formed as well as trace amounts of 3-hydroxymethylacetanilide.

The 4-alkylacetanilides, in contrast, are poor substrates for aryl hydroxylase and do not form detectable amounts of 3- or 4-hydroxy derivatives. Trace amounts of presumably 2-hydroxy derivatives are formed (cf. 3-methylacetanilide). Instead, the 4-alkyl substituent is oxidized to a benzylic alcohol, presumably by another enzyme. 4-Methylacetanilide forms 4-hydroxymethylacetanilide, 4-methylaniline forms the 4-hydroxymethylaniline, and 4-ethylacetanilide forms 4-(1′-hydroxyethyl)acetanilide. The 4-hydroxymethylacetanilide or 4-hydroxymethylaniline are further oxidized, respectively, to 4-acetamidobenzaldehyde or 4-aminobenzaldehyde. Further oxidation of alcohols to ketones or aldehydes is thought to be a function not of microsomal enzymes but of soluble alcohol dehydrogenase.[4] No evidence for the formation of 4-hydroxyacetanilide or 4-hydroxyaniline was obtained, although the conversion by guinea pig microsomes of aniline to hydroxyaniline via an intermediate, 4-hydroxymethylaniline, has been proposed.[20]

The oxidation of arylmethyl derivatives to hydroxymethyl compounds is not an uncommon pathway in drug metabolism.[1, 21–23] McMahon and Sullivan have reported the formation of optically active methylphenylcarbinol from ethylbenzene by microsomal preparations.[24] Although aryl hydroxylation and oxidation of arylmethyl groups probably are functions of different enzyme systems, Ichihara *et al.*[25] have reported that benzoate oxidase forms phthalide from *o*-toluic acid rather than the usual ring hydroxylation to a catechol.

Although the microsomal conversion of a methyl group to hydroxymethyl is not novel, it occurs as a major pathway of microsomal oxidation with 4-methylacetanilide and not with 3-methylacetanilide. It appears likely that both benzylic hydroxylation and aryl hydroxylation are similarly influenced by the position of other aryl substituents, in this case, the acetamido group. Oxidative *O*-dealkylation by microsomes is also known to depend on the nature and position of other aryl substituents. Axelrod[26] reported that 4-methoxyacetanilide is demethylated at a rate seven times the rate of demethylation of 2-methoxyacetanilide.

The migration of ring substituents during enzymatic hydroxylation of aromatic compounds[8] is relevant to studies on drug metabolism. The investigations reported here on microsomal hydroxylations of various substituted anilines and acetanilides have so far not furnished any new examples of alkyl or halogen compounds which undergo the "NIH shift". Two postulates, however, seem warranted on the basis

of the observations: (1) ring hydroxylation of drugs may often be blocked in congeners containing a halogen at the position of preferred aryl hydroxylation; and (2) drugs containing an alkyl substituent at the preferred site of aryl hydroxylation may instead undergo side-chain (benzylic) oxidation.

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