The Metabolism of N-Isopropylaniline-^{14}C Hydrochloride in the Rat

A thesis submitted in partial fulfillment of the requirements for the degree Master of Science

by

Daniel Samuel Sitar

School of Pharmacy

April 1968

ACKNOWLEDGEMENTS

The author wishes to express his appreciation to Dr. W. E. Alexander who personally directed this project, for his assistance, advice and encouragement which were available at all times.

The author is indebted to the University of Manitoba for providing the fellowship enabling him to carry out this project.

ABSTRACT

N-isopropylaniline-^{14}C hydrochloride was synthesized and used for metabolic studies in rats. Three metabolites were identified, 4-hydroxy-N-isopropylaniline, 2-anilinopropionic acid and p-aminophenol. The major metabolic route was found to be p-hydroxylation of the aromatic ring, while dealkylation and ω-oxidation appeared to be minor pathways. The presence of a very water soluble component was also noted in these studies but this fraction has not been characterized.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
A. METABOLIC REACTIONS OF AROMATIC AMINES	3
Site of Metabolism	3
Pathways of Metabolism	5
I. Dealkylation	5
II. Hydroxylation	7
1. Ring Hydroxylation	7
2. Proposed Mechanisms of Hydroxylation	8
3. N-Hydroxylation	13
4. Hydroxylation of N-Substituted Anilines	14
III. Side Chain Oxidation	15
IV. Acetylation	19
V. Conjugation	19
1. Conjugation with Glucuronic Acid	20
2. Conjugation with Sulfuric Acid	20
3. Conjugation with Mercapturic Acid	21
B. EXPERIMENTAL	22
I. Preparation of Compounds	22
II. Reverse Isotope Dilution Experiments	24
III. Animal Experiments	26
IV. Chromatographic Studies	27
1. Methods	27
2. Solvents for Thin-Layer Chromatography	27
3. Detecting Reagents	27
4. Preparation of Thin-Layer Plates	28
V. Detection of Radioactivity	28
1. Liquid Scintillation	28
2. Scanning of Chromatograms	29
3. Materials and Methods	29
C. RESULTS	30
I. Excretion Study	30

Page

C. RESULTS (continued)

II. Chromatography 31

III. Reverse Isotope Dilution Experiments . . . 34

D. DISCUSSION 38

E. CONCLUSION 43

BIBLIOGRAPHY 44

INTRODUCTION

It is now known that many laboratory animals metabolize drugs in a manner similar to man. However, the rate of metabolism of foreign compounds may vary profoundly in various animal species (Brodie 1964), and also the mechanism and end products of metabolism may be entirely different in different species (Koppanyi and Avery 1966).

Drug metabolism reflects the characteristic enzyme systems of different animal species (Koppanyi and Avery 1966). When a foreign compound is introduced into an animal, it is exposed to many enzymes. Most foreign compounds are metabolized, and it appears that the body tries to detoxify or destroy these compounds. By means of tissue enzymes, especially those occurring in the liver, the compound is subjected to oxidation, reduction, hydrolysis or synthesis, the latter involving conjugation with substrates such as glucuronic acid or sulfuric acid. However, the body may make a compound more or less toxic in its attempt to metabolize the substance. For example, phenol is converted to phenyl glucuronide and phenyl sulfate both of which are non-toxic; on the other hand, trifluoroacetic acid is converted to fluorocitrate which is highly toxic to mammalian systems. The latter reaction is referred to as lethal synthesis (Williams 1966).

From recent research, it appears that there are two types of enzymes involved in metabolism; the enzymes of normal metabolic processes, and the drug metabolizing enzymes. Most drug metabolizing enzymes are located in subcellular particles called microsomes, which can be obtained from homogenized liver by centrifugation. These particles are generally believed to be derived from the endoplasmic

reticulum, which consists of a network of tubules extending into almost all regions of the cytoplasm of the liver (Williams 1966). Fouts (1961) separated the "smooth" from the "rough" microsomes, and showed that it was mainly the "smooth" microsomes which were associated with the drug enzyme systems.

Alexander et al. (1968), studying the metabolism of N-secondarybutylaniline-^{14}C, were unable to identify all of the urinary metabolites excreted in twenty-four hours. In fact, only 26% of the administered dose was identified. A considerable amount of water soluble radioactivity was not identified. Thus a study on the metabolism of N-isopropylaniline-^{14}C was begun to determine whether this water soluble component was unique to the metabolism of N-secondarybutylaniline or if this water soluble component was a general occurrence in the metabolism of N-alkylarylamines. To date, no systematic metabolic study of this class of compounds has appeared in the literature.

Recently, it has been found that N-isopropylaniline is a metabolite of the herbicide 2-chloro-N-isopropylacetanilide (Fed. Regist. 1967 a and b). Also, it is well documented that aromatic amines have been implicated as carcinogens in mammalian species (Clayson 1964; Walpole and Williams 1958; Weisburger and Weisburger 1966). Therefore, if residues remain on food products consumed by humans, it is important that the metabolic fate of N-isopropylaniline be determined.

A. METABOLIC REACTIONS OF AROMATIC AMINES

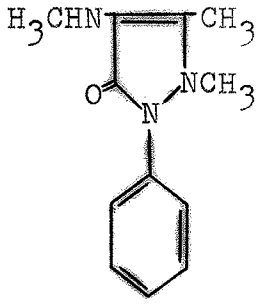
Site of Metabolism

Metabolism of toxic substances by animals occurs mainly in the liver and inactivation of foreign compounds has been found to occur mainly by oxidative processes (Booth et al 1967).

Gillette et al (1957) found that liver microsomes contain an enzyme system involving NADPH oxidase which catalyses the following reaction:

$$NADPH + O_2 \longrightarrow NADP + H_2O_2$$

They implicated this system in oxidative dealkylation of foreign alkylamines such as monomethyl-4-aminoantipyrine (I) by liver microsomes. They also suggested the probable involvement of this system in other types of microsomal drug enzyme systems requiring NADPH and oxygen.



I

Mitoma et al (1956) found that the hydroxylating enzyme system was located in the liver microsomes and required both oxygen and NADPH. Booth and Boyland (1957) had previously demonstrated that an NADPH generating system (NADP, glucose-6-phosphate and glucose-6-phosphate dehydrogenase) could be substituted for NADPH and that such a system would hydroxylate aromatic amines and N-substituted amines as well as benzene and naphthalene.

Kuntzman et al (1966), studying the hydroxylation enzymes of the liver, found the enzyme system in both rat and man to be similar.

Both are localized in the microsomal fraction and require NADPH for activity. They demonstrated enzymes in human liver which metabolize foreign compounds by O-dealkylation, N-dealkylation, hydroxylation and side chain oxidation.

Gram and Fouts (1967) found greater drug metabolizing activity in smooth-surfaced microsomes than in rough-surfaced microsomes in their metabolic studies with hexobarbitone, aniline and aminopyrine. They indicated that these results suggested inequality of enzyme distribution within the hepatic microsomal fraction. They found that their results were independent of the technique of microsomal subfractionation, animal species and the nature of the NADPH generating system.

In their studies on formation of smooth endoplasmic reticulum after drug administration, Remmer and Merker (1965) found an increase in the formation of smooth endoplasmic reticulum. This was accompanied by a threefold increase in cytochrome b_5 and an increase in NADPH. Activity of the same enzymes in the rough membranes was normally lower and only slightly stimulated by phenobarbitone. Activity of the enzymes involved in normal metabolism, for example ATP, NADP and glucose-6-phosphatase, showed little or no increase.

Stitzel and Furner (1967) reported that stress, for example, cold, and administration of phenobarbitone, alone and together, caused an increase in p-hydroxylation of aniline and N-dealkylation of ethylmorphine, the effects of these two factors being additive. Thus one should be careful to control environmental conditions in order that results may be correlated between different animal experiments.

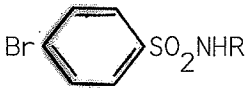
Pathways of Metabolism

I Dealkylation

Gaudette and Brodie (1959) suggested that dealkylation of a host of foreign alkylamine compounds is achieved by relatively few enzymes. Investigating the *in vitro* dealkylation of N-alkylanilines, these workers found dealkylation to occur with N-methyl, N:N-dimethyl, N-ethyl and N-butylaniline by rabbit liver microsomes. They reported that N:N-diethylaniline and N:N-dibutylaniline were not dealkylated by rabbit liver microsomes.

Dealkylation *in vivo* of aromatic (aniline) amines has been demonstrated indirectly with N-methyl, N-ethyl, N-butyl, N-benzyl and N:N-dimethyl compounds (Holzer and Kiese 1960) by spectrophotometric measurement of the corresponding nitroso compounds occurring in the blood. In vivo dealkylation of N-alkylanilines and substituted N-alkylanilines was also demonstrated with N:N-dimethyl- (Smith 1950), N:N-dipropyl- (trifluralin-Emmerson and Anderson (1966)) and N-*sec*-butyl- (Alexander *et al* 1968) compounds.

Smith *et al* (1965) studying the anticonvulsant activity of N-alkyl substituted 4-bromobenzenesuffonamides (II), found the extent



II

of dealkylation of the amides in the mouse to occur as follows:

$$R{=}CH_3 > C_2H_5 > CH_2{-}CH{=}CH_2 > CH(CH_3)_2 > CH_2CH_2CH_3 > CH_2CH_2CH_2CH_3$$

No systematic structure-activity study has been carried out on the N-dealkylation of N-alkylanilines where the alkyl group is

larger than methyl or ethyl (McMahon 1966a).

The overall oxidative N-dealkylation reaction has been represented as:

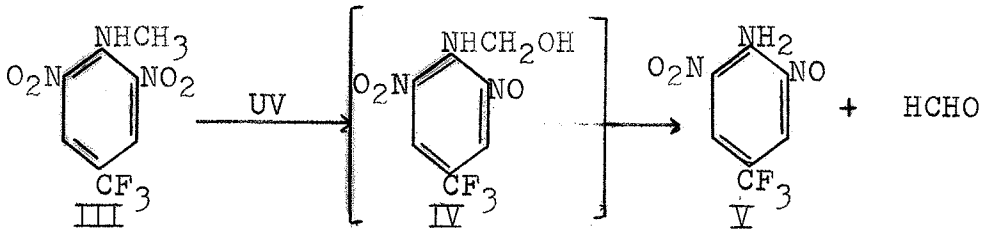
$$R_2NCH_2R^1 \xrightarrow{[O]} R_2NH + R^1CHO \quad \text{(McMahon, 1966a)}$$

McMahon (1966) stated that all of the enzymes concerned are located in the microsomal fraction of rat liver and require NADPH and molecular oxygen. He suggests that the following mechanism

$$RXCH_2R^1 \xrightarrow[\text{oxygen}]{\text{active}} RXCHOHR^1 \longrightarrow RXH + R^1CHO$$

where X=O, S or N is attractive because of the close relationship between the dealkylation and hydroxylation reactions.

At present, dealkylation appears to be a free radical hydroxylation reaction (McMahon 1966a), McMahon (1966b) suggests that the membrane may participate to bring the reactive centres of the molecules in close proximity. He was able to demonstrate the following reaction *in vitro*:



It was found that the N-propyl analogue of this substrate (III) readily underwent the same reaction with propionaldehyde as the other product. McMahon (1966b) suggested this as a plausible non-enzymic mechanism for the liver microsome dealkylase reaction.

II Hydroxylation

1. Ring Hydroxylation

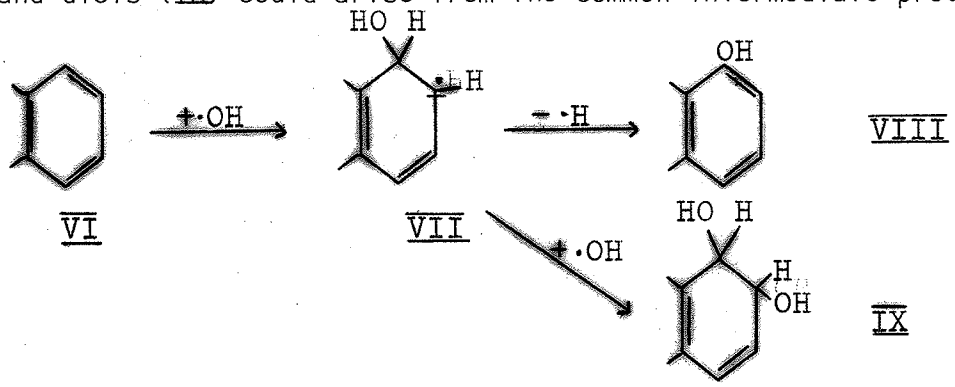
In most species, ring hydroxylation and conjugation are the major pathways of metabolism of foreign aromatic compounds (Bond and Howe 1967, pronethalol; Beckett and Morton 1967, N-alkyloxindoles; Alexander et al 1965, diphenylamine; Parke 1960, aniline; Axelrod 1954, amphetamine; Alexander et al 1968, N-secondary-butylaniline).

Posner et al (1961) performed in vitro hydroxylation studies with rabbit liver microsomes using benzene, naphthalene, quinoline, indole, aniline, diphenyl and coumarin. All of these compounds were hydroxylated by the same microsomal system indicating that enzymatic hydroxylation of a variety of compounds may be carried out by the same enzyme system.

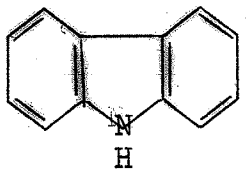
Compounds with acidic side chains such as benzoic acid, phenyl-acetic acid and cinnamic acid have been found to be excreted rapidly, and may thus be removed from the body before hydroxylation can occur (Smith 1950). Smith (1950) also observed that when hydroxylation did occur para-hydroxylation was more common than ortho-hydroxylation. It was considered that this could be due to steric hindrance at the ortho-position. Also, monosubstituted benzene compounds containing ortho- para- directing groups did not hydroxylate in the meta-position, and if the group was meta-directing, hydroxylation occurred in both meta- and para-positions. Parke (1960) found meta-aminophenol as a metabolite of aniline-^{14}C, but only in trace amounts.

2. Proposed Mechanisms of Hydroxylation

Smith (1950) postulated that the hydroxylation substitution pattern found biologically, could more readily be explained by assuming that the substitution was a free radical mechanism and that both phenols (VIII) and diols (IX) could arise from the common intermediate produced (VII).



Johns and Wright (1964) found that carbazole (X) was hydroxylated in the 3-position, the carbon atom in the nucleus having the greatest electron density (Brown and Coller 1959).

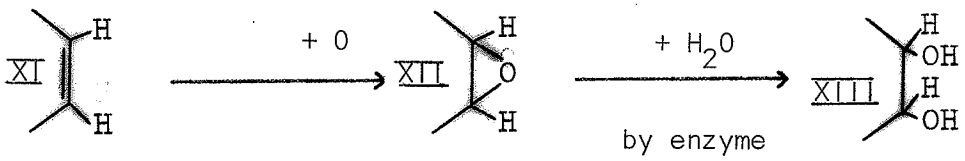


X

From these results, Johns and Wright (1964) suggested the attacking agent to be electron deficient, hence an hydroxyl cation or hydroxyl free radical.

Boyland et al (1964) studied hydroxylation of some aromatic hydrocarbons by both an ascorbic acid model hydroxylating system and by rat liver microsomes. They found that with some compounds studied, different products arose from the two systems, while with others, similar compounds resulted. They suggested that free hydroxy radicals were not involved in the ascorbic acid model hydroxylating system but proposed no other species.

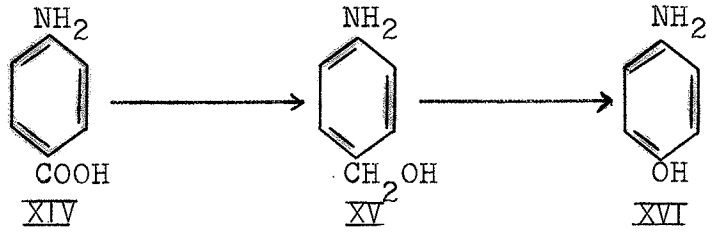
Boyland (1950) postulated an epoxide mechanism for the biological hydroxylation reaction. In formation of the dihydrodiol, the mechanism could be represented as follows (XI-XIII):



If this is the mechanism, then the reaction of hydrolysis of the oxide (XII) must be enzyme catalysed as the products produced are optically active. One would expect a racemic mixture if such were not the case. Posner et al (1961) proposed that phenols and dihydrodiols originated from a common intermediate. Using ^{18}O, they found that atmospheric oxygen was incorporated in the hydroxylation of acetanilide. However, they could not demonstrate a role for hydrogen peroxide in hydroxylation by rabbit liver microsomes, and atoms of isotopic water were not incorporated during hydroxylation. Boyland and Sims (1962) postulated that through intermediate formation of an epoxide, all of the isolated metabolites of phenanthrene could be explained. Booth et al (1960) suggested an epoxide intermediate in the metabolism of 1:2-dihydronaphthalene to trans-1:2-dihydroxy-1:2:3:4-tetrahydronaphthalene. Boyland and Sims (1960) concluded that it is probable that the epoxide is an intermediate in the metabolism of 1:2-dihydronaphthalene. Alexander (1965) stated that it is possible to explain the formation of metabolites for a number of simple substituted aromatic compounds by assuming the intermediate formation of epoxides.

More recently, a new metabolic pathway for p-hydroxylation was

demonstrated by Sloane et al (1963), first in acid-fast bacteria, and later by Sloane (1964; 1965) working with guinea pig microsomes. In acid fast bacteria, the proposed pathway from p-aminobenzoic acid (XIV) was (XIV - XVI):



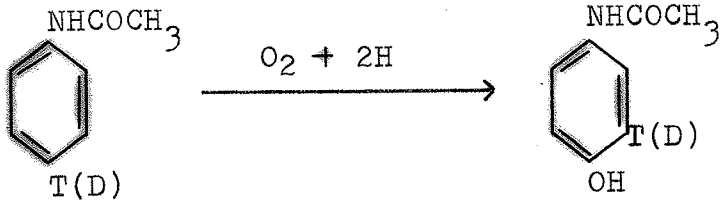
XIV XV XVI

Sloane (1964) demonstrated p-hydroxymethylation (XV) as an intermediate step in p-hydroxylation (XVI) of aniline by guinea pig liver microsomes. However, no p-hydroxyaniline (XVI) was formed from p-aminobenzoic acid (XIV) with this system. The p-aminobenzylalcohol (XV) was not oxidized before hydroxylation. Direct hydroxylation was not disproved as another pathway. In fact, Sloane (1965) showed that hydroxymethylation was a minor pathway, the major pathway appearing to involve direct hydroxylation.

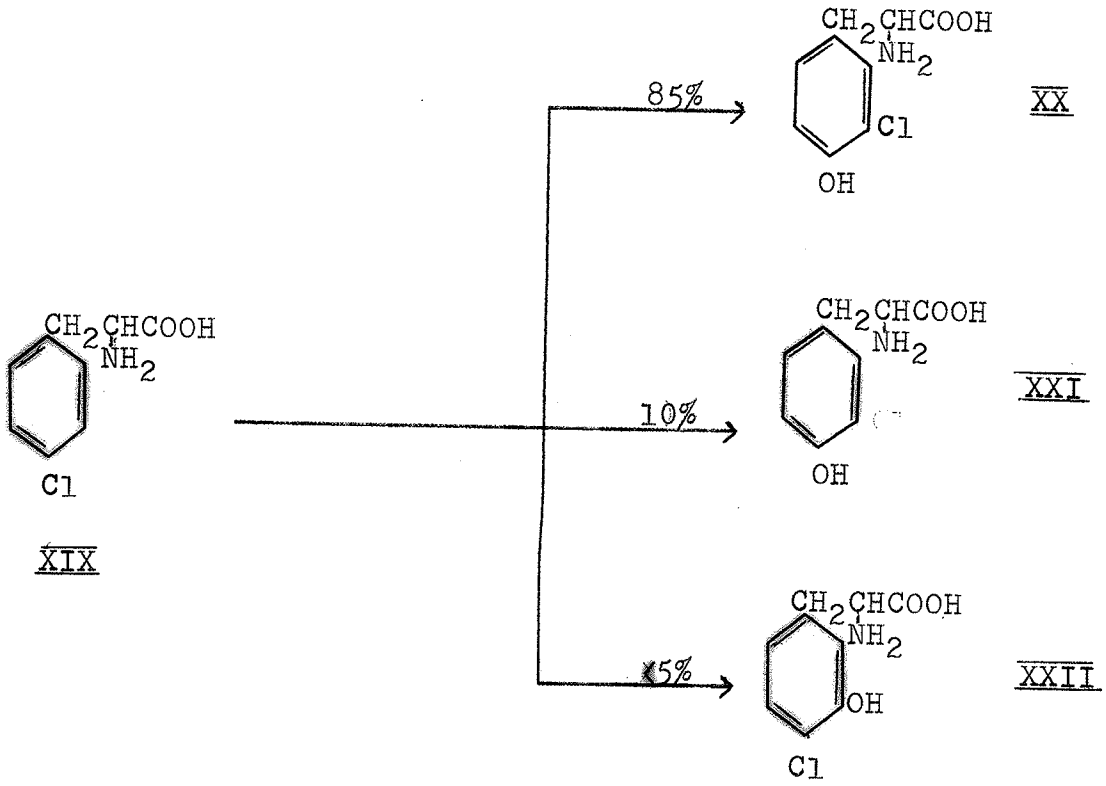
The most recent mechanism proposed is hydroxylation-induced intramolecular migration (NIH Shift) by Guroff et al (1967). Their experiments indicate that a frequent consequence of hydroxylation in aromatic systems is an intramolecular migration or shift of the group displaced by hydroxyl to an adjacent position on the aromatic ring. This contradicts the classical concept by which the group being substituted would be removed by direct displacement.

The NIH shift was demonstrated using acetanilide (XVII) isotopically labelled in the para-position with deuterium (D) or tritium (T) and chemically using p-chlorophenylalamine (XIX). The major reaction

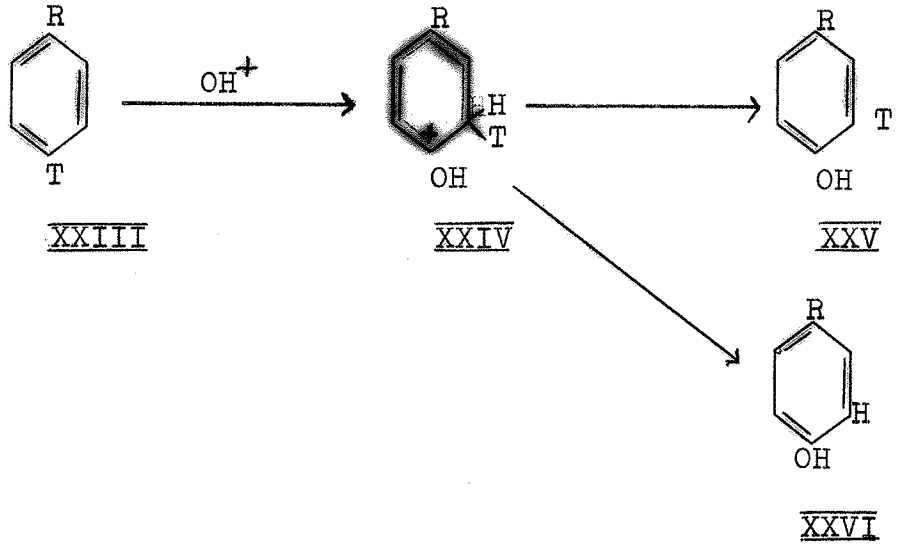
involves a shift of the para-substituent to the meta-position. They found that the amount of tritium retention correlated well with the charge distribution patterns in the postulated hydroxyl containing intermediate (XXIV), and suggest that the "NIH Shift" is a function of the structure of the hydroxyl substituted intermediate rather than a direct consequence of the mechanism of enzyme action. Tanabe et al (1967) found that the rate determining step in the hydroxylation of acetanilide by microsomes was the attachment of oxygen to the para-position or the fixation of oxygen by the enzyme. Tritium was not involved in the rate determining step. The retention of tritium in favor of ordinary hydrogen is explained by the greater strength of the C-T bond over the C-H bond.



XVII XVIII



Proposed mechanism for "NIH Shift" (Guroff et al 1967)[9]



3. N-Hydroxylation

Gaudette and Brodie (1959) stated that N-hydroxylation appears to be a general process in the metabolism of aromatic amines. Miller and Miller (1960) presented evidence to support the hypothesis that N-hydroxy derivatives may rearrange *in vivo* to form the *ortho*-hydroxy derivatives. Booth and Boyland (1964) showed that in metabolism of 4-acetamidobiphenyl, N-oxidation of the arylamine to the N-hydroxy metabolite was a reversible reaction. All N-hydroxy compounds tested by Booth and Boyland (1964) were isomerized to the corresponding *ortho*-hydroxy derivatives by the soluble liver fraction of rats and rabbits as demonstrated by the following:

N-hydroxyacetanilide	*o*-acetamidophenol
4(N-hydroxyacetamido)biphenyl	4-acetamido-3-hydroxybiphenyl
2(N-hydroxyacetamido)naphthalene	2-acetamido-1-naphthol
2(N-hydroxyacetamido)fluorene	2-acetamido-1-hydroxyfluorene

Thus *ortho*-hydroxylation appears to be an enzymic reaction. They found that the cofactors NAD^+, NADH or NADPH were required for this reaction, and stated the possibility that this is a general reaction for all the N-hydroxyacetamidoaryl compounds.

Uhleke (1961) demonstrated the N-hydroxylation of 2-aminofluorene by rat liver microsomes in the presence of NADPH and oxygen. He attributed methemoglobin formation to the production of N-hydroxy compounds from aromatic amines. In later experiments, Uhleke (1963) demonstrated the formation of N-hydroxy compounds in the metabolism of aniline, 2-naphthylamine, 2-aminofluorene and 4-aminobiphenyl.

Kampffmeyer and Kiese (1963) found that N-hydroxylation occurred much more readily with N-alkylanilines than with aniline itself.

Von Jagow et al (1966) found that some aromatic amines, for example, p-aminopropiophenone and 4-aminobiphenyl, were excreted to a large extent as the N-hydroxy derivatives in the urine. They found that urinary excretion of N-hydroxy derivatives was favored in compounds in which the para-position of the aniline derivative was substituted. Boyland and Manson (1966) detected 2-naphthylhydroxylamine as a urinary metabolite of 2-naphthylamine but it was found to be unstable in the urine. It has been established that N-hydroxylation increases the carcinogenic potency of arylamines (Clayson 1964) and therefore this metabolic reaction may be more representative of the lethal synthesis reaction than a detoxification reaction.

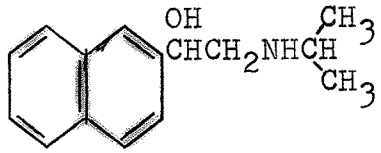
4. Hydroxylation of N-Substituted Anilines

In his studies with aniline-^{14}C in various animal species, Parke (1960) noted that rats hydroxylated aniline in the para-position to a greater extent than in the ortho-position. From studies in the rabbit, he found 51% excretion as p-aminophenol, 9% as ortho-aminophenol and only 0.1% as meta-aminophenol. He was able to account almost quantitatively for the total radioactivity in the twenty-four hour urine. Alexander et al (1965) isolated 4-hydroxydiphenylamine and 4:4'-dihydroxydiphenylamine in the rat, rabbit and man as metabolites of diphenylamine. 2-Hydroxydiphenylamine was found as a metabolite only in rabbits, and then only in trace amounts. Alexander (1965) could not demonstrate ortho-hydroxylated metabolites in the metabolism of N-secondarybutylaniline. The hydroxylated metabolites determined were found to contain the hydroxyl group in the para-position.

III. Side Chain Oxidation

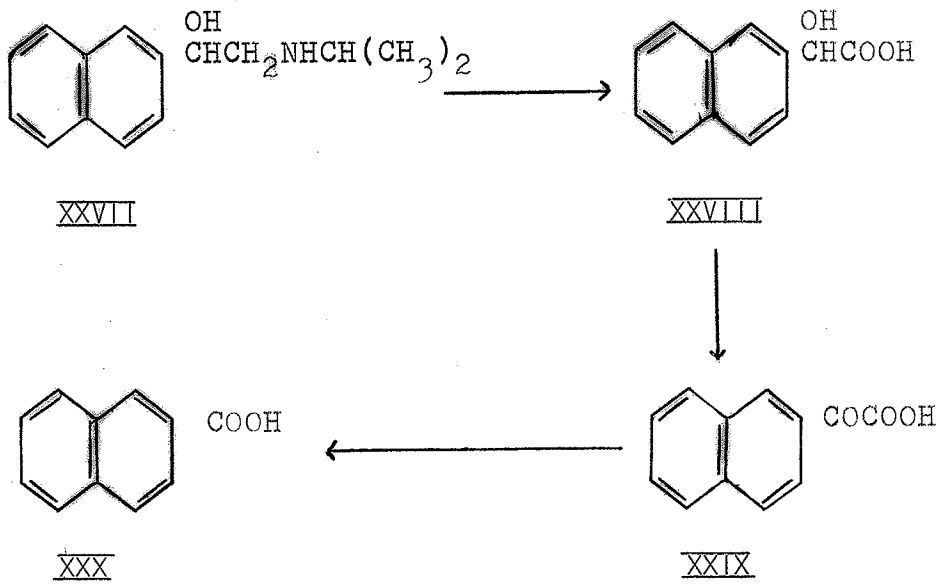
Charalampous and Tansey (1967) stated that when the amino group is located in the side chain, is primary, and is not attached to the aromatic ring, deamination is a major metabolic reaction. Aromatic amines like aniline, in which the amino group is directly attached to the ring are not attacked by amine oxidase (Blaschko et al 1937).

Bond and Howe (1967), using pronethalol (XXVII), a β-adrenergic blocking agent assubstrate, could demonstrate N-dealkylation of the isopropyl group only in vitro.



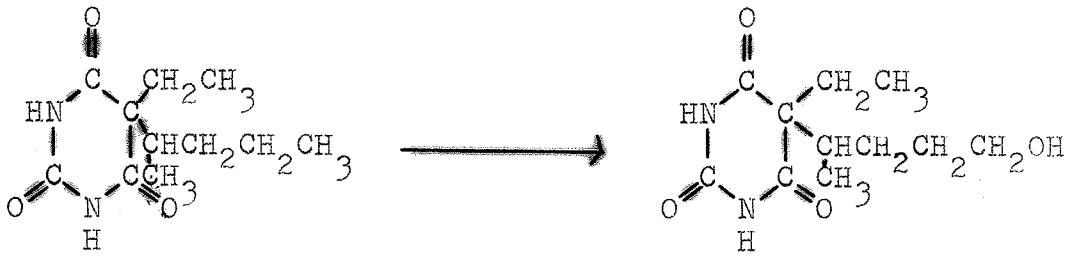
XXVII

Hydroxylation in the C_7 position was found to be the major metabolic route in vivo, except in the guinea pig where side chain oxidation predominated yielding acidic metabolites (XXVIII-XXX) from the hydroxyethyl portion of the side chain after deamination had occurred.



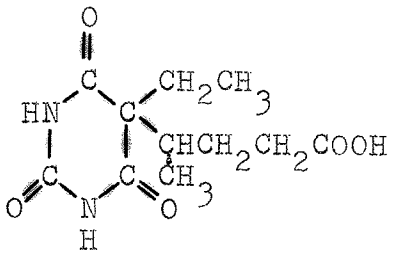
XXVII XXVIII XXIX XXX

Cooper and Brodie (1957) indicated that oxygen barbiturates (barbiturates containing an oxygen function at the 2-position of the nucleus) were metabolized only by the liver, and that generally, barbiturate metabolism by microsomes appeared to involve side chain oxidation rather than ring cleavage. They found that pentobarbitone (XXXI) yielded two metabolites in about equal amounts (XXXII and XXXIII).



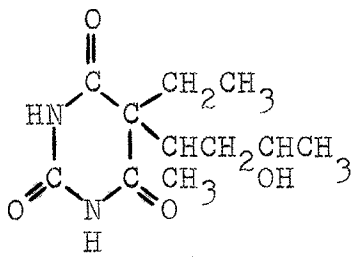
XXXI

XXXII

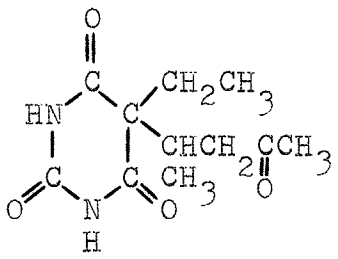


XXXIII

Using thiopentone, the 2-thio analogue of pentobarbitone, as the substrate, these workers found the acid analogue as the major metabolite, the alcohol being only a minor metabolite. Kuntzman et al (1967) suggested the following keto metabolite (XXXV) of pentobarbitone (XXXI) from their in vitro studies, but could not definitely prove it due to the lack of a reference compound. However, they did demonstrate the secondary alcohol (XXXIV) as a metabolite.

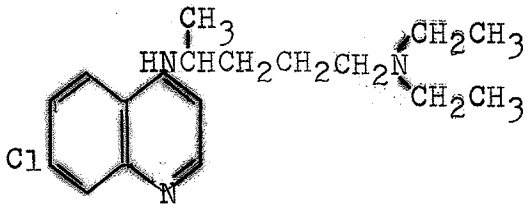


XXXIV

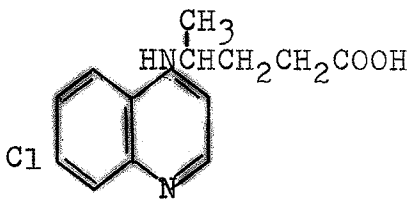


XXXV

Metabolic studies with chloroquine (XXXVI) demonstrated the major acidic metabolite in monkeys as the following compound (XXXVII) formed by dealkylation and deamination of the diethylamino group (McChesney et al 1966).



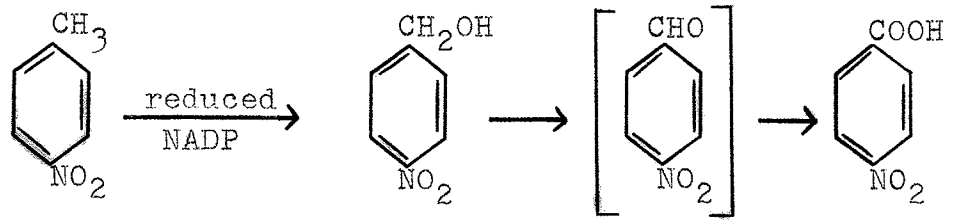
XXXVI



XXXVII

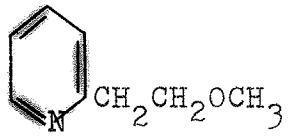
Quantitatively, however, this was only a trace metabolite (McChesney et al 1967). Kuroda (1962) had previously indicated the presence of the alcohol analogue of the above acid (XXXVII) as a metabolite in humans. McChesney et al (1967b) found 1-2% of the acid (XXXVII) as a metabolite of chloroquine in humans.

Other evidence in support of side chain oxidation leading through the alcohol to the acid was demonstrated by Gillette (1959), who showed that p-nitrotoluene (XXXVIII) was metabolized through the alcohol (XXXIX) to p-nitrobenzoic acid (XLI), the aldehyde (XL) also being postulated but not isolated.



XXXVIII XXXIX XL XLI

Gillette (1959) suggested that it is possible that other types of ω-oxidation take place by this pathway. It is to be noted that this is the same pathway later postulated by Burns et al (1967) in their studies on methyridine (XLII) metabolism.



XLII

El Masri et al (1956) demonstrated that, in the metabolism of ethylbenzene, ω-oxidation was not the major metabolic pathway. In fact, the major route of metabolism was ω-1 oxidation (oxidation of the carbon atom attached to the aromatic ring) to give the secondary alcohol. The compound then preferably conjugated with glucuronic acid or was oxidized after demethylation to benzoic acid which then conjugated with glycine. Metabolic studies on propylbenzene by these same workers demonstrated ω-1 oxidation to yield the secondary alcohol also occurred with this compound. However, the major pathway involved oxidation of the carbon atom attached to the aromatic ring to yield the corresponding secondary alcohol (ω-2 oxidation).

Smith et al (1954) found the tertiary alcohol as the major metabolite of isopropylbenzene in rabbits, but ω-oxidation was also encountered yielding the corresponding acidic metabolite.

IV. Acetylation

Acetylation is a potential biological reaction of all types of amino groups. It can only be considered as a general reaction of foreign compounds when the amino group is attached directly to an aromatic ring (Williams 1959a). Enzymes causing deacetylation are found in both liver and kidney, but their occurrence depends upon the species (Williams 1959b).

Booth (1966) stated that acetyl groups can be removed from hydroxamic acids by an acetyl transferase which uses arylamines, for example benzidine, 4-aminoazobenzene, 2-aminofluorene, 2-naphthylamine and 1-naphthylamine, as acceptors and is not affected by fluorides. The acetyl transferase occurs in the soluble fraction of rat liver and requires a thiol, for example cysteine, for maximum activity. With the exception of aniline and aniline derivatives, Booth (1966) found all arylamines which he tested to be effective as acetyl acceptors. Aromatic compounds with side chain amino groups were also found to be inactive. He also showed that acetylcoenzyme A was not the source of the acetyl groups. Von Jagow et al (1966) demonstrated that acetylation of the nitrogen was not necessary for urinary excretion of N-hydroxylation products of aromatic amines. The effect of acetylation in arylamine metabolism is not clear((Booth 1966).

V. Conjugation

The most important and most frequently encountered conjugation reactions occur with glucuronic acid, sulfuric acid and mercapturic acid. Conjugates with glucuronic acid are referred to as glucuronides. Conjugation with sulfuric acid may take place with an amino group causing the formation of a sulfamic acid or with an active oxygen (phenolic OH)

causing formation of ethereal sulfates. Conjugates with mercapturic acid are referred to as mercapturic acids, except when the mercapturic acid is deacetylated, the conjugate then being referred to as a cysteine conjugate.

1. Conjugation with Glucuronic Acid

Axelrod and Inscoe (1957) incubated aniline with guinea pig liver microsomes and uridine diphosphate glucuronic acid (UDPGA). Spectrophotometrically, they found that the free amino group of aniline disappeared and assumed the substrate to be converted to the N-glucuronide. They suggested that this might be an important pathway in the metabolism of amino compounds. It was also noted that other compounds, for example alcohols, phenols and carboxylic acids, could react in the same way. Axelrod et al (1958) later presented evidence to show that conjugation with glucuronic acid was an enzymatic reaction.

3-Hydroxycarbazole conjugated with glucuronic acid was demonstrated to be the major urinary metabolite of carbazole (X) in rats and rabbits (Johns and Wright 1964).

Welch et al (1966) demonstrated that less than 6% of the metabolites of N-acetyl-p-aminophenol was excreted as glucuronide conjugates by cats, whereas man and dog excreted 50-60% of the metabolites as glucuronide conjugates. The ability to form glucuronide conjugates was found to vary with the species involved in the study.

2. Conjugation with Sulfuric Acid

Laidlaw and Young (1948), studying the synthesis of ethereal sulfates in vivo, found that inorganic sulfate which they administered to rats was utilized in the formation of ethereal sulfates.

Boyland et al (1956) stated that aromatic amines are oxidized in animal tissues to ortho- and para-aminophenols which are rapidly

conjugated with sulfate as well as glucuronic acid. They found that 2-amino-1-naphthyl sulfate is not hydrolyzed by any sulfatase, and that aminophenyl sulfates from carcinogenic aromatic amines are generally resistant to the action of sulfatases. They found that sulfates resistant to hydrolysis had a pk_a a 4.3 or higher. There was no correlation between enzymic and acid hydrolysis. Boyland et al (1957) demonstrated the formation of sulfamic acids with aniline, 1-naphthylamine and 2-naphthylamine.

Newell et al (1960), studying the metabolism of monochloroacetanilides, found that sulfate conjugated with the hydroxyl groups on the ring resulting from hydroxylation of these compounds, for example, 3-chloroacetanilide-4-ethereal sulfate and 3-chloroaniline-4-ethereal sulfate. Alexander et al (1964) isolated the sulfate ester of 4-hydroxydiphenylamine as the potassium salt.

Welch et al (1966) found that the cat metabolized less than 2% of N-acetyl-p-aminophenol to the sulfate ester, while Boyland and Manson (1966) found 2-amino-1-naphthyl hydrogen sulfate as the main metabolite of 2-naphthylamine in both the dog and cat.

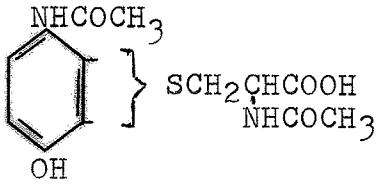
3. Conjugation with Mercapturic Acid

Jagenburg and Toczko (1964) stated that formation of mercapturic acids appeared to be a general metabolic pathway not restricted entirely to aromatic compounds, and that substances known to be metabolized by conjugation with cysteine were believed to be excreted as the corresponding mercapturic acids.

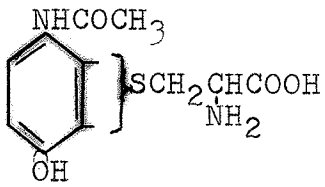
Boyland et al (1963) found (2-amino-1-naphthyl) mercapturic acid as a metabolite of 2-naphthylamine in the urine of dogs and cats. In rats and rabbits dosed with aniline, they found ortho- and para-aminoph-

enyl and para-acetamidophenyl mercapturic acids as urinary metabolites.

Jagenburg and Toczko (1964) found S(1-acetamido-4-hydroxyphenyl) mercapturic acid (XLIII) and S(1-acetamido-4-hydroxyphenyl) cysteine (XLIV) as conjugates in their study on the metabolic fate of acetophenetidin. The cysteine conjugate (XLIV) had not been previously reported as a metabolite of this compound.



XLIII



XLIV

B. EXPERIMENTAL

1. Preparation of Compounds

N-Isopropylaniline Hydrochloride

N-isopropylaniline hydrochloride was prepared by passing dry hydrogen chloride gas through the ethereal solution of commercial N-isopropylaniline (Aldrich) and recrystallized from anhydrous ether/anhydrous ethanol. m. p. 172-173°C.; reported 102°C., (Heilbron and Bunbury 1965). Calculated for $C_9H_{14}NCl$: C, 62.96%; H, 8.22%. Found: C, 62.73%; H, 8.25%. ε (253.5) = 192 (in N/10 hydrochloric acid solution) on the Beckman Model DU spectrophotometer.

N-Isopropylaniline-^{14}C Hydrochloride

N-isopropylaniline-^{14}C hydrochloride was synthesized by modification of the procedures of Titherley (1897), Burger and Schmalz (1954), and Bunnett and Brotherton (1957) for the synthesis of N-alkyl substituted aromatic compounds.

Aniline-^{14}C hydrochloride (2.6 mg.; 0.1 mc) and aniline hydrochloride (total 0.5036g.; 0.0039 moles) were dissolved in water. The solution was made alkaline with ammonia and ether extracted (8x3.0 ml). The ethereal extract was dried over anhydrous magnesium sulfate overnight, filtered, and the magnesium sulfate washed with anhydrous ether. The combined ethereal extract and washings were placed on a water bath under nitrogen to allow removal of the ether. Sodamide (0.2036g.; 0.052 moles) and anhydrous benzene (10 ml) were added. The reaction mixture was refluxed with stirring on an oil bath for two hours. Isopropyl bromide (1.44g.; 0.012 moles) was added through the top of the condenser and the reaction mixture was refluxed for an additional three hours. A small amount of water was added to the reaction mixture to destroy excess sodamide and the reaction mixture was extracted with hydrochloric acid (2N; 6x5 ml). This extract was washed with ether, made alkaline with ammonia, and then ether extracted (6x5 ml). The ethereal extract was dried over anhydrous magnesium sulfate overnight, filtered, and the magnesium sulfate washed with dry ether. Hydrogen chloride gas was passed through the combined filtrate and washings to give the crude product (0.367g; 54.81%). The product was recrystallized to constant count (3.07×10^5 dpm/mg.) from anhydrous ether/anhydrous ethanol. m. p. 169-170°C. (253.5) = ε 193 (in N/10 hydrochloric acid solution) on the Beckman Model DU spectrophotometer. Thin layer chromatography (solvent system benzene) of the ether extract of the aqueous solution after making alkaline with ammonia gave only one radioactive spot which gave an orange color with diazotized sulfanilic acid detecting reagent.

4-Hydroxy-N-Isopropylaniline

This compound was prepared in our laboratory according to the procedure of Major (1931). The product was recrystallized from benzene m. p. 152-153°C found; 155-156°C reported).

4-Hydroxy-N-Isopropylaniline-N:O-Ditosylate

This compound was prepared using p-toluenesulfonyl chloride and 4-hydroxy-N-isopropylaniline according to a procedure by Vogel (1966a). The compound was recrystallized from aqueous methanol. m. p. 149-150°C. Calculated for $C_{23}H_{25}O_5S_2N$; C, 60.10%; H, 5.48%. Found: C, 59.95%; H, 5.57%.

N-Acetyl-p-Aminophenol

This compound was synthesized from p-aminophenol according to a procedure by Vogel (1966b) for acetanilide. The compound was recrystallized from water. m. p. 168-169°C. found and reported.

2-Anilinopropionic Acid

This compound was synthesized in our laboratory according to the procedure of Nastvogel (1890). The compound was recrystallized from water which was acidified with hydrochloric acid when hot. m. p. 161°C; reported 163°C).

II. Reverse Isotope Dilution Experiments

4-Hydroxy-N-Isopropylaniline

4-Hydroxy-N-isopropylaniline (0.2486g.; 0.0016 moles) was added to a urine aliquot (10.00 ml; 2.14×10^5 dpm). Concentrated hydrochloric acid (2 ml) was added and the urine was hydrolysed by heating under pressure for one hour (Presto pressure cooker at 15 psi). The hydrolysed urine was adjusted to pH 6.85 with ammonia and ether extracted (6x5 ml). The ethereal extract was dried over anhydrous sodium sulfate overnight, filtered, and the magnesium sulfate washed with dry ether. The ether was removed from the combined extract and washings on a water bath under nitrogen. Anhydrous pyridine (10 ml) and p-toluenesulfonyl chloride (1.1g; 0.006 moles) were added, and the mixture was refluxed with

stirring on an oil bath for two hours. It was then poured into cold water, filtered, and washed with 2N hydrochloric acid, cold dilute sodium hydroxide and cold water. The N:O-ditosylate was recrystallized to constant activity from aqueous methanol. m. p. 149-150°C.

This experiment was repeated using 0.2514g; 0.0017 moles of 4-hydroxy-N-isopropylaniline. m. p. 149-150°C.

p-Aminophenol

N-Acetyl-p-aminophenol (0.2541g.; 0.0017 moles) was added to a urinary aliquot (25.00 ml; 5.35×10^5 dpm). Concentrated hydrochloric acid (3ml) was added, and the urine was hydrolysed under pressure for one hour (Presto pressure cooker at 15 psi). The hydrolysed urine was adjusted to pH 6.85 with ammonia and ether extracted (6x5 ml). The ether from the extract was removed under nitrogen on a water bath. Anhydrous pyridine (10ml) and a large excess of benzoyl chloride (5.0 ml; 0.043 moles) were added. The mixture was refluxed with stirring on an oil bath for two hours. The reaction mixture was then poured into cold water and ether extracted. The ether extract was washed with sodium bicarbonate solution, dilute hydrochloric acid and water. The ether was then evaporated to leave the crude product (dibenzoate) which was recrystallized from aqueous methanol to constant activity.

This experiment was repeated using 0.3600g.; 0.0024 moles of N-acetyl-p-aminophenol. m.p. 235°C; reported 235°C. (Alexander 1965).

2-Anilinopropionic Acid

2-Anilinopropionic acid (0.5010g; 0.0030 moles) was added to an aliquot of urine (20.00 ml; 1.05×10^6 dpm). Concentrated hydrochloric acid (5 ml) was added and the urine was hydrolysed under pressure for one hour (Presto pressure cooker at 15 psi). The urine

was then adjusted to pH 2.1 with ammonia and continuously ether extracted for eight hours. The ether was then removed on a water bath, leaving the crude product which was recrystallized to constant count from water acidified with hydrochloric acid when hot. m.p. 160°C.

III. Animal Experiments

Male Sprague Dawley rats (325-345g) were each injected intraperitoneally (15 mg/Kg) with an aqueous solution of N-isopropylaniline-^{14}C hydrochloride adjusted to contain the required amount in approximately 1 ml. of solution. The rats were placed in metabolism cages (Acme), and urine was collected every twenty-four hours for a total of ninety-six hours. The radioactivity present in each twenty-four hour sample was determined by liquid scintillation counting. The twenty-four hour urine samples from four rats were bulked and made up to 200.0 ml. in a volumetric flask. Aliquots of this solution were used for distribution studies and identification of metabolites. The identification of 2-anilinopropionic acid in the twenty-four hour urine was conducted on an aliquot of the twenty-four hour urine from a single rat.

Hydrolysis and Extraction of Urine Samples

Urinary aliquots of 5.00 ml. were used for the distribution studies. The aliquots were hydrolysed for one hour under pressure (Presto pressure cooker at 15 psi) using varying concentrations of hydrochloric acid, the acid concentration being varied from a volume which would give a solution of pH 1 to a total volume of 2.00 ml. of acid/5.00 ml. of urine.

The hydrolysed urine was adjusted to pH values of 2.1, 5.0 and 6.85 with ammonia and extracted with ether, butanol and methylene dichloride. All extracts were examined for radioactivity by the procedure of liquid scintillation and thin-layer chromatography.

IV. Chromatographic Studies

1. Methods

Urine (5.00 ml) was spotted on Whatman No. 3 chromatographic paper utilizing an Agla micrometer syringe. The paper was developed by descending chromatography utilizing benzene/acetic acid/water (4:1:5) as the solvent system. The papers were scanned for radioactivity and the radioactive areas located. The radioactivity was then removed from the paper by Soxhlet extraction using 50% $^{v}/v$ methanol/water. The solvent was then removed on a Rinco rotary evaporator and the residues dissolved in distilled water and subjected to distribution studies after acid hydrolysis as previously described.

The organic extractable fractions from the distribution studies after acid hydrolysis were spotted on thin-layer plates of fluorescent silica gel (Silica Gel GF_{254}- Merck; 0.25 mm thick) and developed in various chromatographic solvent systems to give a qualitative indication of metabolites of N-isopropylaniline that might be present. The plates were scanned for radioactivity and sprayed with various detecting reagents. All plates were developed a distance of 10 cm.

2. Chromatographic Solvents for Thin-Layer Chromatography

The following solvent systems were utilized in thin-layer chromatographic studies: benzene, 5% $^{v}/v$ isopropanol/benzene, 25%$^{v}/v$ isopropanol/benzene, benzene/methanol/acetic acid (90/16/8), and distilled water.

3. Detecting Reagents

The following spray reagents were used to detect N-isopropylaniline and derivatives:

1) diazotized sulfanilic acid prepared freshly by mixing equal

volumes of 1%^{W}/v sulfanilic acid in N/1 hydrochloric acid and 5%^{W}/v aqueous sodium nitrite solution followed by neutralization with an equal volume of 10% ^{W}/v sodium carbonate solution.

2) dimethylaminobenzaldehyde: 1%^{W}/v in N/1 hydrochloric acid.

3) Acetone silver nitrate solution prepared by adding two drops of a saturated aqueous solution of silver nitrate to 10 ml of acetone.

4) Aqueous ferric chloride solution 10%^{W}/v.

4. Preparation of Thin-Layer Plates

Thin layer plates were prepared using fluorescent silica gel (Silica Gel GF_{254} acc. to Stahl-Merck) on a Quickfit apparatus. All plates were 0.25 mm thick, plate thickness being governed and kept constant by a fixed aperture spreader.

V. Detection of Radioactivity

1. Liquid Scintillation

Solid samples (approximately 1 mg) from reverse isotope dilution studies were weighed on a Cahn Gram Electrobalance. They were directly transferred to a glass vial and dissolved in (2.0 ml) methanol. Scintillation fluid (8.0 ml) was added and the resulting solution counted in a Nuclear Chicago Unilux Liquid Scintillation Counter.

Organic extracts (0.10 ml) were dissolved directly in scintillation fluid (10.0 ml) and counted as above.

Aqueous samples (0.1 ml) were suspended in scintillation fluid (10.0 ml) by means of thixotropic gel (250 mg/vial) and counted as above.

All counts were converted to dpm by the internal standard technique using a solution of ^{14}C-benzoic acid in toluene.

Scintillation Fluid

The following formula was utilized to prepare all of the required scintillation fluid for all experiments:

PPO (2:5-diphenyloxazole)	2.0 g.
Dimethyl POPOP (1:4-bis-2(4-methyl-5-phenyloxazolyl) benzene)	0.5 g.
Isopropanol	400.0 ml.
Toluene q.s.	1000.0 ml.

2. Scanning of Chromatograms

All thin layer chromatograms were scanned for radioactivity on a Nuclear Chicago Thin Layer Chromatogram Scanner attachment connected to a Nuclear Chicago Actigraph III. Paper chromatrograms were scanned for activity on a Nuclear Chicago Actigraph III.

3. Materials and Methods

Benzoic acid- ^{14}C (6.6×10^3 dpm/mg) and aniline- ^{14}C hydrochloride (0.1 mc) uniformly labelled were purchased from Nuclear Chicago Corp. Scintillation compounds were purchased from Nuclear Enterprises. Other materials were obtained commercially or prepared as previously described. All melting points are uncorrected.

Accurate volumes of less than 1.0 ml. were delivered with an Agla Micrometer Syringe. Solid samples of less than 10 mg. were weighed on a Cahn Gram Electrobalance.

C. RESULTS

1. Excretion Study

A major proportion of the administered dose of N-isopropylaniline was found to be excreted in the urine in the first twenty-four hours. However, detectable radioactivity could still be measured after ninety-six hours (Table I).

Rat	Dose in dpmx10^5	^{14}C-excretion as %				
		24 hr.	48 hr.	72 hr.	96 hr.	Total
1	15.4	74.87	8.86	3.74	1.31	88.78
2	15.4	50.66	14.37	7.15	4.72	76.90
3	15.4	68.57	5.91	2.56	2.23	79.27
4	15.4	70.54	9.51	4.86	2.30	87.21
5*	18.4	71.39				
6*	7.7	69.86				

Table I: Excretion data for N-isopropylaniline metabolism in rats. *Excretion data was only determined for 24 hr. urine samples.

Distribution Study

Hydrolysis with varying concentrations of hydrochloric acid and extraction at different pH values with various solvents gave the following results (Table II).

Urine Aliquot (5.0 ml)	Conditions of Hydrolysis	pH of Extraction	% Extractable Radioactivity			
			Ether	Butanol	Methylene Dichloride	Total
A	unhydrolysed	not adjusted	1.49	57.12		58.61
B	to pH 1 with conc. HCl	6.85	62.62	8.32		70.94
C	2 ml conc.HCl	6.85	65.14	5.61		70.75
D	1 ml conc.HCl	6.85	67.85		0.65	68.50
E	1 ml conc.HCl	5.00	30.84			

Table II: Distribution study on urinary metabolites of N-isopropylaniline

Extraction with ether at pH 5.0 gave onlyaa lesser amount of extractable activity which chromatographed similarly to ether extracts at pH 6.85. Methylene dichloride was found to be a less efficient solvent than butanol when extracting at pH 6.85.

II. Chromatography

The chromatographic behavior of N-isopropylaniline and a number of related compounds suspected as metabolites are summarized in Table III along with their color reactions when sprayed with different detecting reagents.

Potential Metabolites	R_f values x 100 in solvent systems					Color reactions with spray reagents			
	1	2	3	4	5	A	B	C	D
Aniline	10	43	59	52	0	light yellow	yellow		green
p-Aminophenol	0	8	38	9	80	brown	orange	black	purple
o-Aminophenol	0	18	55	23	80	orange	yellow	brown	brown
N-Isopropylaniline	27	73	75	67	19	orange		grey-brown	light yellow
2(4-Hydroxyanilino)*-propionic Acid	0	0	0	13	92		faint yellow	black	
2-Anilinopropan-1-ol*	0	20	58	44	65	orange		pale grey	light yellow
4-Hydroxy-N-isopropyl-aniline	0	19	57	57	53			grey	
2-Anilinopropionic Acid	0	0	5	51	84	orange	yellow	pale grey	

Table III: Chromatographic properties of potential N-isopropylaniline metabolites.
For details see overleaf.

Table III: Chromatographic properties of potential N-isopropylaniline metabolites. Solvent systems: 1-benzene, 2-5% v/v isopropanol/benzene, 3-25% isopropanol/benzene, 4-benzene/methanol/acetic acid (90/16/8) and 5-distilled water. Spray reagents: A-diazotized sulfanilic acid, B-p-dimethylamino-benzaldehyde, C-acetone silver nitrate and D-ferric chloride. *These compounds have not yet been definitely confirmed by analysis.

Paper chromatography of the unhydrolysed urine as previously described resulted in the separation of two radioactive spots having R_f values of 0.00 (Spot A) and 0.50 (spot B). The majority of the radioactivity was found to be located in spot B. Soxhlet extraction of spots A and B yielded 53% of the spotted radioactivity. Acid hydrolysis of these extracts followed by extraction at pH 6.85 yielded both ether and butanol soluble material from both spots. However, some radioactivity from both spots remained in the aqueous phase. This indicated that the water soluble fraction of the distribution on whole urine was present in both spots. Adjustment of the aqueous solution from spot B to pH 2.1 after the above extraction yielded a small quantity of butanol extractable radioactivity, indicating the presence of an acidic metabolite. This aspect was not further investigated.

Little information could be obtained from the thin-layer chromatography experiments. It appeared that the presence of extraneous material in the extracts interfered with the chromatographic properties of most of the suspected metabolites, thus rendering the results somewhat questionable. This interference occurred to a lesser degree with the ether extracts and the chromatographic identification of two metabolites from the extractable radioactivity is presented in Table IV.

Component	R_f x 100 in solvents		Color with acetone silver nitrate
	1	2	
4-Hydroxy-N-isopropyl-aniline	57	53	Black
p-Aminophenol	38		Black
Ether extractable radioactivity **			
a)	58	51	Black
b)	39		*Not detectable

Table LV: Thin layer chromatographic results of ether extracts from hydrolysed urine. Solvent systems: 1-25% v/v isopropanol/ benzene and 2-distilled water. *The radioactive peak matched the standard although it did not give a color reaction itself. **R_f determined by scanning.

Due to difficulties encountered in thin-layer chromatography, the technique of reverse isotope dilution was adopted for qualitative and quantitative determination of metabolites.

III. Reverse Isotope Dilution Studies

4-Hydroxy-N-Isopropylaniline

Duplicate experiments demonstrated 61.44% of the radioactivity from the combined twenty-four hour urine samples of four rats was

due to 4-hydroxy-N-isopropylaniline (Table V).

Recrystallization	Experiment (dpm/mg)	
	I	II
1	190	191
2	187	182
3	191	182
4	193	181
5	192	181
% Radioactivity in the 24 hr. urine	62.38	60.50
Average	61.44	

Table V: Recrystallization to constant count of 4-Hydroxy-N-isopropylaniline-N:O-ditosylate.

p-Aminophenol

Duplicate experiments were carried out to determine the radioactivity due to p-aminophenol. However, insufficient material was recovered in experiment I to allow recrystallization to constant count (Table VI).

Recrystallization	Experiment (dpm/mg)	
	I	II
1	576	-
2	297	-
3	181	61
4	137	57
5	132	54
6	-	56
% radioactivity in the 24 hour urine	Not to constant count	7.91

Table VI: Recrystallization to constant count of p-aminophenol-N:O-dibenzoate.

2-Anilinopropionic Acid

Only one experiment was carried out to determine if and in what quantity 2-anilinopropionic acid occurred in the twenty-four hour urine. Results are shown in Table VII.

Recrystallization	dpm/mg
1	-
2	16
3	12
4	11
% radioactivity in the 24 hour urine	</%

Table VII: Recrystallization to constant count of 2-anilinopropionic acid.

D. DISCUSSION

The melting point of N-isopropylaniline hydrochloride (172-173°C) prepared from commercial samples of N-isopropylaniline did not agree with the melting point in the literature (m.pt. 102°C; Heilbron and Bunbury 1965). The analysis of the compound prepared in our laboratory confirms that the melting point reported in the literature is incorrect. The physical data of the prepared radioactive compound correlated well with the N-isopropylaniline-^{14}C hydrochloride.

From this study, N-isopropylaniline appears to be rapidly metabolized and readily excreted. However, detectable radioactivity was being excreted even up to ninety-six hours after dosing the animals, when the experiment was terminated.

The pathways of hydroxylation, dealkylation and ω-oxidation have been demonstrated in metabolic studies with N-isopropylaniline-^{14}C hydrochloride in rats. However, all of these pathways have been previously demonstrated in metabolic studies with other compounds.

To date, the metabolism of only one other branched chain N-alkylaniline has been reported i.e. N-sec-butylaniline (Alexander et al 1968). In their study, the same metabolic pathways were demonstrated as encountered in metabolic studies with N-isopropylaniline. However, in N-isopropylaniline metabolism, hydroxylation appears to have a much greater function while dealkylation appears to play a lesser role (Table VIII).

N-sec-butyl-aniline	N-isopropyl-aniline	Metabolites
10%	41.6%	p-hydroxy parent compound
13%	5.4%	p-aminophenol
3%	<1%	Acid from ω-oxidation of N-alkyl chain
71%	67.7%	Average 24 hr. urinary excretion

Table VIII: Comparative metabolites in N-sec-butylaniline and N-isopropylaniline metabolic studies. All values are derived from experiments on the 24 hour urine and expressed as a percent of administered dosage.

In the study of N-sec-butylaniline metabolism, distribution studies revealed a considerable proportion of the twenty-four hour excreted radioactivity to be extremely water soluble. This was also encountered in distribution studies with urinary metabolites of N-isopropylaniline, although there appears to be a lesser proportion of water soluble metabolites after ether extraction in the case of N-isopropylaniline (Table IX). From these results it appears likely that elucidation of the water soluble component(s) of either of these two compounds will lead to characterization of the water soluble component(s) of the other.

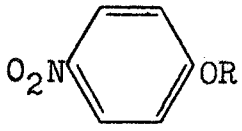
	N-isopropyl-aniline	N-sec-butyl-aniline
% of administered dose excreted in 24 hr.	67.7 a)	70.6 b)
Ether extractable activity as a % of 24 hr. excretion.	65.2 c)	53.0
Ether/Butanol extractable activity as a % of 24 hr. excretion	70.8 d)	-
Water soluble metabolites by difference	29.2	47.0
% Radioactivity in identified metabolites	69.4	36.6

Table IX: Comparative distribution between N-sec-butylaniline and N-isopropylaniline metabolites. a) average from seven rats (59.7-80.7%) b) average from six rats (50.7-74.9%) c) average from three experiments (62.6 - 67.8%) d) average from two experiments (70.75 - 70.94%) of total radioactivity in the sample taken as 100%.

Ring hydroxylation in the metabolism of N-isopropylaniline to give p-hydroxy=N-isoppopylaniline was an expected metabolic reaction as it has been demonstrated with other N-alkylanilines eg. N-secondarybutylaniline (Alexander et al. 1968), N:N-dimethylaniline (Horn 1936b) and N:N-diethylaniline (Horn 1937). All of the above compounds were p-hydroxylated. No ortho-hydroxylated metabolites have been demonstrated either with N-secondarybutylaniline or with N-isopropylaniline. Horn (1936a) demonstrated the formation of o-aminophenol from N:N-dimethylaniline and N:N-dimethylaniline-N-oxide in dogs. Parke (1960) found that male rats and female rabbits

hydroxylated aniline preferably in the *para* position giving a *para*/*ortho* ratio of about 6:1 in both cases. Creaven *et al* (1964) could not demonstrate *o*-hydroxylation of biphenyl by rat liver microsomes which were not pretreated with hydroxylation stimulants such as benzpyrene and phenobarbitone. Thus, if *o*-aminophenol is a metabolite of N-isopropylaniline in rats, it would be expected to occur in such small quantities as to make it a trace metabolite and thus not the water soluble component which as yet remains unidentified.

Concerning dealkylation, N-isopropylaniline appears to be metabolized through this route to a significantly lesser extent than is N-secondarybutylaniline (Table VIII). Horn (1936b) demonstrated dealkylation of N:N-dimethylaniline to N-methylaniline in herbivores and *o*-aminophenol in dogs (Horn 1936a) but did not demonstrate these reactions with N:N-diethylaniline in either dogs or rabbits (Horn 1937). McMahon *et al* (1963) demonstrated dealkylation of *p*-nitrophenyl ethers (XLV) in rats.



XLV

When R was methyl, ethyl, propyl, or isoppopyl, dealkylation was found to be a major reaction (76-87%). A significant decrease in dealkylation was demonstrated only when R was butyl or a longer alkyl chain. Smith *et al* (1965) showed that dealkylation decreased with increased chain length in their studies on dealkylation of N-alkyl-4-bromobenzene sulfonamides (II) in mice. They found that the N-isopropyl group dealkylated more readily than the N:N-dimethyl, N-propyl or N-butyl analogues. Significant decreases in the rate of dealkylation occurred even with the N-ethyl compound when compared to the N-methyl

compound. However, insufficient information exists on dealkylation of N-alkylanilines to warrant any conclusion being drawn here other than that at present, N-dealkylation appears to play a minor role in the metabolism of N-isopropylaniline.

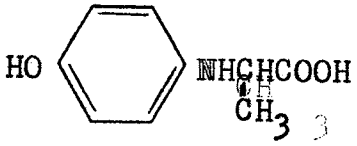
The ω-oxidation of the alkyl side chain of N-isopropylaniline to give 2-anilinopropionic acid appears also to be a minor metabolic pathway at present. This route has been previously demonstrated in metabolic studies with pentobarbitone (XXXIX) (Cooper and Brodie 1957) and isopropyl benzene (Smith et al 1954) which also undergo ω-oxidation to give the corresponding carboxylic acid metabolites.

From the above evidence, it appears that oxidation of the N-alkyl side chain must be involved in the metabolism of N-isopropylaniline to give as yet unidentified metabolites. Another potential metabolite which might be investigated is 2-anilinopropan-1-ol. In the laboratory, continuous ether extraction of an aqueous alkaline solution of this compound for one week was required to extract most of the compound. Also, it was found that milligram quantities of the alcohol were quite susceptible to oxidation. Alexander (1965) could not demonstrate the ω-alcohol from oxidation of the alkyl side chain of N-secondarybutylaniline. However, the alcohol metabolite would be expected from the metabolic pathway,

$$-CH_3 \longrightarrow -CH_2OH \longrightarrow -COOH$$

which has been demonstrated by Gillette (1959) with p-nitrotoluene (XXXVIII) and Burns et al (1967) with methyridine (XLII). It may be that oxidation of the alcohol is an important factor in the failure to demonstrate this metabolite in N-alkylaniline metabolism to date.

Another potential metabolite might be 2(4-hydroxyanilino) propionic acid (XLVI), as ω-oxidation has been demonstrated in the metabolism of N-isopropylaniline and p-hydroxylation was demonstrated to be a major metabolic route in the metabolism of N-isopropylaniline.



XLVI

Thus the problem of the water soluble component still appears to remain. Although this study confirms the metabolic pathways of N-secondarybutylaniline (Alexander et al 1968), it does not appear to shed any new light on the composition of the water soluble fraction other than that it does not separate into a single component spot by the technique of paper chromatography as previously described.

E. CONCLUSION

The metabolism of N-isopropylaniline-^{14}C hydrochloride in rats has been studied. To date, the following three urinary metabolites have been demonstrated: p-aminophenol, p-hydroxy-N-isopropylaniline and 2-anilinopropionic acid. These are analagous to the metabolites of Alexander et al (1968) from N-secondarybutylaniline-^{14}C hydrochloride. The N:O- ditosylate of p-hydroxy-N-isopropylaniline is a new compound. Thus the pathways of p-hydroxylation, dealkylation and ω-oxidation have been confirmed in the metabolism of these branched chain N-alkylarylamines. However, the water soluble component which could not be extracted remains to be identified.

BIBLIOGRAPHY

ALEXANDER, W. E. (1965) Ph.D. Diss., University of Sydney, Australia.

ALEXANDER, W.E., RYAN, A. J. and WRIGHT, S. E. (1964) Metabolism of Diphenylamine in the Rat and Rabbit. Experientia 20, 223.

ALEXANDER, W. E., RYAN, A. J. and WRIGHT, S. E. (1965) Metabolism of Diphenylamine in the Rat, Rabbit and Man. Fd. Cosmet. Toxicol, 3, 571.

ALEXANDER, W. E., RYAN, A. J. and WRIGHT, S. E. (1968) The Metabolism of N-Secondarybutylaniline in the Rat. Can. J. Pharm. Sci. In press.

AXELROD, J. (1954) Studies on Sympathomimetic Amines. II The Biotransformation and Pharmacological Disposition of D-Amphetamine, D-p-Hydroxyamphetamine and D-Methamphetamine. J. Pharmacol. Exptl. Therap. 110, 315.

AXELROD, J. and INSCOE, J. K. (1957) Enzymatic Synthesis of N-Glucuronic Acid Conjugates. Nature 179, 538.

AXELROD, J., INSCOE, J. K. and TOMKINS, G. M. (1958) Enzymatic Synthesis of N-Glucosyluronic Acid Conjugates. J. Biol. Chem. 232, 835.

BECKETT, A. H. and Morton, D.M. (1967) The Metabolism of N-Alkyloxindoles. Biochem. Pharmacol, 16, 1787.

BLASCHKO, H., RICHTER, D. AND SCHLOSSMANN, H. (1937) The Oxidation of Adrenaline and Other Amines. Biochem. J. 31, 2187.

BOND, P.A. and HOWE, R. (1967) The Metabolism of Pronethalol. Biochem. Pharmacol. 9 (1), 84.

BOOTH, J. (1966) Acetyl Transfer in Arylamine Metabolism. Biochem. J. 100, 745.

BOOTH, J. and BOYLAND, E. (1957) The Biochemistry of Aromatic Amines, 3. Enzymatic Hydroxylation by Rat Liver Microsomes. Biochem. J. 66, 73.

BOOTH, J. and BOYLAND, E. (1964) The Biochemistry of Aromatic Amines, 10. Enzymatic N-Hydroxylation of Arylamines and Conversion of Arylhydroxylamines into ortho-Aminophenols. Biochem. J. 91, 362.

BOOTH, J., BOYLAND, E. and COOLING, C. (1967) The Respiration of Human Liver. Biochem. Pharmacol. 16, 721.

BOOTH, J., BOYLAND E., SATO, T. and SIMS, P. (1960) Metabolism of Polycyclic Compounds. 17. The Reaction of 1:2-Dihydronaphthalene and 1:2-Epoxy-1:2:3:4-tetrahydronaphthalene with Glutathione Catalysed by Tissue Preparations. Biochem. J. 77, 182.

BOYLAND, E. (1950) 4. The Biological Significance of Metabolism of Polycyclic Compounds. Biochem. Soc. Symp. No. 5, 40.

BOYLAND, E. and MANSON, D. (1966) The Biochemistry of Aromatic Amines. The Metabolism of 2-Naphthylamine Derivatives. Biochem. J. 101, 84.

BOYLAND, E., MANSON, D. and NERY, R. (1963) The Biochemistry of Aromatic Amines. 9. Mercapturic Acids as Metabolites of Aniline and 2-Naphthylamine. Biochem. J. 86, 263.

BOYLAND, E., MANSON, D., SIMS, P. and WILLIAMS, D.C. (1956) The Biochemistry of Aromatic Amines. The Resistance of Some o-Aminoaryl Sulfates to Hydrolysis by Aryl Sulfatases. Biochem. J. 62, 68.

BOYLAND, E. and SIMS, P. (1960) Metabolism of Polycyclic Compounds, 16. The Metabolism of 1:2-Dihydronaphthalene and 1:2-Epoxy-1:2:3:4-tetrahydronaphthalene. Biochem. J. 77, 175.

BOYLAND, E. and SIMS, P. (1962) Metabolism of Polycyclic Compounds, 21. The Metabolism of Phenanthrene in Rabbits and Rats: Dihydroxy Compounds and Related Glucosiduronic Acids. Biochem. J. 84, 571.

BRODIE, B. B. (1964) Distribution and Fate of Drugs: Therapeutic Implications. In: Absorption and Distribution of Drugs, edited by T. B. Binns. Baltimore: Williams and Wilkins pp. 199-251; through BRODIE, B. B. and REID, W. D. (1967) Some Pharmacological Consequences of Species Variation in Rates of Metabolism. Fed. Proc. 26 (4), 1062.

BROWN, R. D. and COLLER, A. W. (1959) A Theoretical Study of the Chemistry of Furan, Pyrrole, Benzofuran, Indole, Dibenzofuran and Carbazole. Australian J. Chem. 12(2), 152.

BUNNETT, J. F. and BROTHERTON, T. K. (1957) Preparation of Dialkylanilines by the Reaction of Bromobenzene with Sodium Amide and Dialkylamines. J. Org. Chem. 22, 832.

BURGER, A. and SCHMALZ, A. C. (1954) Some Derivatives of Phenothiazine II. J. Org. Chem. 19, 1841.

BURNS, J., DUNCAN, W. A. M. and SCALES, B. (1967) Absorption, Distribution and Metabolism of the Anthelmintic Methyridine. Biochem. Pharmacol. 16, 463.

CHARALAMPOUS, K. D. and TANSEY, W. L. (1967) Metabolic Fate of β-(3:4-Dimethoxyphenyl)ethylamine in Man. J. Pharmacol. Exptl. Therap. 155 (2), 318.

CLAYSON, D. B. (1964) The Aromatic Amines. Brit. Med. Bull. 20(2), 115

COOPER, J. R. and BRODIE, B. B. (1957) Enzymatic Oxidation of Pentobarbital and Thiopental. J. Pharmacol. Exptl. Therap. 120, 75.

CREAVEN, P. J., PARKE, D. V. and WILLIAMS, R. T. (1964) Differential Stimulation of the o- and p-Hydroxylation of Biphenyl by Liver Microsomes. Biochem. J. 91, 12P.

EL MASRI, A. M., SMITH, J. N. and WILLIAMS, R. T. (1956) Studies in Detoxication 69. The Metabolism of Alkylbenzenes: n-Propylbenzene and n-Butylbenzene with Further Observations on Ethylbenzene. Biochem. J. 64, 50.

EMMERSON, J. L. and ANDERSON, R. C. (1966) Metabolism of Trifluralin in the Rat and Dog. Toxicol. Appl. Pharmacol. 9(1), 84.

Fed. Regist. (1967a) 32, 8816 through C.A. (1967) 67, 63032r.

Fed. Regist. (1967b) 32, 14944 through C.A. (1968) 68, 1998x.

FOUTS, J. R. (1961) The Metabolism of Drugs by Subfractions of Hepatic Microsomes. Biochem. Biophys. Res. Commun. 6 (5), 373.

GAUDETTE, L. E. and BRODIE, B. B. (1959) Relationship Between the Lipid Solubility of Drugs and their Oxidation by Liver Microsomes. Biochem. Pharmacol. 2 (1), 89.

GILLETTE, J. R. (1959) Side Chain Oxidation of Alkyl Substituted Ring Compounds. I Enzymatic Oxidation of para-Nitrotoluene. J. Biol. Chem. 234, 139.

GILLETTE, J. R., BRODIE, B. B. and La Du, B. N. (1957) The Oxidation of Drugs by Liver Microsomes: On the Role of TPNH and Oxygen. J. Pharmacol. Exptl. Therap. 119, 532.

GRAM, T. E. and FOUTS, J. R. (1967) Further Studies on the Metabolism of Drugs by Subfractions of Hepatic Microsomes. III Effects of the NADPH-Generating System. J. Pharmacol. Exptl. Therap. 158 (2), 317.

GUROFF, G., DALY, J. W., JERINA, D. M., RONSON, J., WITKOP, B. and UDENFRIEND, S. (1967) Hydroxylation-Induced Migration: The NIH Shift. Sci. 157, 1524

HEILBRON, I. and BUNBURY, H. M. (1965) Dictionary of Organic Compounds, Vol. 3, Eyre and Spottiswoode, London, p. 1932.

HOLZER, N. and KIESE, M. (1960) Bildung von Nitrosobenzol, Anilin und Hamiglobin in Katzen und Hunden nach intravenöser Injection von N-Alkylanilinen. Arch. exp. Path. Pharmak. 238, 546.

HORN, F. (1936a) The Breakdown of Dimethylaniline and Dimethylaniline Oxide in the Animal Body. Z. Physiol. Chem. 238, 84 through C.A. 30, 18674

HORN, F. (1936b) The Decomposition of Dimethylaniline in Herbivores. Z. Physiol. Chem. 242, 23 through C.A. 30, 76908.

HORN, F. (1937) The Fate of Diethylaniline and Diethylaniline Oxide in the Animal Body. Z. Physiol. Chem. 249, 82 through C.A. 31, 24445.

JAGENBURG, O. R. and TOCZKO, K. (1964) The Metabolism of Acetophenetidine. Isolation and Characterization of S(1-Acetamido-4-Hydroxyphenyl)-Cysteine, A Metabolite of Acetophenetidine. Biochem. J. 92, 639.

JOHNS, S. R. and WRIGHT, S. E. (1964) The Metabolism of Carbazole in Rats and Rabbits. J. Med. Chem. 7, 158.

KAMPFFMEYER, H. and KIESE, M. (1963) Enige Eigenschaften der Anilinhydroxylierenden Enzyme in Mikrosomen. Arch. exp. Path. Pharmak. 244 387 through Fd. Cosmet. Toxicol. (1964) 2, 129.

KOPPANYI, T. and AVERY, M. A. (1966) Species Differences and the Clinical Trial of New Drugs: A Review. Clin. Pharmacol. Ther. 7(2), 250.

KUNTZMAN, R., MARK, L. C., BRAND, L., JACOBSON, M., LEVIN, W. and CONNEY, A. H. (1966) Metabolism of Drugs and Carcinogens by Human Liver Enzymes. J. Pharmacol. Exptl. Therap. 152(1), 151.

KUNTZMAN, R., IKEDA, M., JACOBSON, M. and CONNEY, A. H. (1967) A Sensitive Method for the Determination of Pentobarbital-^{14}C Metabolites. Pharmacol. Exptl. Therap. 157 (1), 220.

KURODA, K. (1962) Detection and Distribution of Chloroquine Metabolites in Human Tissues. J. Pharmacol. Exptl. Therap. 137, 156.

LAIDLAW, J. C. and YOUNG, L. (1948) Studies on the Synthesis of Ethereal Sulfates *in vivo*. Proc. Biochem. Soc. Biochem. J. 42(4), I.

MAJOR, R. T. (1931) Catalytic Reduction of Mixtures of *para*-Nitro and Nitrophenols with Aldehydes and Ketones. J. Am. Chem. Soc. 53, 1901.

McCHESNEY, E. W., CONWAY, W. D., BANKS Jr., W. F., ROGERS, J. E. and SHEKOSKY, J. M. (1966) Studies on the Metabolism of some Compounds of the 4-Amino-7-chloroquine Series. J. Pharmacol. Exptl. Therap. 151 (3), 482.

McCHESNEY, E. W., SHEKOSKY, J. M. and HERNANDEZ, P. H. (1967) Metabolism of Chloroquine-3-^{14}C in the Rhesus Monkey. Biochem. Pharmacol. 16, 2444.

McCHESNEY, E. W., FASO, M. J. and BANKS Jr., W. F. (1967b) The Metabolism of Chloroquine in Man During and After Repeated Oral Dosage. J. Pharmacol. Exptl. Therap. 158, 323

McMAHON, R. E. (1966a) Microsomal Dealkylation of Drugs. Substrate Specificity and Mechanism. J. Pharm. Sci 55, 457.

McMAHON, R. E. (1966b) The Photochemical Demethylation of N-Methyl-2:6-dinitro-4-trifluoromethylaniline. A Possible Model for Enzymic Dealkylation. Tetrahedron Letters No. 21, 2307.

McMAHON, R. E., CULP, H. W., MILLS, J. and MARSHALL, F. J. (1963) Demethylation Studies. IV. The *in vitro* and *in vivo* Cleavage of Alkyl- and Arylalkyl-*p*-nitrophenyl Ethers. J. Med. Chem. 6, 343.

MILLER, E. C. and MILLER, J. A. (1960) A Mechanism of *ortho*-Hydroxylation of Aromatic Amines *in vivo*. Biochem. Biophys. Acta 40, 380.

MITOMA, C., POSNER, H. S., REITZ, H. C. and UDENFRIEND, S. (1956) Enzymatic Hydroxylation of Aromatic Compounds. Arch. Biochem. Biophys. 61, 431.

NASTVOGEL, O. (1890) Weitere Beiträge zur Kenntniss der α-Anilidopropionsäure und α-Anilidonormalbuttersäure. Ber. 23, 2010.

NEWELL, M., ARGUS, M. and RAY, F. E. (1960) Routes of Metabolism of ^{36}Cl Ring Substituted Monochloroacetanilides. Biochem, Pharmacol. 5, 30.

PARKE, D. V. (1960) Studies in Detoxication 84. The Metabolism of ^{14}C Aniline in the Rabbit and Other Animals. Biochem. J. 77, 493.

POSNER, H. S., MITOMA, C., ROTHBERG, S. and UDENFRIEND, S. (1961) Enzymic Hydroxylation of Aromatic Compounds. III Studies on the Mechanism of Microsomal Hydroxylation. Arch. Biochem. Biophys. 94, 280.

REMMER, H. and MERKER, H. J. (1965) Part II. Metabolic Aspects of the Toxicity of Drugs. Effect of Drugs on the Formation of Smooth Endoplasmic Reticulum and Drug-Metabolizing Enzymes. Ann. N.Y. Acad. Sci. 123, 79.

SLOANE, N. H. (1964) Microsomal Hydroxymethylation of the Benzene Ring, an Intermediate Step in Enzymic Hydroxylation. Biochim. Biophys. Acta 81, 408.

SLOANE, N. H. (1965) Hydroxymethylation of the Benzene Ring. I. Microsomal Formation of Phenol via Prior Hydroxymethylation of Benzene. Biochim. Biophys. Acta 107, 599.

SLOANE, N. H., UNTCH, K. G. and JOHNSON, A. W. (1963) Metabolites of p-Aminobenzoic AciddResulting in the Sequential Formation of p-Aminobenzyl Alcohol and p-Hydroxyaniline. Biochim. Biophys. Acta 78, 588 .

SMITH, J. N. (1950) 2. The Orientation of Biochemically Introduced Hydroxyl Groups in Benzene Derivatives in the Animal Body, Biochem. Symp. No. 5, 15.

SMITH, J. N., SMITHIES, R. H. and WILLIAMS, R. T. (1954) Studies in Detoxication 56. The Metabolism of Alkylbenzenes. Stereochemical Aspects of the Biological Hydroxylation of Ethylbenzene to Methylphenylcarbinol, Biochem. J. 56, 317.

SMITH, D. L., KEASLING, H. H. and FORIST, A. A. (1965) The Metabolism of N-Alkyl-4-bromobenzenesulfonamides in the Mouse, Correlation with Anticonvulsant Activity. J. Med. Chem. 8, 520.

STITZEL, R. E. and FURNER, R. L. (1967) Stress-Induced Alterations in Microsomal Drug Metabolism in the Rat. Biochem. Pharmacol. 16, 1489.

TANABE, M., YASUDA, D., TAGG, J. and MITOMA, C. (1967) Absence of Isotope Effects in the Microsomal Hydroxylation of Acetanilide. Biochem. Pharmacol. 16, 2230.

TITHERLEY, A. W. (1897) XLV. Sodamide and Some of its Substitution Derivatives. J. Chem. Soc. 71, 464.

UHLEKE, H. (1961) N-Hydroxylierung von 2-Aminofluoren durch Lebermikrosomen. Experientia 17, 557.

UHLEKE, H. (1963) N-Hydroxylation of Carcinogenic Amines *in vivo* and *in vitro* with Liver Microsomes. Biochem. Pharmacol. 12, 219.

VOGEL, A. I. (1966) Practical Organic Chemistry, 3rd Edition. John Wiley and Sons Inc., New York, N.Y. a) p. 684 b) p. 577.

von JAGOW, R., KIESE, M. and RENNER, G. (1966) Urinary Excretion of N-Hydroxy Derivatives of Some Aromatic Amines by Rabbits, Guinea Pigs and Dogs. Biochem. Pharmacol. 15, 1889.

WALPOLE, A. L. and WILLIAMS, M. H. C. (1958) Aromatic Amines as Carcinogens in Industry. Brit. Med. Bull. 14, 141.

WELCH, R. M.,CONNEY, A. H. and BURNS, J. J. (1966) The Metabolism of Acetophenetidin and N-Acetyl-p-aminophenol in the Cat. Biochem. Pharmacol. 15, 521.

WEISBURGER, J. H. and WEISBURGER, E. K. (1966) Chemicals as Causes of Cancer. Chem. Eng. News 44 (Feb. 7), 124.

WILLIAMS, R. T. (1959) Detoxication Mechanisms, 2nd Ed., Chapman and Hall Ltd., London, a) p. 435 b) p. 439.

WILLIAMS, R. T. (1966) Biochemical Toxicology. Science J. 2(1), 57.