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# METABOLISM OF ACETANILIDES AND ANISOLES WITH RAT LIVER MICROSOMES

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**Abstract**—The metabolism of various substituted (F, Cl, Br, I, $CF_3$, $NO_2$) acetanilides and anisoles have been studied with 3-methylcholanthrene-induced rat liver microsomes. Metabolism of acetanilides and anisoles, which occurs primarily at the position *para*- to the acetamido or methoxy function, is markedly influenced by the presence of other substituents in the *ortho*-, *meta*- or *para*-positions. A novel loss of an adjacent substituent during hydroxylation *ortho*- to an iodo group was observed in the conversion of 4-iodoanisole to 3-hydroxyanisole. Migration of substituents (the NIH Shift) was not a significant metabolism with any of these substrates.

DURING the course of studies on the migration of ring substituents during hydroxylation of aromatic substrates,[1, 2] the metabolism of a variety of substituted anisoles and acetanilides have been investigated with preparations of liver microsomes. The metabolism of these substrates by liver microsomes from 3-methylcholanthrene-treated rats is reported in this paper.

## MATERIALS AND METHODS

The substrates were from commercial sources or were prepared by standard procedures. Reference phenolic products were from commercial sources or were prepared by standard procedures including hydroxylation with Fenton's reagent.* Livers from Sprague–Dawley male rats pretreated with 3-methylcholanthrene[4] were homogenized with 3 vol. (w/v) of isotonic KCl, centrifuged at 20,000 *g* for 20 min to afford a crude microsomal suspension. Incubations were carried out as described in the footnotes to the tables, and the products identified by comparison to reference standards. Products were arbitrarily classified as major, minor and trace metabolites, as described in the tables. The remainder of the substrate (>90 per cent) was recovered unchanged.

Typical identifications for products obtained after incubations of 2-, 3- and 4-fluoroacetanilide and of 2-, 3- and 4-fluoroanisole are given as examples.

*2-Fluoroacetanilide.* Two trace metabolites were detected by paper chromatography ($R_f$s 0·15 and 0·62). Insufficient matter (<0·1 μmole) was obtained for accurate quantitation by ultraviolet spectroscopy. Both compounds gave color reactions typical of phenols with Folin's reagent and diazotized *p*-nitroaniline. The product with $R_f$ 0·62 gave a color reaction with Gibbs' reagent indicative of a phenol unsubstituted in the *para*-position. The high $R_f$ and color reactions of this product are compatible only

* Extensive loss of fluorine to form 4-hydroxyanisole was noted during hydroxylation of 4-fluoroanisole with Fenton's reagent (Table 1, footnote[||]; *cf.* ref. 3).

TABLE 1. METABOLISM OF ANISOLE AND HALO-SUBSTITUTED ANISOLES WITH RAT LIVER MICROSOMES*

| Substrates | *p*-Hydroxylation | | | *m*-Hydroxylation | | | *o*-Hydroxylation | | | Demethylation | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Product | Conversion† | Retention time (Temp.)‡ | Product | Conversion† | Retention time (Temp.)‡ | Product | Conversion† | Retention time (Temp.)‡ | Conversion† | Retention Time (Temp.)‡ |
| Anisole§ | 4-OH | Major (4) | 12·5 min (138°) | | | | 2-OH | Minor (0·8) | 2·5 min (138°) | Minor (0·2) | 2·8 min (138°) |
| 2-Fluoroanisole | 4-OH | Major (2) | 16·3 min (130°) | | | | 6-OH | Minor (0·4) | 2·1 min (130°) | | |
| 3-Fluoroanisole | 4-OH | Major (3) | 4·8 min (130°) | | | | 6-OH | Minor (0·5) | 2·6 min (130°) | Trace | 3·8 min (130°) |
| 4-Fluoroanisole‖ | | | | 3-OH | Trace | 4·7 min (130°) | 2-OH | Major (4) | 2·3 min (130°) | Major (3) | 3·5 min (130°) |
| 2-Chloroanisole | 4-OH | Major (2) | 30·5 min (138°) | | | | 6-OH | Major (1) | 5·1 min (138°) | | |
| 3-Chloroanisole¶ | 4-OH | Trace | 6·7 min (138°) | | | | 2-OH | Major (4) | 6·7 min (138°) | Trace | 10·6 min (138°) |
| 4-Chloroanisole¶ | | | | 3-OH | Minor (0·4) | 6·7 min (138°) | 2-OH | Major (4) | 7·2 min (138°) | Major (3) | 10·4 min (138°) |
| 2-Bromoanisole | 4-OH | Major (2) | Broad 41 min (148°) | | | | 6-OH | Major (2) | 4·4 min (148°) | | |
| 3-Bromoanisole¶ | 4-OH | Minor (0·5) | 5·1 min (148°) | | | | 2-OH | Major (6) | 5·1 min (148°) | Trace | 9·6 min (148°) |
| 4-Bromoanisole¶ | | | | 3-OH | Minor (0·3) | 4·8 min (148°) | 2-OH | Major (3) | 5·8 min (148°) | Major (3) | 9·7 min (148°) |

| | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 2-Iodoanisole | 4-OH | Major (2) | Broad 37 min (162°) | | | | 6-OH | Major (3) | 4·3 min (148°) | | |
| 3-Iodoanisole | 4-OH | Major (4) | 12·6 min (148°) | | | | 6-OH | Trace | 10·9 min (148°) | Trace | 19·2 min (148°) |
| 4-Iodoanisole | 4-OH (Loss I) | Major (2) | 7·6 min (148°) | 3-OH (Loss I) | Major (2) | 8·1 min (148°) | | | | | |
| | | | | 3-OH | Major (1) | 12·2 min (148°) | 2-OH | Major (3) | 13·0 min (148°) | Major (3) | 19·6 min (148°) |
| 2,4-Dichloroanisole | | | | 5-OH | Minor (0·3) | 2·3 min (148°) | 2-OH | Minor (0·3) | 5·3 min (148°) | | |

* Incubations were carried out at 37° for 15 min with 10 ml of microsomal preparation, 3·5 ml of 0·5 M Tris buffer, pH 8·0, 15 μmoles ATP, 50 μmoles glucose-6-phosphate, 10 units glucose-6-phosphate dehydrogenase, 2 mg Tween 80 and 50 μmoles of substrate added in 0·1 ml acetone in a final volume of 15 ml. Solution was extracted with equal volume of ethyl acetate. The extract was dried $Na_2SO_4$, concentrated *in vacuo* and the phenolic products isolated by thin-layer chromatography, silica gel GF, chloroform, benzene, ethyl acetate (65:15:15). Detection was with Gibb's or Folin's reagent. Phenols were extracted from silica gel scrapings into ethyl acetate, concentrated and subjected to combined gas chromatography–mass spectrometry with a LKB-9000 combination gas chromatograph–mass spectrometer.

† Conversions for major and minor products (μmoles in parentheses) are estimates based on gas chromatography. Trace products were formed in quantities of less than 0·2 μmole.

‡ Retention times are on a 6-ft column with 3 per cent ECNSS-M on Gas Chrom Q at the temperature indicated.

§ Pretreatment of rats with 3-methylcholanthrene appeared to enhance 2- and 4-hydroxylation of anisole but not demethylation to phenol.

‖ 4-Fluoroanisole was converted with Fenton's reagent to a mixture of 4-hydroxyanisole (loss fluorine) and 2-hydroxy-4-fluoroanisole. A small amount of 4-fluorophenol and a trace of 3-hydroxy-4-fluoroanisole were also formed.

¶ The phenolic products were also converted to phenol and hydroxyanisoles by reductive dehalogenation using palladium on charcoal catalyst, hydrogen gas and a trace of triethylamine in tetrahydrofuran for 16 hr. The amounts of phenol and hydroxyanisoles were then analyzed by gas chromatography–mass spectrometry.

with the *ortho*-hydroxylated acetanilide, 6-hydroxy-2-fluoroacetanilide. This product, after isolation by paper chromatography, gave the expected mass spectrum (parent ion, m/e 169; base peak, m/e 127). The identity of this compound was confirmed by comparison with synthetic material. The other product appeared on the basis of $R_f$ value, color reactions and mass spectrum (parent ion, m/e 169; base peak, m/e 127) to be 4-hydroxy-2-fluoroacetanilide. Comparison of $R_f$ values, color reactions and mass spectrum with those of synthetic 4-hydroxy-2-fluoroacetanilide confirmed its identity.

3-*Fluoroacetanilide*. One major metabolite ($R_f$ 0·23) and a trace metabolite ($R_f$ 0·45) were detected by paper chromatography. The major product gave $R_f$ values, color reactions, ultraviolet spectrum ($\lambda_{max}$ 250 m$\mu$, $\epsilon_{max}$ 8800) and mass spectrum (parent ion, m/e 169; base peak, m/e 127) identical to those of synthetic 4-hydroxy-3-fluoroacetanilide. Based on ultraviolet spectroscopy, 1·4 $\pm$ 0·1 $\mu$moles of this product were formed in 15 min from 25 $\mu$moles of substrate. The trace metabolite ($<0{\cdot}1$ $\mu$mole) had an $R_f$ value and gave color reactions with Folin's reagent, diazotized sulfanilic acid and Gibbs' reagent typical of an *ortho*-hydroxyacetanilide. It was, therefore, either 6-hydroxy-3-fluoroacetanilide or 2-hydroxy-3-fluoroacetanilide. The mass spectrum exhibited the required parent ion, m/e 169 and base peak, m/e 127.

4-*Fluoroacetanilide*. Three metabolites ($R_f$s 0·10, 0·18 and 0·48) were detected by paper chromatography. These were identified by comparison to synthetic compounds as, respectively, 4-hydroxyacetanilide (mass spectrum; parent ion, m/e 151; base peak, m/e 109), 3-hydroxy-4-fluoroacetanilide (mass spectrum; parent ion, m/e 169; base peak, m/e 127) and 2-hydroxy-4-fluoroacetanilide (mass spectrum: parent ion, m/e 169; base peak, m/e 127).

2-*Fluoroanisole*. Phenolic metabolites were isolated by thin-layer chromatography and subjected to combined gas chromatography–mass spectrometry (Table 1). Two products were detected with retention times of 2·1 and 16·3 min. The mass spectra of these products (parent ion, m/e 142; base peak, m/e 127) identified them as *ortho*- or *para*-hydroxyanisoles since *meta*-hydroxyanisoles exhibit, in addition to those peaks, a significant parent-30 peak in their mass spectra. The low retention time (2·1 min) of the minor component is typical of *ortho*-hydroxyanisoles, indicating that this product is 6-hydroxy-2-fluoroanisole. The major product with retention time of 16·3 min must be 4-hydroxy-2-fluoroanisole. This was confirmed by comparison to synthetic material. Approximate conversions were estimated as 0·2 and 4 $\mu$moles, respectively, by gas chromatographic comparison with standard solutions of hydroxyanisoles or hydroxyfluoroanisoles.

3-*Fluoroanisole*. Phenolic products were isolated by thin-layer chromatography and analyzed by combination gas chromatography–mass spectrometry. Three metabolites were detected with retention times of 2·6, 3·8 and 4·8 min. The mass spectrum (parent ion, m/e 142; base peak, m/e 127) of the minor metabolite in conjunction with its low retention time of 2·6 min indicated it was one of the two possible fluorinated *ortho*-hydroxyanisoles. Its identity as 6-hydroxy-3-fluoroanisole was confirmed by comparison (retention time, mass spectrum) with synthetic material. The trace metabolite with retention time of 3·8 min was identified as 3-fluorophenol (mass spectrum: parent ion and base peak, m/e 112) by comparison with synthetic material. The major metabolite with retention time of 4·8 min was identified as 4-hydroxy-3-fluoroanisole by comparison with synthetic material.

4-*Fluoroanisole.* Phenolic products were isolated by thin-layer chromatography and analyzed by combination gas chromatography–mass spectrometry. Three metabolites were detected with retention times of 2·3, 3·5 and 4·7 min. These were identified as 2-hydroxy-4-fluoroanisole (major), 4-fluorophenol (major), and 3-hydroxy-4-fluoroanisole (trace) by comparison with synthetic compounds. With the latter compound, the mass spectrum with peaks at m/e 142, 127 and 112 had provided initial evidence for a *meta*-hydroxyanisole since the parent ion rather than the parent-15 peak was the base peak and a parent-30 peak, typical of *meta*-hydroxyanisoles, was present. This identification was confirmed by comparison with synthetic material.

## RESULTS AND DISCUSSION

The metabolism *in vitro* of various acetanilides and anisoles with 3-methylcholanthrene-induced rat liver microsomes are presented in Tables 1 to 6. The semiquantitative results presented in the tables are in substantial agreement with earlier findings[1] that the extent and position of enzymatic aryl hydroxylation with microsomal preparations is correlated with the reactivity of the aromatic ring to electrophilic attack and that attack at a preferred ring position may be blocked by a halo or alkyl substituent.[5] Steric factors cause the microsomal metabolism of aromatic rings to favor *para-* over *ortho*-hydroxylation. It is apparent in the present study that steric factors are indeed very important in determining the extent and direction of metabolism of aromatic substrates.

*Acetanilides.** For example, the effect of *ortho*-substituents (F, Cl, Br, I, $CH_3$, $CF_3$) in blocking the normal *para*-hydroxylation of acetanilides was particularly striking (Tables 2 and 3). The extent of this effect appears to be much greater than would have been predicted from consideration of ring deactivation because of steric effects of the *ortho*-substituent on electron donation by the acetamido group. The effect of *ortho*-F, Cl, Br or $CH_3$ substituents on *para*-hydroxylation of acetanilides was similar. With 2-iodo or 2-trifluoromethylacetanilide, the blocking effect was greater and no *para*-hydroxylated or other metabolites could be detected. In the case of 2-methoxyacetanilide, the *ortho*-substituent effect on *para*-hydroxylation was less pronounced.

No hydroxylation at the 5-position (*para-* to methoxy) was observed. The same *ortho*-substituent effect was also noted in acetanilides where the *para*-directed metabolism was by either alkyl hydroxylation (4-methylacetanilides) or by dealkylation (4-methoxyacetanilides). Thus, presence of a 2-methyl or 2-chloro substituent markedly reduced the hydroxylation of the 4-methyl group (Table 3) or the dealkylation of the 4-methoxy substituent in acetanilides. In both types of metabolisms, the 2-methyl group was more effective than the 2-chlorogroup in blocking attack of the *para*-substituent. It appears likely that steric effects of the *ortho*-substituent, which may involve the relative configuration of the acetamido group and the aromatic ring, prevent proper binding of acetanilides to the enzyme(s) and thus prevent metabolism by *para*-attack.

*Meta*-substituents either had no effect ($CH_3$) on the *para*-hydroxylation of acetanilides or caused only a slight reduction (F, Cl, Br, I, $CF_3$, $OCH_3$) in this metabolism. With 3-methoxyacetanilide, hydroxylation *para-* to the methoxy group yielded the 6-hydroxy derivative as a minor metabolite. With a disubstituted compound, such as

* For acetanilides, the terms *ortho*, *meta* and *para* are referenced to the acetamido group, while for anisoles they are referenced to the methoxyl group.

TABLE 2. METABOLISM OF ACETANILIDE AND HALO-SUBSTITUTED ACETANILIDES WITH RAT LIVER MICROSOMES*

| Substrate | *p*-Hydroxylation | | | *m*-Hydroxylation | | | *o*-Hydroxylation | | |
|---|---|---|---|---|---|---|---|---|---|
| | Product | Conversion† | $R_f$‡ | Product | Conversion† | $R_f$‡ | Product | Conversion† | $R_f$‡ |
| Acetanilide§ | 4-OH | Major (2·6) | 0·10 | | | | 2-OH | Trace | 0·38 |
| 2-Fluoroacetanilide | 4-OH | Trace | 0·15 | | | | 6-OH | Trace | 0·62 |
| 3-Fluoroacetanilide§ | 4-OH | Major (1.4) | 0·23 | | | | 6(2)-OH‖ | Trace | 0·45 |
| 4-Fluoroacetanilide | 4-OH (Loss F) | Trace | 0·10 | 3-OH | Trace | 0·18 | 2-OH | Trace | 0·48 |
| 2-Chloroacetanilide | 4-OH | Trace | 0·28 | | | | | | |
| 3-Chloroacetanilide§ | 4-OH | Major (2·0) | 0·23 | | | | 6(2)-OH‖ | Trace | 0·56 |
| 4-Chloroacetanilide§ | 4-OH (Loss Cl) | Trace (Minor) | 0·11 | 3-OH | Trace | 0·22 | 2-OH | Trace | 0·44 |
| | 4-OH-3-Cl | Trace (Minor) | 0·23 | | | | | | |
| 2-Bromoacetanilide | 4-OH | Trace | 0·33 | | | | | | |
| 3-Bromoacetanilide | 4-OH | Major (2·0) | 0·27 | | | | 6(2)-OH‖ | Trace | 0·62 |
| 4-Bromoacetanilide | 4-OH (Loss Br) | Trace | 0·11 | 3-OH | Trace | 0·32 | 2-OH | Trace | 0·62 |
| 2-Iodoacetanilide¶ | | | | | | | | | |
| 3-Iodoacetanilide | 4-OH | Major (1·8) | 0·27 | | | | 6-OH | Trace | 0·63 |
| 4-Iodoacetanilide | | | | 3-OH | Trace | 0·28 | 6-OH | Trace | 0·60 |
| 3,5-Dichloroacetanilide | 4-OH | Trace | —** | | | | | | |

* Incubations were carried out at 37° for 15 min with 10 ml of microsomal preparation, 3·5 ml 0·5 M Tris buffer, pH 8·0, 15 μmoles ATP, 50 μmoles glucose 6-phosphate, 10 units glucose 6-phosphate dehydrogenase, 2 mg Tween 80 and 25 μmoles of substrate added in 0·2 ml acetone in a final volume of 15 ml. Solution was saturated with NaCl and extracted two times with equal volume of ethyl acetate. The extract was dried $Na_2SO_4$, concentrated *in vacuo* and the products isolated by paper chromatography and identified by mass spectrometry using a Hitachi–Perkin–Elmer RMU-6D mass spectrometer.

† Conversions in μmoles product have been estimated from ultraviolet spectra of the major products (μmoles in parenthesis). Trace products (0·2 μmole or less) have been, in addition, designated as minor when the amount is barely detectable (<0·1 μmole).

‡ $R_f$ values, Whatman No. 1, benzene–acetic acid–water (2:2:1). Many products were then subsequently chromatographed on $SiO_2$-GF thin-layer chromatoplates with benzene–methanol–acetic acid (90:16:8).[5, 6] In most cases $R_f$ values were compared with authentic reference compounds. Gibbs' reagent, Folin's (phenol) reagent and diazotized *p*-nitroaniline were used to detect phenols and in the case of the Gibbs' reagent to distinguish between *ortho*- and *meta*-substituted, which give a positive color reaction and *para*-substituted phenols, which do not.

§ Previously reported hydroxylations[5] with *rabbit* liver microsomes gave similar results except that 4-chloroacetanilide did not form detectable amounts of 4-chloro-3-hydroxyacetanilide or 3-chloro-4-hydroxyacetanilide. (See text.)

‖ Identification of this phenolic product is tentative. Other possible isomers in parentheses. ¶ No metabolites detected.

** Chromatography carried out on $SiO_2$-GF thin-layer chromatoplates, benzene–ethyl acetate (4:5), $R_f$ for 3,5-dichloro-4-hydroxyacetanilide, 0·54. Identification by reductive dehalogenation to 4-hydroxyacetanilide using palladium on charcoal, hydrogen gas and a trace of triethylamine in tetrahydrofuran for 16 hr.

TABLE 3. METABOLISM OF ACETANILIDE AND METHYL- AND TRIFLUOROMETHYL-SUBSTITUTED ACETANILIDES WITH RAT LIVER MICROSOMES*

| Substrate | *p*-Hydroxylation | | | *m*-Hydroxylation | | | *o*-Hydroxylation | | |
|---|---|---|---|---|---|---|---|---|---|
| | Product | Conversion† | $R_f$‡ | Product | Conversion† | $R_f$‡ | Product | Conversion† | $R_f$‡ |
| Acetanilide§ | 4-OH | Major (2·6) | 0·10 | | | | 2-OH | Trace | 0·38 |
| 2-Methylacetanilide | 4-OH | Trace | 0·25 | | | | 6-OH | Trace | 0·55 |
| 3-Methylacetanilide§ | 4-OH | Major (2·5) | 0·19 | | | | 6(2)-OH‖ | Trace | 0·41 |
| 4-Methylacetanilide§ | 4-$CH_2OH$ | Major (1·6) | 0·16 | 3-OH | Trace | 0·28 | 2-OH | Trace (Minor) | 0·45 |
| 2,4-Dimethylacetanilide | 4-$CH_2OH$ | Trace | 0·19 | | | | | | |
| 2-Chloro-4-methylacetanilide | 4-$CH_2OH$ | Major (0·5) | 0·38 | | | | | | |
| 2-Trifluoromethylacetanilide¶ | | | | | | | | | |
| 3-Trifluoromethylacetanilide | 4-OH | Major (1·9) | 0·23 | | | | | | |
| 4-Trifluoromethylacetanilide¶ | | | | | | | | | |

*,†,‡,§, ‖, ¶ See footnotes to Table 2.

3,5-dichloroacetanilide, steric effects may well have been responsible for the marked reduction in the extent of *para*-hydroxylation.

*Para*-substituents either reduced the extent of *para*-hydroxylation of the acetanilides to a trace metabolism (F, Cl, Br) or completely blocked this route of metabolism (I, $CH_3$, $CF_3$, $OCH_3$). With certain *para*-substituted acetanilides, hydroxylation *meta*- to the acetamido group was now noted either as a trace (F, Cl, Br, $CH_3$, I) or minor ($OCH_3$) metabolic route. With 4-methyl- or 4-methoxyacetanilides, oxidative attack on the *para*-substituent was the major metabolism.[5] *Para*-hydroxylation of 4-haloacetanilides was accompanied by loss of halogen (F, Cl, Br) or by migration of halogen (Cl), but in all cases these were quantitatively insignificant metabolisms. The migration of halogen during hydroxylation of 4-chloroacetanilide and 4-bromoacetanilide with benzypyrene-induced rat liver microsomes has been reported[6] and the conversions reported were much higher than those obtained in the present study. Migration of halogen, however, in both the present study and the earlier report was found to be the least significant metabolic route. *Meta*- and *ortho*-hydroxylation and loss of halogen during *para*-hydroxylation were the major pathways. The results with 4-chloroacetanilide in both studies are in at least qualitative agreement as to the ratio of metabolites which are formed. In another study[5] using normal rabbit liver microsomes and 4-chloroacetanilide, only *para*-hydroxylation with loss of halogen could be detected. The mechanism of oxidative dehalogenation with *para*-haloacetanilides presumably involves formation of an oxidized intermediate which undergoes reductive loss of halogen. Such a mechanism has been proposed for the conversion of *para*-halophenylalanines to tyrosine.[7]

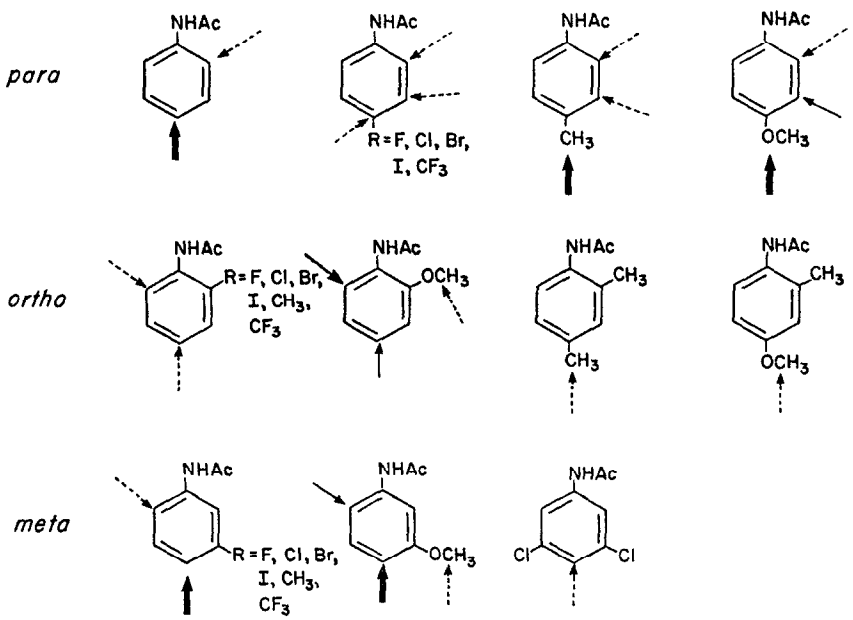


FIG. 1. Effect of substituents on hydroxylation of acetanilides with rat liver microsomes. (bold arrow), major metabolism; →, minor metabolism; and ——→, trace or no metabolism.

TABLE 4. METABOLISM OF ACETANILIDE AND METHOXY-SUBSTITUTED ACETANILIDES WITH RAT LIVER MICROSOMES*

| Substrate | *p*-Hydroxylation | | | *m*-Hydroxylation | | | *o*-Hydroxylation | | |
|---|---|---|---|---|---|---|---|---|---|
| | Product | Conversion† | $R_f$‡ | Product | Conversion† | $R_f$‡ | Product | Conversion† | $R_f$‡ |
| Acetanilide§ | 4-OH | Major (2·6) | 0·10 | | | | 2-OH | Trace | 0·38 |
| 2-Methoxyacetanilide | 4-OH‖ | Minor (0·3) | 0·21¶ | | | | 2-OH (desmethyl) | Trace | 0·36 |
| | | | | | | | 6-OH | Major (0·8) | 0·50 |
| 3-Methoxyacetanilide | 4-OH | Major (0·8) | 0·22¶ | 3-OH (Desmethyl) | Trace | 0·14 | 6-OH | Minor (0·2) | 0·37 |
| 4-Methoxyacetanilide§ | 4-OH (Desmethyl) | Major (0·8) | 0·11 | 3-OH | Minor (0·2) | 0·10 | 2-OH | Trace | 0·30 |
| 2-Methyl-4-methoxyacetanilide | 4-OH (Desmethyl) | Trace | 0·29 | | | | | | |
| 2-Chloro-4-methoxyacetanilide | 4-OH (Desmethyl) | Minor (0·2) | 0·26 | | | | | | |

*, †, ‡, § See footnotes to Table 2.

‖ This product was identified by conversion to 2,4-dimethoxyacetanilide with diazomethane. Analysis by TLC (silica gel, benzene, ethyl acetate, $CHCl_3$, 2:1:1) distinguished 2,4-dimethoxyacetanilide ($R_f$ 0·36) from 2,5-dimethoxyacetanilide ($R_f$ 0·45).

¶ These *para*-substituted phenols gave a positive Gibb's reaction.

*Ortho*-hydroxylation of acetanilides was observed in the present study as a significant metabolism only with 2- and 3-methoxyacetanilide. The results of these studies on metabolism of substituted acetanilides are presented in Fig. 1 and Tables 2–4.

*Anisoles.* The pathways of metabolism of anisole with microsomal preparations from rats that have been induced with 3-methylcholanthrene are by *para*-hydroxylation (major), *ortho*-hydroxylation (minor) and by *O*-demethylation (minor). With normal rat or rabbit[1] microsomes, the major metabolisms are *para*-hydroxylation and *O*-demethylation.

Most *ortho*-substituents (F, Cl, Br, I, $CH_3$) greatly reduced metabolism by *O*-demethylation but had little effect on *para*-hydroxylation. The ratio of *ortho*- to *para*-hydroxylation, however, tended to increase from 0·2 in anisole and 0·2 in 2-fluoroanisole to 0·25 in 2-methylanisole, 0·5 in 2-chloroanisole, 1·0 in 2-bromoanisole, and 1·5 in 3-iodoanisole, in spite of the fact that one less *ortho*-position was available for hydroxylation in these *ortho*-substituted anisoles. If this is because of the same steric effect which was noted in the *ortho*-substituted acetanilides, it is much less effective with the *ortho*-substituted anisoles in blocking metabolism by *para*-hydroxylation.

*Meta*-substitution with F, $CH_3$, or I substituents had little effect on *ortho*- or *para*-ring hydroxylation of anisoles. However, with Cl or Br substituents in the *meta*-position, *ortho*-hydroxylation (*para*- to the halogen) was greatly enhanced with a concomitant decrease in *para*-hydroxylation. This is in contrast to acetanilide metabolism where F, Cl, Br, I, $CH_3$, and $OCH_3$ substituents in the *meta*-position had little effect on *para*-hydroxylation. The explanation of this rather remarkable effect of *meta*-Cl or Br substituents in the anisole series might involve steric interactions which result

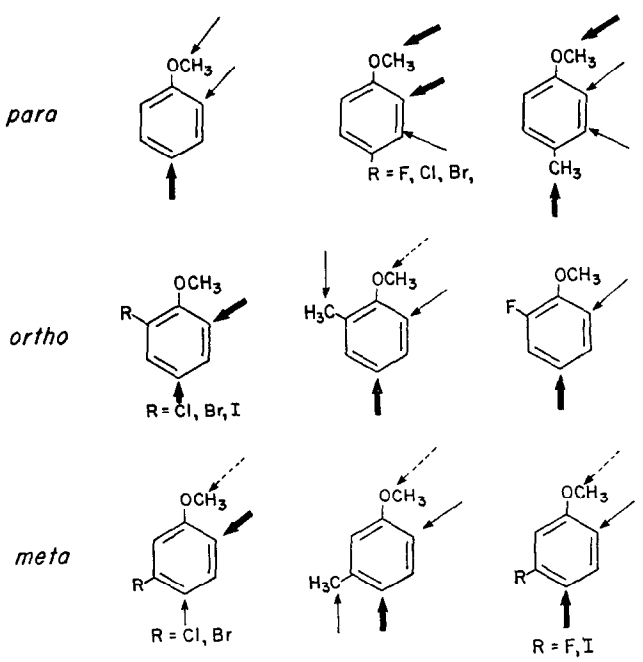


FIG. 2. Effect of substituents on the hydroxylation of anisoles with rat liver microsomes. (bold arrow), major metabolism; →, minor metabolism; and ⟶, trace or no metabolism.

TABLE 5. METABOLISM OF ANISOLE AND METHYL-SUBSTITUTED ANISOLES WITH RAT LIVER MICROSOMES*

| Substrates | *p*-Hydroxylation | | | *m*-Hydroxylation | | | *o*-Hydroxylation | | | Demethylation | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | Product | Conversion† | Retention time (148°)‡ | Product | Conversion† | Retention time (148°)‡ | Product | Conversion† | Retention time (148°)‡ | Conversion† | Retention time (148°)‡ |
| Anisole§ | 4-OH | Major (4) | 4·8 min | | | | 2-OH | Minor (0·8) | 1·2 min | Minor (0·2) | 1·5 min |
| 2-Methylanisole | 4-OH | Major (4) | 6·2 min | | | | 2-OH | Minor (0·8) | 1·3 min | Trace | 1·3 min |
| 3-Methylanisole | 4-OH | Major (5) | 5·6 min | | | | 6-OH | Minor (1) | 1·6 min | Trace | 1·5 min |
| 4-Methylanisole | | | | 3-OH | Minor (0·2) | 5·9 min | 2-OH | Minor (0·6) | 1·4 min | Major (3) | 1·8 min |
| 2,3-Dimethylanisole | 4-OH | Minor (0·8) | 7·6 min | | | | 6-OH | Minor (0·2) | 2·5 min | Trace | 2·8 min |
| 2,4-Dimethylanisole | | | | 5-OH | Major (0·8) | 7·7 min | 6-OH | Minor (0·4) | 2·6 min | Trace | 2·7 min |
| 2,5-Dimethylanisole | 4-OH | Major (3) | 6·3 min | | | | 6-OH | Trace | 2·5 min | Trace | 2·6 min |
| 2,6-Dimethylanisole | 4-OH | Trace | 7·0 min | 3-OH | Trace | 5·3 min | | | | Minor (0·2) | 1·6 min |
| 3,4-Dimethylanisole | | | | | | | 6-OH | Major (2) | 3·2 min | Trace | 3·4 min |
| 3,5-Dimethylanisole | 4-OH | Major (1·5) | 5·6 min | | | | 2-OH | Trace | 2·9 min | Minor (0·2) | 3·0 min |
| 3-Methyl-4-chloroanisole | | | | 5-OH | Trace | ~7 min | 6-OH | Minor (1) | 5·1 min | Major (4) | 6·8 min |

*, †, ‡, § See footnotes to Table 1. Nonphenolic metabolites were formed by hydroxylation of ring-methyl substituents in all cases but these metabolites were not examined after separation by the thin-layer chromatography.

in differing modes of binding only with Cl and Br. Anisoles containing *meta*-substituents (F, Cl, Br, I, $CH_3$) exhibited greatly decreased metabolism by *O*-demethylation.

In 3,5-dimethylanisole, *para*-hydroxylation between the methyl groups was still a major metabolism. In other dimethylanisoles, *para*-hydroxylation was blocked in 2,4-, and 2,6-dimethylanisoles, reduced in 2,3-dimethylanisole and unaffected in 2,5-dimethylanisole.

*Para*-substitution (F, Cl, Br, $CH_3$) in the anisole series blocked *para*-hydroxylation of the ring and markedly stimulated *O*-demethylation. This enhanced *O*-demethylation was blocked by a second substitutent *ortho*- to the methoxy group (2,4-dichloroanisole, 2,4-dimethylanisole) and, in one case, by a second substituent *meta*- to the methoxy group (3,4-dimethylanisole). In another case of 3,4-substitution, 3-methyl-4-chloroanisole, *O*-demethylation was still a major metabolism. *Ortho*-hydroxylation of anisole appeared to be stimulated by the presence of a *para*-halogen substituent (F, Cl, Br, I) but not by a *para*-methyl group. No migration or loss of F, Cl or Br substituents from the *para*-position was observed in the anisoles in distinction to the metabolism of 4-haloacetanilides (see above). *Meta*-hydroxylation was observed in anisoles which contained Cl, Br, I, or $CH_3$ substituents in the *para*-position. The metabolism of halo- and methyl-substituted anisoles is summarised in Fig. 2 and Tables 1, 4 and 5. With many of the isomeric halogenated hydroxyanisoles, separation by GLC or TLC was difficult and final identification often necessitated conversion of the products to *ortho*-, *meta*- and *para*-hydroxyanisoles by reductive dehalogenation (Table 1, footnote ¶). The mass spectra of hydroxyanisoles were also useful in identification of the isomeric hydroxyanisoles, since the *ortho*- and *para*-isomers tend to lose methyl to form the most intense peak in the mass spectrum. This loss is not as prominent with *meta*-hydroxyanisoles and an alternate fragmentation involving loss of $OCH_3$ is present.

The results obtained with microsomal metabolism of 4-iodoanisole were unexpected. In contrast to the other 4-haloanisoles, formation of 4-hydroxyanisole with loss of iodide was observed. In addition, 3-hydroxyanisole was a major metabolite as were 2-hydroxy-4-iodoanisole and 3-hydroxy-4-iodoanisole (Fig. 3). Incubation of iodinated hydroxyanisoles with microsomes did not result in deiodination which demonstrates that the 3-hydroxy-4-iodoanisole was not an intermediate in the formation of

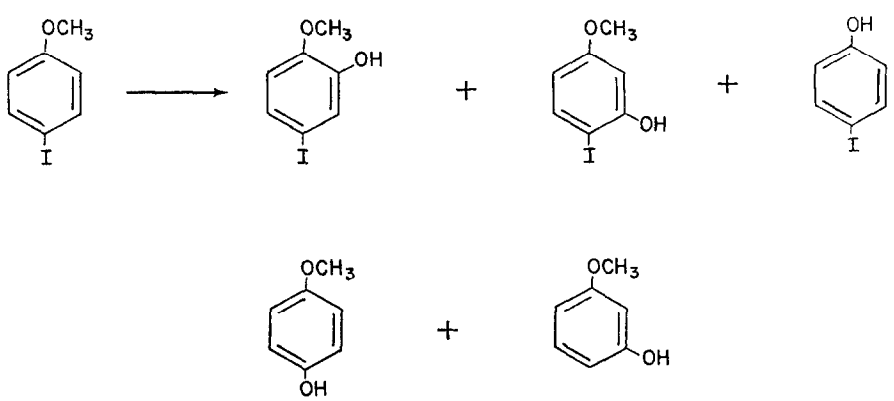


FIG. 3. Metabolism of iodoanisole with rat liver microsomes. The products are formed in a ratio of 3:1:3:2:2 respectively.

TABLE 6. METABOLISM OF NITROANISOLES WITH HEPATIC MICROSOMES FROM RATS PRETREATED WITH 3-METHYLCHOLANTHRENE*

| Substrate | Product | Conversion† | $R_f$* | Color‡ | Gibbs‡ |
|---|---|---|---|---|---|
| 2-Nitroanisole | 2-Nitro-4-hydroxyanisole | Trace | 0·26 | | Faint green |
| | 2-Nitro-6-hydroxyanisole | Trace | 0·35 | | Faint blue |
| | 2-Aminoanisole | Minor | 0·50 | | Light purple |
| | 2-Nitrophenol | Major | 0·86 | Yellow | |
| 3-Nitroanisole | 3-Nitro-6-hydroxyanisole | Minor | 0·22 | Yellow | |
| | 3-Aminoanisole | Major | 0·32 | | Blue-purple |
| | 3-Nitrophenol | Minor | 0·45 | Light yellow | Light green |
| 4-Nitroanisole | 4-Nitro-2-hydroxyanisole | Major | 0·26 | Light yellow | Light green |
| | 4-Nitrophenol | Major | 0·35 | Yellow | |
| | 4-Aminoanisole | Trace | 0·46 | | Blue-purple |

* Incubations carried out as described in Table 1. Ethyl acetate extracts chromatagraphed on thin-layer chromatoplates, $SiO_2$-GF, benzene–ethyl acetate, 95:5. Detection with Gibbs' or Folin's reagent. Products were extracted from plate into ethyl acetate for estimation by ultraviolet spectroscopy and identification by mass spectrometry.

† Major product, $>1$ μmole; trace product, $<0{\cdot}1$ μmole.

‡ *Ortho-* and *para*-nitrophenols are yellow compounds, which give no color with Gibbs' reagent.

3-hydroxyanisole. At the present time, the mechanism of the novel formation of 3-hydroxyanisole from 4-iodoanisole is unknown. This type of reaction was not observed with 4-iodoacetanilide. The only report of such a reaction is the conversion of 3-fluoroaniline to 4-hydroxyaniline with microsomes.[8]

*Nitroanisoles.* The metabolism of the three nitroanisoles was examined (Table 6). Ring hydroxylation, reduction of the nitro group to an amine and dealkylation were observed and the fractionation between these pathways varied with the substitution pattern. The major metabolism with 2-nitroanisole was dealkylation. The principal metabolism of 3-nitroanisole was reduction to 3-aminoanisoles, but ring hydroxylation *para-* to the nitro group and dealkylation were also observed as minor pathways. 4-Nitroanisole underwent both dealkylation and ring hydroxylation, *ortho* to the methoxy group, as major metabolisms (see Fig. 4).

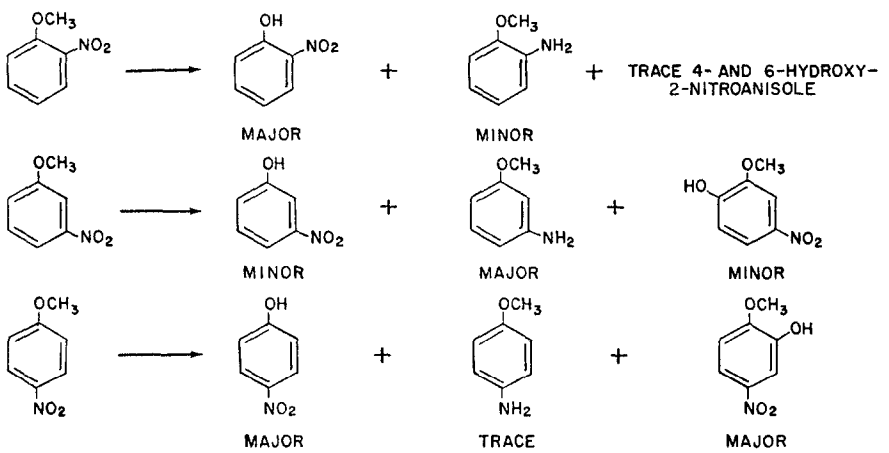


FIG. 4. Metabolism of nitroanisoles with rat liver microsomes.

The results presented in this paper further delineate the complex of reactions catalyzed by drug-metabolising enzymes and indicate the importance of both electronic and steric factors in determining the metabolism of aromatic substrates. It is noteworthy that *methyl* or *halogen* migration has not been observed as a major metabolic route with any of these substrates. This is in contrast to the results obtained with 4-methyl-[9] and 4-chlorophenylalanine[10] with the highly specific enzyme, phenylalanine hydroxylase, where migration of the substituent is the major metabolic pathway. As yet there is only one report[11] of the "NIH Shift" of halogen as a relatively major metabolism during oxidation of an aromatic substrate by nonspecific microsomal enzymes. In all other cases[4, 5, 12, 13] the migration of halogen during metabolism by nonspecific enzymes has been a minor route or has not been observed. The significance of these observations and their relation to the role of arene oxides in the hydroxylation of aromatic substrates are under investigation.[14–16]

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## REFERENCES

1. J. DALY, D. JERINA and B. WITKOP, *Archs Biochem. Biophys.* **128,** 517 (1968).
2. J. DALY and D. JERINA, *Archs Biochem. Biophys.* **134,** 226 (1969).
3. G. M. K. HUGHES and B. C. SAUNDERS, *J. chem. Soc.* 4360 (1954).
4. J. DALY, D. JERINA, J. FARNSWORTH and G. GUROFF, *Archs. Biochem. Biophys.* **131,** 238 (1969).
5. J. W. DALY, G. GUROFF, S. UDENFRIEND and B. WITKOP, *Biochem. Pharmac.* **17,** 31 (1968).
6. U. ULLRICH, J. WOLF, E. AMADORI and H. STAUDINGER, *Hoppe-Seyler's Z. physiol. Chem.* **349,** 85 (1968).
7. S. KAUFMAN, *Biochim. biophys. Acta* **51,** 619 (1961).
8. J. RENSON and U. BOURDON, *Archs int. Pharmacodyn. Ther.* **171,** 240 (1968).
9. J. DALY and G. GUROFF, *Archs. Biochem. Biophys.* **125,** 136 (1968).
10. G. GUROFF, K. KONDO and J. DALY, *Biochem. biophys. Res. Commun.* **25,** 622 (1966).
11. D. M. FOULKES, *Nature, Lond.* **221,** 582 (1969).
12. J. K. FAULKNER and D. WOODCOCK, *J. chem. Soc.* 1187 (1965).
13. E. W. THOMAS, B. C. LOUGHMAN and R. G. POWELL, *Nature, Lond.* **204,** 884 (1964).
14. D. JERINA, J. DALY, B. WITKOP, P. ZALTZMAN-NIRENBERG and S. UDENFRIEND, *Archs Biochem. Biophys.* **128,** 176 (1968).
15. D. JERINA, J. DALY and B. WITKOP, *J. Am. chem. Soc.* **90,** 6523 (1968).
16. D. JERINA, J. DALY, B. WITKOP, P. ZALTZMAN-NIRENBERG and S. UDENFRIEND, *J. Am. chem. Soc.* **90,** 6525 (1968).