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

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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

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

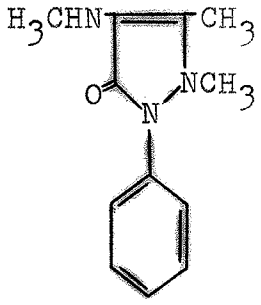
Site of Metabolism

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.

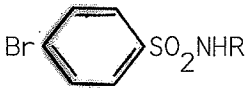
Pathways of Metabolism

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:

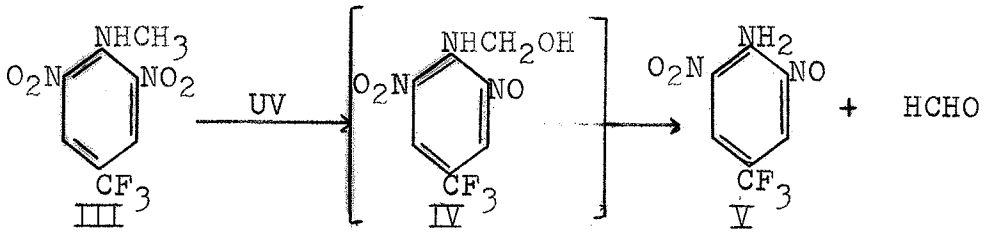
$$R_2NCH_2R^1 \xrightarrow{[O]} R_2NH + R^1CHO \quad \text{(McMahon, 1966a)}$$

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

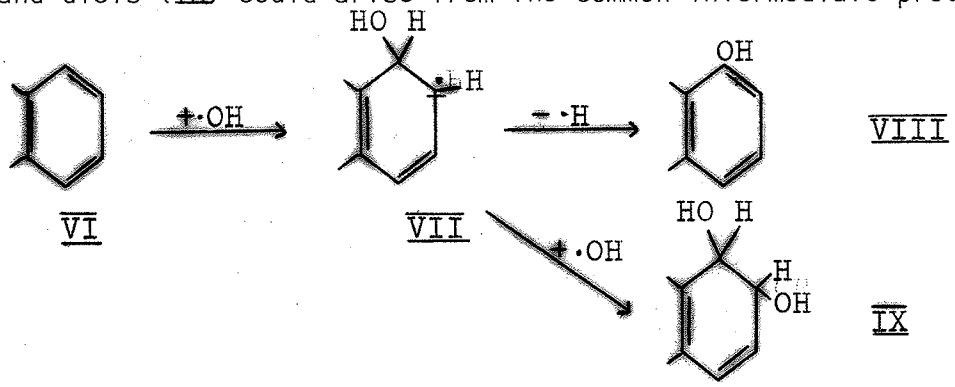
In most species, ring hydroxylation and conjugation are the major pathways of metabolism of foreign aromatic compounds (Bond and Howe 1967, pronethalol; Beckett and Morton 1967, N-alkyloxindoles; Alexander et al 1965, diphenylamine; Parke 1960, aniline; Axelrod 1954, amphetamine; Alexander et al 1968,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

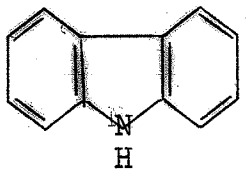
Compounds with acidic side chains such as benzoic acid, phenyl-acetic acid and cinnamic acid have been found to be excreted rapidly, and may thus be removed from the body before hydroxylation can occur (Smith 1950). Smith (1950) also observed that when hydroxylation did occur para-hydroxylation was more 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).

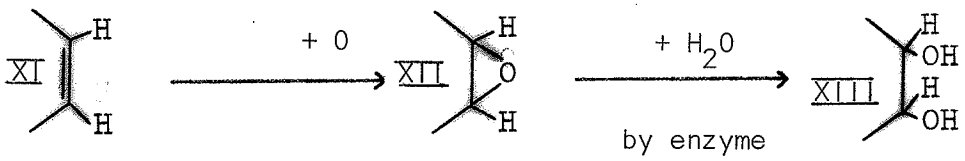


X

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.

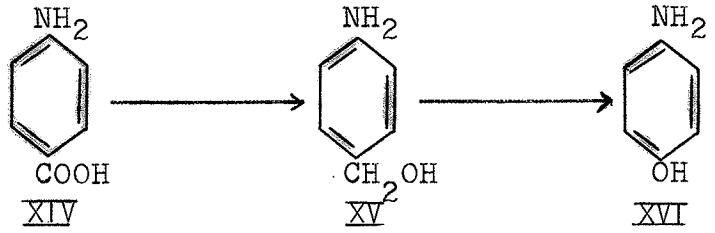
Boyland (1950) postulated an epoxide mechanism for the biological hydroxylation reaction. In formation of the dihydrodiol, the mechanism could be represented as follows (XI-XIII):



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):



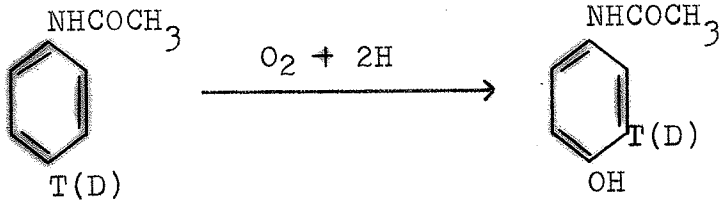
XIV XV XVI

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.

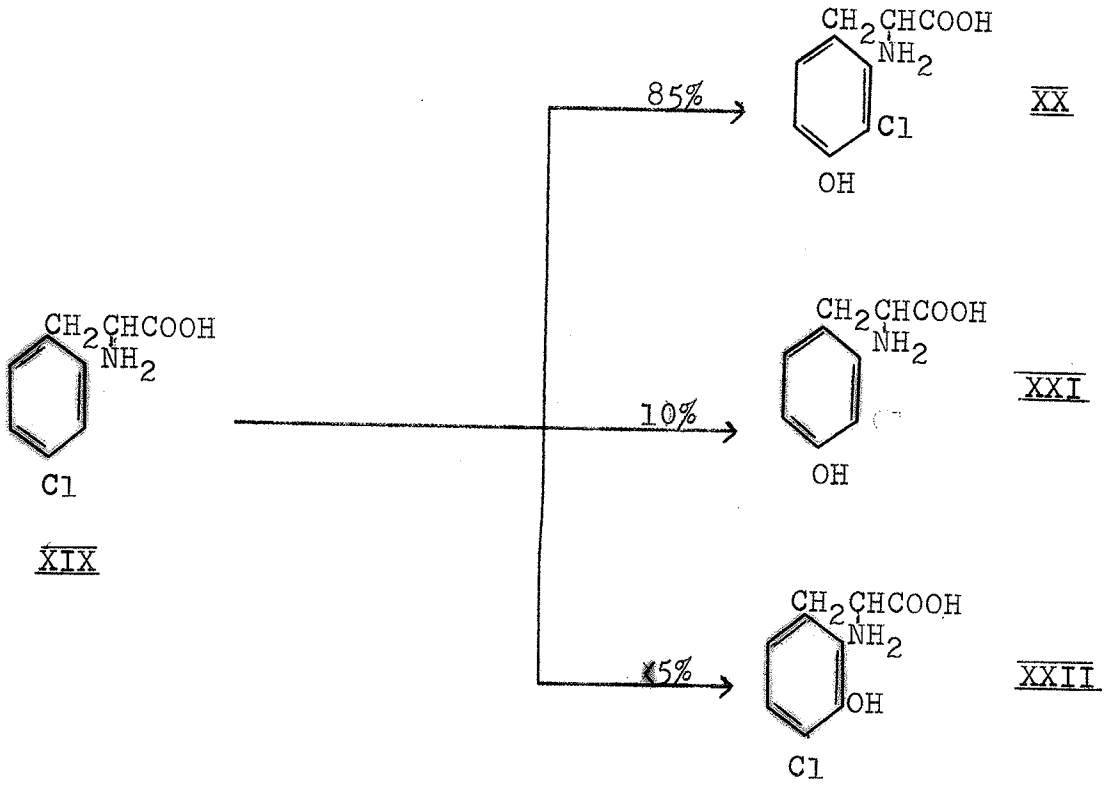
The most recent mechanism proposed is hydroxylation-induced intramolecular migration (NIH Shift) by Guroff et al (1967). Their experiments indicate that a frequent consequence of hydroxylation in aromatic systems is an intramolecular migration or shift of the group displaced by hydroxyl to an adjacent 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

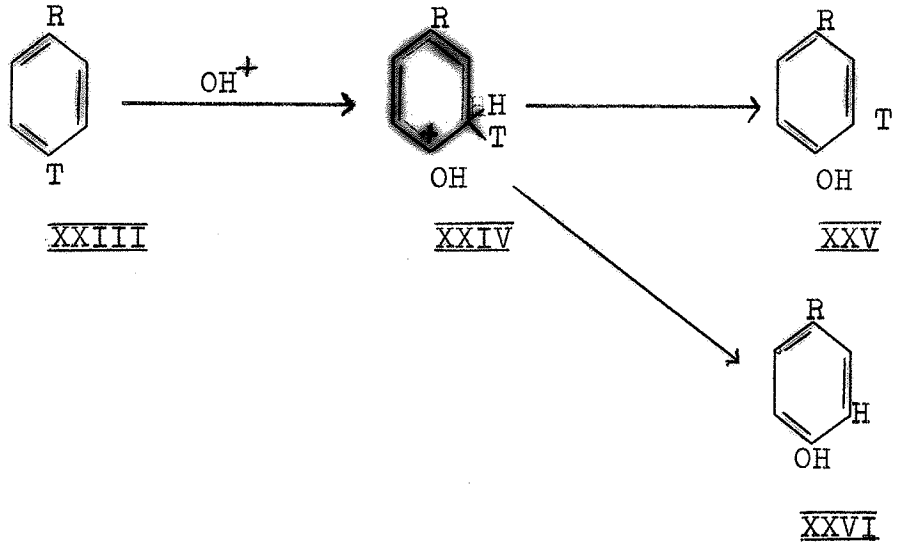
involves a shift of the para-substituent to the meta-position. They found that the amount of tritium retention correlated well with the charge distribution patterns in the postulated hydroxyl containing intermediate (XXIV), and suggest that the "NIH Shift" is a function of the structure of the hydroxyl 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.

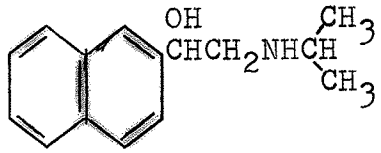
4. Hydroxylation of N-Substituted Anilines

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

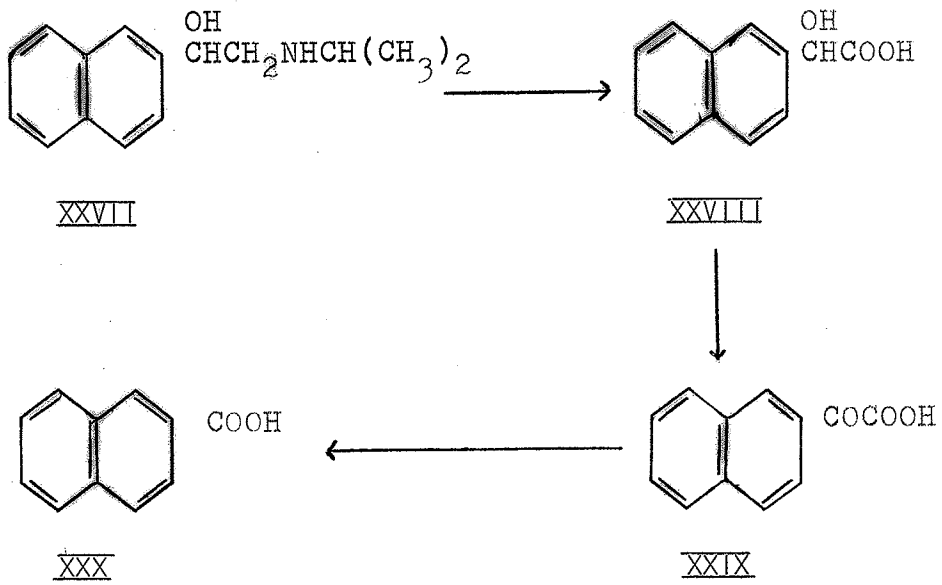
Charalampous and Tansey (1967) stated that when the amino group is located in the side chain, is primary, and is not attached to the aromatic ring, deamination is a major metabolic reaction. Aromatic amines like aniline, in which the amino group is directly attached to the ring are not attacked by amine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



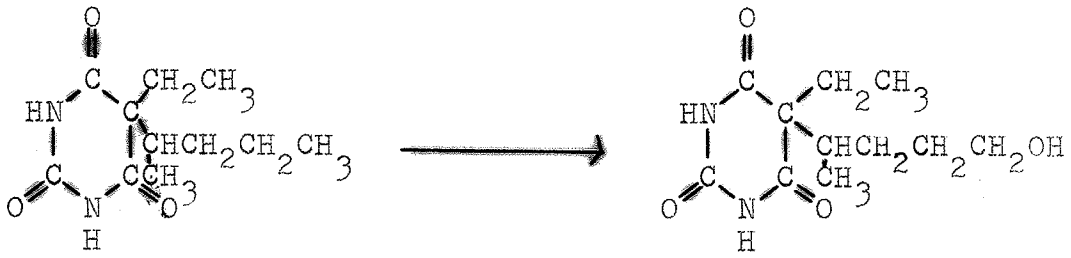
XXVII

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.



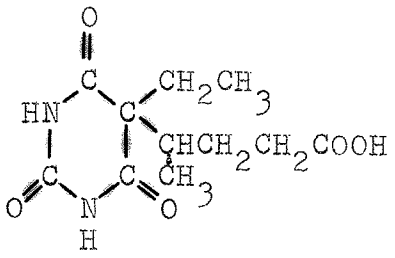
XXVII XXVIII XXIX XXX

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).



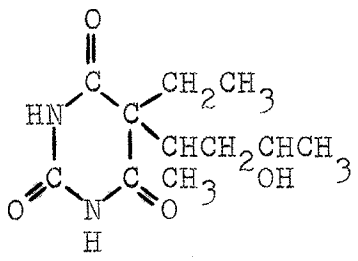
XXXI

XXXII

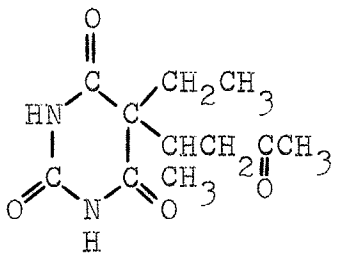


XXXIII

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.

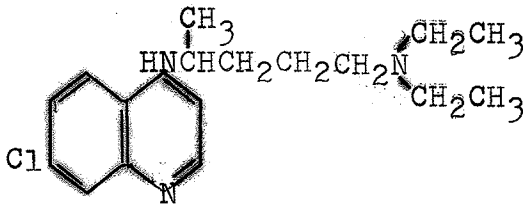


XXXIV

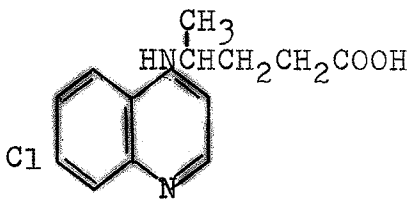


XXXV

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).



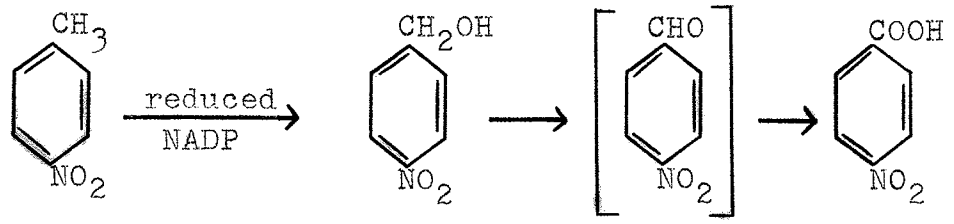
XXXVI



XXXVII

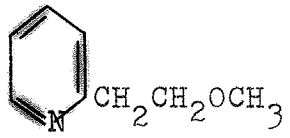
Quantitatively, however, this was only a trace metabolite (McChesney et al 1967). Kuroda (1962) had previously indicated the presence of the alcohol analogue of the above acid (XXXVII) as a metabolite in humans. McChesney et al (1967b) found 1-2% of the acid (XXXVII) as a metabolite of chloroquine in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