THE METABOLISM OF SELECTED N-ALKYLAROMATIC AMINES IN THE RAT

by

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ABSTRACT

N-isobutylaniline-^{14}C hydrochloride was synthesized and its metabolic fate investigated in the rat. Two metabolites were observed which accounted for 93.1% of the twenty-four hour urinary metabolites. These metabolites were 4-hydroxy-N-isobutylaniline (37.4%) and p-aminophenol (55.7%), illustrating the participation of the metabolic pathways: aromatic hydroxylation and N-dealkylation. N-phenylglycine, 2-anilinopropionic acid and o-aminophenol were eliminated as the unknown polar metabolites of N-isopropylaniline. These unknown metabolites remain uncharacterized.

ACKNOWLEDGEMENTS

I wish to express my appreciation to Dr. W. E. Alexander who personally directed this project, for his continual assistance, advice and encouragement while preparing this thesis.

I am indebted to the University of Manitoba and the Medical Research Council who provided financial assistance which enabled me to carry out this project.

I wish to thank the Chemistry Department, University of Manitoba, for performing the nuclear magnetic resonance analysis.

Finally, I wish to express a deeply felt gratitude to my wife for her continual encouragement, thoughtfulness and assistance while completing this project.

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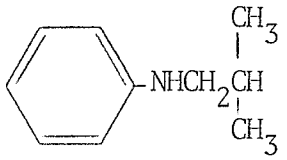
INTRODUCTION

Many compounds foreign to the body are now employed by man. It is important to know what happens to these compounds when they enter the body for it is of great necessity that the body eliminate them without being damaged in the process. Thus, metabolism has gained great prominence in recent years, with interest centered around the chemical nature of the metabolites, the site of metabolism, the biological implications in metabolism, and the mechanism of metabolism.

Most compounds foreign to the body undergo metabolic transformation in the body. The principle site for this transformation is the liver but other tissues are also known to participate (Goldstein et al., 1969). Oxidation is one of the most general reactions of foreign compounds in the body (Williams, 1959a). Aromatic amines are a class of compounds which have found considerable application in the manufacture of dyestuffs and drugs. Interest in the School of Pharmacy, University of Manitoba, has centered around the metabolism of N-alkylaromatic amines. Of the oxidative metabolic transformation reactions, the two which are known to occur with N-alkylaromatic amines are: (1) the hydroxylation of the aromatic ring system, and (2) oxidative dealkylation (Williams, 1959b).

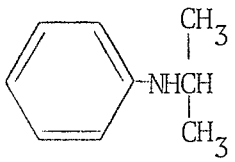
Alexander and Sitar (1969), working with the metabolism of N-alkylaromatic amines, observed a regularity with respect to

structure-activity relationships in the N-dealkylation reaction. It was observed that branching at the α-carbon* on the alkyl chain caused a marked reduction in dealkylation. The question as to the effect of branching on the β-carbon** of the alkyl chain was thus a natural outgrowth in the metabolism of these compounds. As a result the project concerning the metabolism of N-isobutylaniline (I.) was initiated with special interest in the extent of N-dealkylation.



I.

Another problem which had arisen in the metabolic investigations of N-isopropylaniline (II.) and N-sec.butylaniline (III.), was the formation of very polar metabolite(s) which were not identified.



II.

III.

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* Carbon adjacent to the -NH- group.

** Carbon atom, one carbon removed from the -NH- group.

In addition to the extent of N-dealkylation, N-isobutylaniline was thus investigated metabolically to determine whether this compound was metabolized in a manner analogous to N-isopropylaniline and N-sec.butylaniline with respect to formation of the polar metabolites. If metabolic differences were found, then this might assist in understanding what metabolic sequences were involved in the latter two compounds which would be directly related to the structure of the N-alkyl side chain. Work was also initiated to determine the unknown polar metabolite(s) in the metabolism of N-isopropylaniline (II.).

GENERAL METABOLIC REACTIONS OF

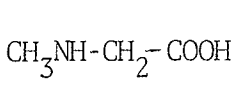
FOREIGN ORGANIC MOLECULES

In general, metabolic transformation of foreign organic molecules result in the formation of compounds more polar than the parent compound, thus facilitating their removal from the body (Goldstein et al., 1969). These authors mention that many of the drug metabolism reactions are reversible and that a polar compound could yield a less polar product. Although this reaction is rare it can be observed in the deacetylation of acetanilide to aniline. Organic compounds foreign to the animal body may undergo one or even all of four basic metabolic transformation reactions: oxidation, reduction, hydrolysis and synthesis (conjugation). Various literature sources summarize these reactions and the metabolic pathways will be dealt with briefly (Goldstein et al., 1969; Williams, 1959; Brodie et al., 1958; Gillette, 1966).

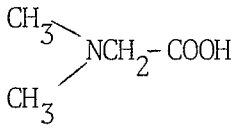
I. OXIDATION:

Most foreign organic compounds undergo oxidation in the body and therefore oxidation is one of the most general metabolic reactions. It includes reactions such as: the oxidation of alcohols and aldehydes to acids, the hydroxylation of ring systems, oxidation of alkyl groups and chains to alcohols and acids, oxidative deamination of amines, oxidative dealkylation, oxidation of sulphur

compounds to sulphoxides and sulphones, the oxidative splitting of rings, dehalogenation, and a variety of other reactions. The liver is the principle site of metabolic reactions but not the only site. The enzymes in the liver which mediate the oxidative reactions are situated in the microsomal fraction of the cell but there are exceptions as well, to the site of oxidative metabolism. La Du et al. (1955), Brodie et al. (1958), and Gaudette and Brodie (1959) have determined that the oxidative demethylation of sarcosine (IV.) and dimethylglycine (V.) takes place in the mitochondrial fraction of mammalian liver cells.



IV.



V.

The requirements for metabolic oxidation in the majority of cases have been noted to be the microsomes, supernatant solution, $NADP^+$, Mg^{+2}, nicotinamide and oxygen. The supernatant solution furnishes the enzymes necessary to reduce $NADP^+$ to NADPH, since the latter compound is able to supplant the supernatant fraction. Oxidation reactions may often produce a center for conjugation thus subjecting the transformed compound to an additional metabolic reaction.

II. REDUCTION:

Reduction is a less common metabolic reaction than oxidation

and found to occur in various body tissues. Reduction reactions which have been observed are: the conversion of some aldehydes to alcohols, reduction of ketones to secondary alcohols, reduction of nitro groups to hydroxylamines and amines, reduction of disulphides to sulphydryl derivatives, and other reactions. Frequently, as found with oxidation, reduction results in a center at which conjugation may occur. (for reference see Page 4).

III. HYDROLYSIS:

Drug metabolism by hydrolysis is found to be restricted to esters and amides. The enzymes responsible for hydrolysis are found in blood plasma and other tissues, including the liver, usually in the soluble fraction of the cells and are called esterases and amidases. (for reference see Page 4).

IV. SYNTHESIS (CONJUGATION):

Certain molecules in the body can react with foreign organic molecules, or their partial transformation products which in formation have developed centers of conjugation. These synthetic reactions include the formation of glucuronides, ribosides and riboside phosphates, mercapturic acids, sulphuric acid esters, ethereal sulphates and a number of other compounds. These synthetic reactions result in the formation of more polar compounds which are more readily excreted by the kidney. Of the synthetic reactions, conjugation with glucuronic acid is one of the most common reactions.

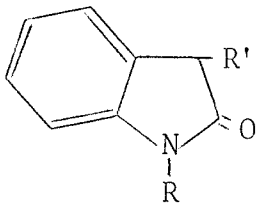
The liver contains enzymes that catalyze the formation of uridine diphosphate-α-D-glucuronic acid (UDPGA) which serves as a donor of glucuronic acid to various acceptors. By the mediation of transferases, glucuronic acid from UDPGA is conjugated with the hydroxyl group in phenols and aliphatic alcohols, the carboxyl group of acids, the nitrogen of aromatic amines and even occasionally a sulphydryl group. Sulfate conjugated metabolites also occur to some extent. An activated form of sulphate, as part of 3'-phosphoadenosine-5'-phosphosulphate (PAPS), is capable of forming ethereal sulphates by reaction with aromatic and aliphatic hydroxyl groups, and of certain amino groups. Alexander et al. (1964) isolated the sulphate ester of 4-hydroxy-diphenylamine as the potassium salt. Newell et al. (1960) discovered that sulphate conjugated with the hydroxylated metabolites of monochloroacetanilides. In the metabolism of 2-naphthylamine in both the dog and the cat, Boyland and Manson (1966) found 2-amino-1-naphthyl hydrogen sulphate as the main metabolite (for reference see Page 4).

METABOLISM OF AROMATIC AMINES

The metabolic fate of various aromatic amines has been investigated and two predominant transformation reactions have been observed as was indicated earlier: (1) the hydroxylation of the aromatic ring system, and (2) oxidative dealkylation whenever the nitrogen was N-alkylated (Williams, 1959b). Other reactions have been observed and there have also been exceptions to the two predominant general reactions. In accordance with the compounds investigated and purpose of this project, the metabolic reactions of aromatic amines will be discussed briefly, with special emphasis on N-dealkylation reactions, hydroxylation reactions and reactions involving side chain oxidation, giving examples of N-alkylaromatic amines where possible.

I. N-DEALKYLATION:

N-dealkylation has been observed with numerous N-alkyl substituted compounds both *in vivo* and *in vitro*. Since the interest in structure-activity relationship in dealkylation of N-alkylaromatic amines prompted this project, the literature was consulted for analogous studies. Beckett and Morton (1967) found that an increase in chain length caused a decrease in rate of N-dealkylation in the metabolism of N-alkyloxindoles (VI.).

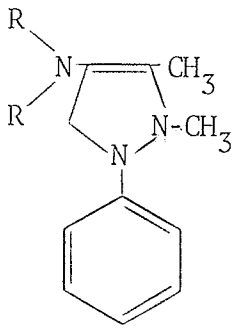


R' = H or C_2H_5

R = CH_3, C_2H_5, n-C_3H_7

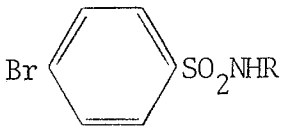
VI.

Similar results were obtained by La Du et al. (1955) with substituted 4-aminoantipyrines (VII.).



VII.

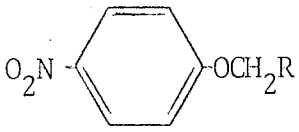
They found that as chain length increased, the percent dealkylation decreased. Also, dialkyl substituted compounds were dealkylated less than monoalkyl compounds. Of the compounds investigated, dealkylation was found to occur in the following order: dibutyl < monobutyl < diethyl < monoethyl < dimethyl < monomethyl. For N-alkyl-4-bromobenzene sulphonamides (VIII.) Smith et al. (1965) discovered that the degree of N-dealkylation was dependent on the structure of the N-alkyl chain and occurred as follows:



VIII.

R: CH_3 > C_2H_5 > $CH_2CH{=}CH_2$ > $CH(CH_3)_2$ > $(CH_2)_2CH_3$ > $(CH_2)_3CH_3$

McMahon et al. (1963), studying the O-dealkylation of alkyl- and arylalkyl-p-nitrophenylethers (IX.) in the rat found that, similar to N-dealkylation, increasing chain length of normal alkyl groups decreased the rate of dealkylation.



IX.

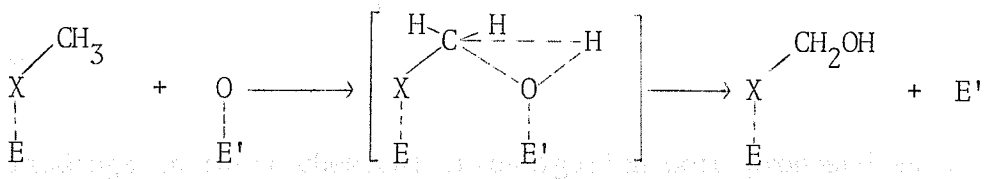
N-dealkylation of N-alkylanilines has been demonstrated in vitro by Gaudette and Brodie (1959) with the compounds N-methyl, N,N-dimethyl, N-ethyl and N-butylaniline using rabbit liver microsomes. In vivo dealkylation of N-alkylanilines and substituted N-alkylanilines was also demonstrated with N,N-dimethyl- (Smith, 1950), N,N-dipropyl- (Emmerson and Anderson, 1966) and N-sec.butyl- (Alexander et al., 1968) compounds. However, no systematic structure-activity study has been observed in the N-dealkylation of N-alkylanilines where the alkyl group is larger than methyl or ethyl (McMahon, 1966).

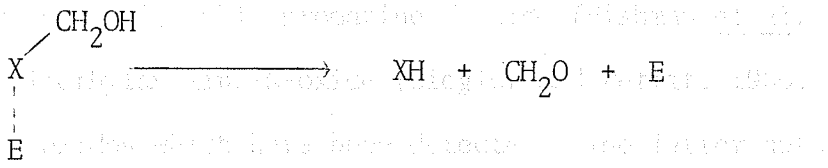
The overall dealkylation reaction has been considered to occur via the following mechanism:

$$RXCH_2R' \xrightarrow{(O)} RXH + R'CHO$$

X = NH, O or S

Two basic mechanisms have been proposed for the dealkylation reaction and there is no evidence at present that either of these represents the only mechanism. McMahon and Sullivan (1964), proposed that dealkylation was initiated by an oxidative attack on the α-carbon in the following way:





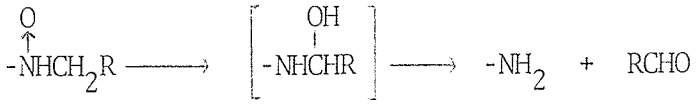
X = NH, O, S

E = Enzyme

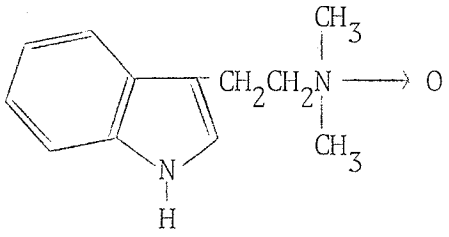
E'= Enzyme

This type of mechanism was attractive because it emphasized the relationship of dealkylation to hydroxylation which it resembled so closely with respect to cofactor requirements, response to inhibitors and inductibility. The other mechanism proposes an initial oxidative attack on the nitrogen atom. The N-hydroxymethyl, if present, then arises from the rearrangement of the alkylamine

N-oxide intermediate:

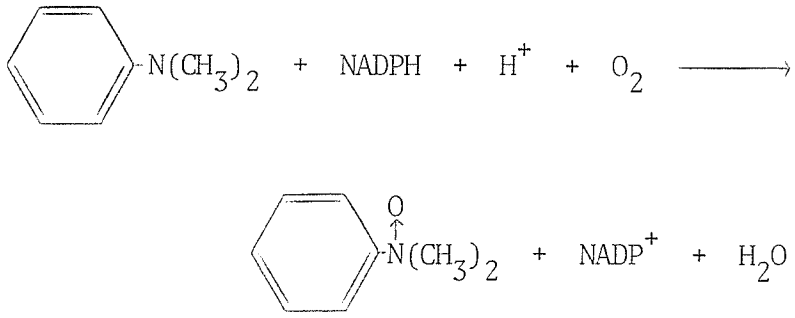


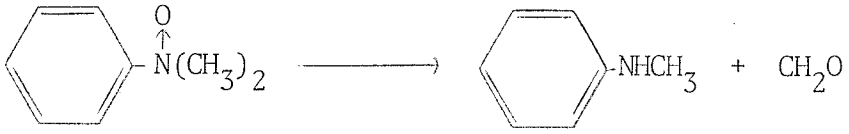
This sequence was observed in the chemical investigation of N,N-dimethyltryptamine-N-oxide (X.) by Fish et al. (1956).



X.

The findings in their chemical investigation were proposed as a model for biological systems. Trimethylamine-N-oxide (Baker and Chaykin, 1962), Chlorpromazine-N-oxide (Fishman et al., 1962) and N,N-dimethylaniline-N-oxide (Ziegler and Pettit, 1966) are some of the N-oxides which have been detected. The latter authors, working with N,N-dimethylaniline proposed the following metabolic sequence:

(1) 

(2) 

The two mechanisms thus differ as to the site of oxidative attack, being the α-carbon in one mechanism and the nitrogen atom in the other mechanism.

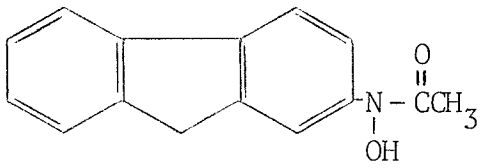
II. HYDROXYLATION:

Various investigations with foreign organic compounds in a variety of species have indicated that ring hydroxylation and subsequent conjugation are significant metabolic pathways: (Bond and Howe, 1967 - pronethalol; Alexander et al., 1965 - diphenylamine; Axelrod, 1954 - amphetamine; Alexander et al., 1968 - N-sec.butylaniline; Alexander and Sitar, 1969 - N-isopropylaniline).

Rabbit liver microsomes were used to perform in vitro hydroxylation studies with the compounds benzene, naphthalene, quinoline, indole, aniline, diphenyl and coumarin (Posner et al., 1961). These compounds were all hydroxylated by the same microsomal system indicating that enzymatic hydroxylation of a variety of compounds might be carried out by the same enzyme system.

Hydroxylation has been observed to take place at the ortho-, para- and even meta-positions, with the preferred point, or at times the only point of oxidative attack being the para-position. This has been shown with aniline (Parke, 1960), N, N-dimethylaniline (Horn, 1936) and N-isopropylaniline (Alexander and Sitar, 1969). However, acetanilide is extensively deacetylated and mainly excreted as o-aminophenol in the dog (Williams, 1959c).

Aromatic amines are also known to undergo N-hydroxylation. N-hydroxylation of both acetylated and free aromatic amines has been found to be a general reaction. This type of reaction was observed with 2-acetylaminofluorene *in vitro* resulting in the formation of N-hydroxy-2-acetylaminofluorene (XI.) (Uheke, 1961).



XI.

In the rat, this compound was excreted as the O-glucuronide (Cramer *et al.*, 1960). N-hydroxy derivatives have also been identified with 4-acetamidobiphenyl and N-acetylbenzidine after incubation of the parent amine with rabbit liver microsomes (Booth and Boyland, 1964). Thus the enzyme system responsible for the N-hydroxylation is located in the liver microsomal fraction. Booth and Boyland (1964) demonstrated that the N-hydroxy derivatives of acetanilide, 2-acetylaminofluorene, 2-acetamidonaphthalene and 4-acetamidobiphenyl were isomerized to the corresponding *ortho* hydroxylated products by enzymes in the soluble fraction of rabbit liver in the presence of NAD, NADH or NADPH. It was therefore suggested that the *ortho* hydroxylated amines are formed by the rearrangement of the corresponding N-hydroxy derivatives.

Various mechanisms have been postulated for the hydroxylation reaction. These include:

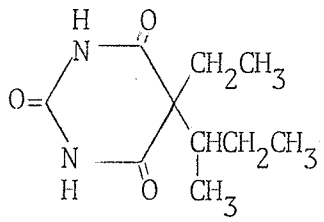
(a) hydroxylation proceeding via a free radical mechanism (Smith, 1950).

(b) hydroxylation proceeding via an epoxide mechanism (Boyland, 1950).

(c) hydroxylation proceeding via a hydroxylation-induced intramolecular migration - NIH Shift (Guroff et al., 1967).
In addition to these proposed mechanisms Daly et al. (1968) have indicated that the degree to which aromatic substrates are hydroxylated by microsomal hydroxylases appears to be directly related to the relative reactivity of the ring to electrophilic substitution.

III. SIDE-CHAIN OXIDATION:

Since this project involved N-alkyl substituted anilines, the possibility of metabolic attack on the alkyl group appeared feasible. Various examples exist in the literature in which there is a report of side-chain oxidation. In the metabolism of butabarbital (XII.) (Maynert and Losin, 1955), the metabolite 5-ethyl-5(1-methyl-2-carboxyethyl)-barbituric acid (XIII.) was detected exemplifying side chain oxidation.



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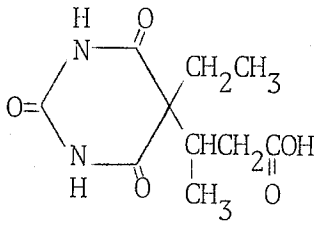
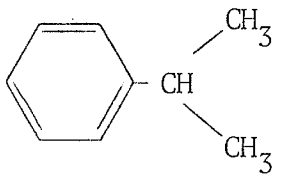
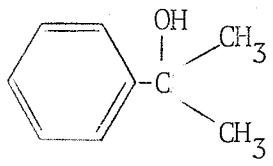


XIII.

In the metabolism of isopropylbenzene (XIV.), Robinson et al. (1955) were able to detect three metabolites which were products of side chain oxidation. These products, excreted as the glucuronides, were 2-phenylpropan-2-ol (XV.), hydrotropyl alcohol (XVI.) and hydrotropic acid (XVII.).



XIV.



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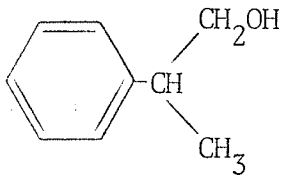
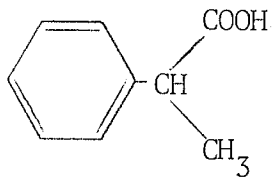
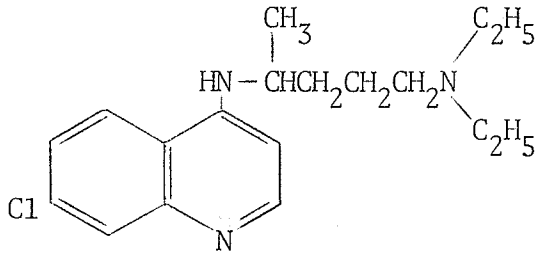


XVI.

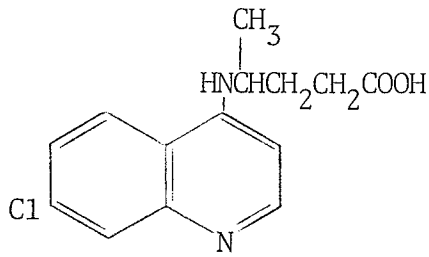


XVII.

Metabolic studies with chloroquine (XVIII.) demonstrated an acidic metabolite (XIX.) formed by dealkylation and deamination of the diethylamine group (McChesney et al., 1966).



XVIII.



XIX.

This last investigation is the only example observed in which the alkyl side chain was linked to an aromatic nucleus via a nitrogen atom. Yet, this example does not shed light on side chain oxidation of N-alkylaromatic amines since its structure facilitated oxidative deamination.

IV. SYNTHESIS (CONJUGATION):

Conjugation also occurs with aromatic amines both at the nitrogen atom and with hydroxylated aromatic ring systems. The amino group has been observed to conjugate directly with glucuronic acid and inorganic sulfate to form N-glucuronides and N-sulphates as shown by Parke (1960) in his studies on the metabolism of aniline. Boyland et al. (1956) have 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. In addition aromatic amines have been observed to undergo acetylation on the nitrogen atom as was observed by Lipmann (1945) in his work with sulphanilamide. Coenzyme A is the requirement for enzymic acetylation of aromatic amines and probably for all other biological acetylation processes (Williams, 1959d). The general reactions of acetylation may be represented as follows:

$$CH_3COX + CoASH \longrightarrow HX + CoASCOCH_3$$

$$CoASCOCH_3 + HY \longrightarrow CoASH + CH_3COY$$

CH_3COX, the source of acetyl groups, may be pyruvate, acetyl phosphate or acetate in the presence of ATP. HY is the acceptor

of acetyl groups and may be an amine or some other compound native to the biological system.

From the brief discussion on the metabolism of aromatic amines, various typical metabolic reactions have been mentioned: N-dealkylation, ring hydroxylation, N-hydroxylation, side-chain oxidation, sulphate and glucuronide conjugation and acetylation. Certain of these reactions have been observed in this project with N-isobutylaniline and the experimental work will illustrate these observations.

EXPERIMENTAL

I. PREPARATION OF COMPOUNDS:

A) N-Isobutylaniline Hydrochloride:

Dry hydrogen chloride gas was passed through an ethereal solution of commercial N-isobutylaniline (K. & K. Laboratories, Inc.) and recrystallized from super dry ethanol/dry ether.

m.p. 202-203°C.; reported 192°C. (Beilstein, 1950). Logϵ (245) = 4.025 (in equal quantities of 95% alcohol and 0.008 N sodium hydroxide); reported logϵ (245) = 4.02 (Rumpf, P. and Girault, G., 1954). The instrument used in determining logϵ (245) was the Beckman model DU spectrophotometer.

B) N-Isobutylaniline-^{14}C Hydrochloride:

N-isobutylaniline-^{14}C hydrochloride was synthesized according to a modification of the procedure by Schellenberg (1963). Aniline-^{14}C hydrochloride* (3.3 mg.; 0.125 mC.) and aniline hydrochloride (total 0.4028 g.; 0.003108 moles) were dissolved in water (22 ml.). The solution was made just alkaline to litmus by the addition of sodium hydroxide solution, followed by the addition of 95% ethanol (4.5 ml.). Then glacial acetic acid (1.1 ml.) was added along with trihydrated sodium acetate (2.7 g.). The mixture was stirred to effect the solution of the sodium acetate. The solution was then cooled to 0°C. by setting the container in an ice/salt bath. One minute after the addition of redistilled isobutyraldehyde (0.64g.; 0.0089 moles) and water (1.0 ml.), sodium borohydride (1.4 g.; 0.037 moles) was added in 30 mg. portions

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* Uniformly labelled (New England Nuclear Corp., Boston, Massachusetts).

to the stirred solution over a period of 10 minutes while maintaining the temperature near $0^{o}C$. The mixture was stirred an additional 10 minutes. Then the mixture was made alkaline with ammonia and extracted with 5 x 5 ml. portions of ether. Following a washing with saturated sodium chloride solution, the ethereal extract was dried over anhydrous sodium sulfate and filtered. The ether was evaporated under nitrogen and the resulting liquid spotted on thin layer chromatographic plates (1.00 mm.) of silica gel GF-254 via an Agla micrometer syringe, and allowed to develop in a solvent system of 40% petroleum ether (30-60)/60% benzene. The plates were air dried and the band corresponding to N-isobutylaniline (Rf = 0.5) was scraped from the plates. The resulting silica gel scrapings were placed in a column and eluted with dry ether (50 ml.). Dry hydrogen chloride gas, passed through the ethereal eluent resulted in the crude product (0.390 g.; 67.6%). The crude product was recrystallized to constant specific activity [3.8048×10^{5} disintegrations per minute per mg. (dpm./mg.)] from super dry ethanol/dry ether (Table I). m.p. $202\text{-}203^{o}C$. $\text{Log}\epsilon$ (245) = 4.026 (in equal parts 95% ethanol and 0.008 N sodium hydroxide) on the Beckman model DU spectrophotometer. Thin layer chromatography of this compound by converting it to the free base exhibited one radioactive spot corresponding to N-isobutylaniline (Rf = 0.48) after developing in 40% petroleum ether (30-60)/60% benzene. Cold N-isobutylaniline hydrochloride was synthesized similarily and analyzed, viz.

Calculated for $C_{10}H_{16}NCl$: C, 64.68%; H, 8.69%.

Found for $C_{10}H_{16}NCl$: C, 64.47%; H, 8.60%.

C) N-Isopropylaniline-^{14}C Hydrochloride:

N-isopropylaniline-^{14}C hydrochloride was synthesized according to a modification of the procedure by Schellenberg (1963). Aniline-^{14}C hydrochloride* (3.3 mg.; 0.125 mC.) and aniline hydrochloride (total 0.4039 g.; 0.003116 moles) were dissolved in water (17.5 ml.). The solution was made just alkaline to litmus by the addition of sodium hydroxide solution, followed by the addition of 95% ethanol (6.0 ml.). Then glacial acetic acid (2.0 ml.) was added along with trihydrated sodium acetate (2.7 g.). The mixture was stirred to effect the solution of the sodium acetate. The solution was then cooled to $0^{o}C$. by setting the container in an ice/salt bath. One minute after the addition of acetone (2.4 g.; 0.041 moles), sodium borohydride (1.6 g.; 0.042 moles) was added in 30 mg. portions to the stirred solution over a period of 15 minutes while maintaining the temperature near $0^{o}C$. The mixture was stirred an additional 10 minutes. Then the mixture was made alkaline with ammonia and extracted with 5 x 5 ml. portions of ether. Following a washing with saturated sodium chloride solution, the ethereal extract was dried over anhydrous sodium sulfate and filtered. The ether was evaporated under nitrogen and the resulting liquid spotted on thin layer chromatographic plates (1.00 mm.) of silica

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* Uniformly labelled (New England Nuclear Corp., Boston, Massachusetts).

gel GF-254 via an Alga micrometer syringe, and allowed to develop in a solvent system of 40% petroleum ether (30-60)/60% benzene. The plates were air dried and the band corresponding to N-isopropylaniline (Rf = 0.3) was scraped from the plates. The resulting silica gel scrapings were placed in a column and eluted with dry ether (50 ml.). Dry hydrogen chloride gas, passed through the ethereal eluent resulted in the crude product (0.420 g.; 78.5%). The crude product was recrystallized to constant specific activity (5.19586×10^5 dpm./mg.) from super dry ethanol/dry ether (Table I.). m.p. 172-173°C.; reported 169-170°C. (Alexander and Sitar, 1969). Thin layer chromatography of this compound by converting it to the free base exhibited one radioactive spot corresponding to N-isopropylaniline (Rf = 0.29) after developing in 40% petroleum ether (30-60)/60% benzene. Non-radioactive N-isopropylaniline hydrochloride reported m.p. 172-173°C. (Alexander and Sitar, 1969).

D) 4-Hydroxy-N-Isobutylaniline Hydrochloride:

This compound was prepared according to the procedure of Emmerson and Uraneck (1941). 4-Nitrophenol (13.9 g.; 0.100 moles), isobutyraldehyde (7.21 g.; 0.100 moles), glacial acetic acid (10 ml.), 95% ethanol (150 ml.) and platinum oxide (100 mg.) were placed in a Parr Pressure Reaction Apparatus, subjected to hydrogen (1800 lbs./in.^{2}), and the reaction allowed to proceed with shaking for 4 hours. The resulting mixture was filtered, the alcohol removed on a Rinco Rotary Evaporator, dried and the dry residue dissolved in dilute hydrochloric acid. This

acid solution was then washed with ether, neutralized with sodium hydroxide solution and again extracted with ether. The ether extract was dried over anhydrous sodium sulfate, filtered and dry hydrogen chloride gas was passed through the filtrate to yield the crude product. This crude product was recrystallized from super dry ethanol/dry ether. m.p. 196-197°C.

Calculated for $C_{10}H_{16}NOCl$: C, 59.55%; H, 8.00%.

Found for $C_{10}H_{16}NOCl$: C, 59.25%; H, 7.97%.

E) 4-Hydroxy-N-Isobutylaniline-N,O-Ditosylate:

The procedure by Vogel (1966a) was employed in the preparation of this compound. The starting materials used were 4-hydroxy-N-isobutylaniline hydrochloride and p-toluenesulphonyl chloride. The crude product was recrystallized from two solvent systems; methanol/water and ethyl acetate/petroleum ether (30-60). m.p. 109-110°C.

Calculated for $C_{24}H_{27}S_2O_5N$: C, 60.87%; H, 5.75%.

Found for $C_{24}H_{27}S_2O_5N$: C, 60.66%; H, 5.73%.

F) p-Aminophenol-N,O-Ditosylate:

The procedure by Vogel (1966a) was employed using p-toluenesulphonyl chloride and p-aminophenol to prepare this compound. The crude compound was recrystallized from methanol/water and ethyl acetate/petroleum ether (30-60). m.p. 170-171°C. Reported: 169°C. from benzene (Beilstein, 1930a).

G) N-Acetyl-p-Aminophenol:

This compound was synthesized from p-aminophenol

according to the procedure by Vogel (1966b) for acetanilide. The crude product was recrystallized from water. m.p. 168-169°C. Reported: 168°C. (Heilbron and Bunbury, 1963a).

H) p-Aminophenol Hydrochloride:

This compound was prepared in our laboratories by dissolving a maximum quantity of p-aminophenol in hot concentrated hydrochloric acid and allowing to cool. The crude product was recrystallized from concentrated hydrochloric acid until the optical extinction coefficient (ϵ) remained constant: ϵ (245) = 1424 (in N/100 hydrochloric acid) on the Beckman model DU Spectrophotometer. m.p. 304-305°C. (decomp.). Reported m.p. 306°C. (decomp.) (Heilbron and Bunbury, 1963b).

I) N-Phenylglycine Methyl Ester Hydrochloride:

This compound was prepared by adding N-phenylglycine (Eastman Organic Chemicals) to ethereal diazomethane (prepared from diazald according to the method by Aldrich Chemicals). The ethereal solution was heated to destroy excess diazomethane and then dried over anhydrous sodium sulfate. After filtering, dry hydrogen chloride gas was bubbled through the ethereal filtrate to yield the crude product. This crude product was recrystallized from ethyl acetate/methanol/petroleum ether (30-60). m.p. 173-175°C.

Calculated for $C_9H_{12}NO_2Cl$: C, 53.61%; H, 6.00%.

Found for $C_9H_{12}NO_2Cl$: C, 53.37%; H, 6.20%.

J) Methyl-2-Phenylaminopropanoate Hydrochloride:

2-Phenylaminopropionic acid, synthesized in our

laboratory, was dissolved in ethyl alcohol and methylated with diazomethane (prepared from diazald according to the method by Aldrich Chemicals). The solution was concentrated on a Rinco Rotary Evaporator, the resulting residue dissolved in ether and the methyl ester isolated as the hydrochloride salt by passing dry hydrogen chloride gas. The product was recrystallized from super dry ethanol/dry ether. m.p. 171-172°C. found and reported (Alexander, 1965).

K) 2-Anilinopropionic Acid:

This compound was synthesized in our laboratories 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.

L) o-Aminophenol-N,O-Dibenzoate:

This compound was synthesized by way of the Schotten-Baumann reaction according to the procedure of Vogel (1966c) using o-aminophenol and benzoyl chloride. The compound was recrystallized from ethanol, chloroform, and benzene. m.p. 182-183°C. Reported: 182°C. (Beilstein, 1930b).

II. ANIMAL EXPERIMENTS:

Male Sprague-Dawley rats (310-390 g.) were each injected intraperitoneally with an aqueous solution (1 ml.) of N-isobutylaniline-^{14}C hydrochloride (15 mg. free base/Kg.). The rats were each isolated in individual metabolism cages (Acme), and urine samples were collected at intervals of twenty-four hours over a total period of seventy-two hours. The urine samples collected were then each centrifuged, made up to 25.0 ml. with water in a volmetric flask and refrigerated. The radioactivity present in each twenty-four hour urine sample was determined by liquid scintillation counting. Aliquots of the first twenty-four hour urine sample were taken and used in the distribution study and in the identification of metabolites.

A similar procedure was followed, employing male Sprague-Dawley rats (325-465 g.), after the injection of an aqueous solution (1 ml.) of N-isopropylaniline-^{14}C hydrochloride (15 mg. free base/Kg.) and the collection of twenty-four hour urine samples. In the identification of N-phenylglycine and 2-anilinopropionic acid as possible metabolites, the first twenty-four hour urine samples of three rats were bulked, centrifuged, made up to 50.0 ml. in a volumetric flask and refrigerated. Aliquots of this urine were then used in the determination of the metabolites.

III. DISTRIBUTION STUDIES:

Twenty-four hour urine samples (5.00 ml.) were used for the distribution studies. The samples were hydrolyzed under varying conditions employing a Presto pressure cooker at 15 p.s.i. or the process of refluxing. The hydrolyzed urine was then adjusted to a desired pH using hydrochloric acid or ammonium hydroxide. After the addition of 5 ml. of appropriate buffer, the samples were extracted first with ether and then with butanol (4 x 5 ml. solvent extraction volumes with an extraction period of 30 minutes /5 ml. volume). The extractions were performed in 13 ml. centrifuge tubes on a mechanical shaker. The ether and butanol extractions were made up to 25.0 ml. in a volumetric flask and the amount of radioactivity present determined by liquid scintillation counting.

IV. REVERSE ISOTOPE DILUTION STUDIES:

The technique of reverse isotope dilution was utilized in the identification of suspected metabolites. This technique involved the addition of the suspected metabolite or a hydrolyzable derivative of it in a non-radioactive state to an aliquot of twenty-four hour urine containing a known quantity of radioactivity. The urine was hydrolyzed and the metabolite reisolated by ether extraction at a desired pH and finally characterized as a derivative. This derivative was then recrystallized to constant specific activity as determined by liquid scintillation counting.

In determining when an isolated derivative was at constant specific activity, the following procedure was followed. Recrystallization was performed on each isolated derivative and samples of each recrystallization retained. These samples were then uniformly dried under vacuum over sulphuric acid. Duplicate samples of each recrystallization were then counted for radioactivity. A sample was assumed to be at constant specific activity when the disintegrations per minute per mg. (dpm./mg.) of the last three recrystallizations were within 2% of the average. These determinations required a solvent change after the first of these last three recrystallizations.

The pH for maximum extraction with ether of a metabolite was determined by dissolving the metabolite or its salt in water. The aqueous solution, placed in numerous centrifuge tubes, was then adjusted to various pH values with base or acid and ether extracted on a mechanical shaker. The ether extract yielding the largest amount of compound indicated the optimum pH for extraction.

A) 4-Hydroxy-N-Isobutylaniline:

4-Hydroxy-N-isobutylaniline hydrochloride (407.5 mg.; 0.002020 moles) was added to 20.00 ml. of twenty-four hour urine (1.7468×10^6 dpm.) obtained from a rat injected with N-isobutylaniline-^{14}C hydrochloride. Concentrated hydrochloric acid (4 ml.) was added and the urine hydrolyzed for one hour

(Presto pressure cooker at 15 p.s.i.). The urine was then adjusted to pH 7.8 with ammonium hydroxide and continuously ether extracted for three hours. After drying the ethereal solution over anhydrous sodium sulphate and filtering, the ether was evaporated under nitrogen. p-Toluenesulphonyl chloride (6.0 g.; 0.016 moles) and anhydrous pyridine (15 ml.) were added and the mixture refluxed for two hours after which it was poured into cold water. The water was ether extracted and the ethereal extract washed with cold hydrochloric acid (5%), cold sodium hydroxide (5%), saturated sodium chloride solution, and dried over anhydrous sodium sulphate. After filtration and evaporation of the ether under nitrogen, the remaining crude residue was recrystallized to constant specific activity from methanol/water and ethyl acetate/petroleum ether (30-60). m.p. 109-110°C.

The experiment was repeated with different twenty-four hour urine samples obtained from different rats:

20.00 ml. urine (1.4928×10^6 dpm.) and 4-hydroxy-N-isobutylaniline hydrochloride (401.4 mg.; 0.001990 moles). m.p. 109-110°C.

10.00 ml. urine (8.699×10^5 dpm.) and 4-hydroxy-N-isobutylaniline hydrochloride (398.1 mg.; 0.001974 moles). m.p. 109-110°C.

B) p-Aminophenol:

N-acetyl-p-aminophenol (413.5 mg.; 0.002737 moles) was added to 20.00 ml. of twenty-four hour urine (7.925×10^5 dpm.)

obtained from a rat injected with N-isobutylaniline-^{14}C hydrochloride. Concentrated hydrochloric acid (3ml.) was added and the urine hydrolyzed for one hour (Presto pressure cooker at 15 p.s.i.). The urine was then adjusted to pH 7.0 with ammonium hydroxide and continuously ether extracted for three hours. After drying the ethereal solution over anhydrous sodium sulphate and filtering, the ether was evaporated under nitrogen. p-Toluenesulphonyl chloride (6.0 g.; 0.016 moles) and anhydrous pyridine (15 ml.) were added and the mixture was refluxed for two hours after which it was poured into cold water. The water was ether extracted and the ethereal extract was successively washed with cold hydrochloric acid (5%), cold sodium bicarbonate (5%), saturated sodium chloride solution, and dried over anhydrous sodium sulphate. After filtration and evaporation of the ether under nitrogen, the remaining residue was recrystallized to constant specific activity from methanol/water and ethyl acetate/petroleum ether (30-60). m.p. 170-171°C.

The experiment was repeated with different twenty-four hour urine samples from different rats:

10.00 ml. urine (8.810×10^5 dpm.) and N-acetyl-p-aminophenol (400.7 mg.; 0.002652 moles). m.p. 170-171°C.

10.00 ml. urine (8.668×10^5 dpm.) and N-acetyl-p-aminophenol (407.1 mg.; 0.002694 moles). m.p. 170-171°C.

8.00 ml. urine (6.479×10^5 dpm.) and N-acetyl-p-aminophenol (407.0 mg.; 0.002694 moles). m.p. 170-171°C.

8.00 ml. urine (6.479×10^5 dpm.) and p-aminophenol hydrochloride (392.0 mg.; 0.002692 moles). m.p. 170-171°C.

C) N-Phenylglycine:

N-phenylglycine (Eastman Organic Chemicals) (502.3 mg.; 0.003322 moles) was added to 20.00 ml. twenty-four hour urine (2.6368×10^6 dpm.) obtained from rats injected with N-isopropyl-aniline-^{14}C hydrochloride. Concentrated hydrochloric acid (6 ml.) was added and the urine hydrolyzed for one hour (Presto pressure cooker at 15 p.s.i.). The urine was then adjusted to pH 3.5 with sodium hydroxide solution (20%) and continuously ether extracted for eight hours. After evaporating the ether under nitrogen, the residue was dried under vacuum over sulphuric acid. Ethereal diazomethane (prepared from diazald according to the method by Aldrich Chemicals) was added to the dried residue. The ethereal solution was boiled to destroy excess diazomethane and reduced to a minimum volume on a Rinco Rotary Evaporator. The resulting liquid was spotted on thin layer chromatographic plates (1.00 mm.) of silica gel GF-254 via an Agla micrometer syringe, and allowed to develop in a solvent system of 5% petroleum ether (30-60)/95% benzene. The plates were air dried and the band corresponding to N-phenyl-glycine methyl ester (Rf = 0.30) was scraped from the plates. The resulting silica gel scrapings were placed in a column and eluted with dry ether. Dry hydrogen chloride gas, passed through the ethereal eluent resulted in the crude product. The crude product was recrystallized once from super dry ethanol/dry ether and counted to determine the presence of radioactivity. m.p. 169-171^{o}C. Nuclear magnetic resonance analysis was performed on this sample in D_2O.

D) 2-Anilinopropionic Acid:

2-Anilinopropionic acid (503.0 mg.; 0.003045 moles) was added to 20.00 ml. of twenty-four hour urine (2.6368×10^6 dpm.) obtained from rats injected with N-isopropylaniline-^{14}C hydrochloride. Concentrated hydrochloric acid (6 ml.) was added and the urine hydrolyzed for one hour (Presto pressure cooker at 15 p.s.i.). The urine was then adjusted to pH 3.0 with sodium hydroxide solution (20%) and continuously ether extracted for eight hours. After evaporating the ether under nitrogen, the residue was dried under vacuum over sulphuric acid. Ethereal diazomethane (prepared from diazald according to the method by Aldrich Chemicals) was added to the dried residue. The ethereal solution was boiled to destroy excess diazomethane and reduced to a minimum volume on a Rinco Rotary Evaporator. The resulting liquid was spotted on thin layer chromatographic plates (1.00 mm.) of silica gel GF-254 via an Agla micrometer syringe, and allowed to develop in a solvent system of 5% petroleum ether (30-60)/95% benzene. The plates were air dried and the band corresponding to 2-anilinopropionic acid methyl ester (Rf = 0.35) was scraped from the plates. The resulting silica gel scrapings were placed in a column and eluted with dry ether. Dry hydrogen chloride gas, passed through the ethereal eluent resulted in the crude product. The crude product was recrystallized once from super dry ethanol/dry ether and counted to determine the presence of radioactivity. m.p. 169-171^{o}C. Nuclear magnetic resonance analysis was performed on this sample in D_2O.

E) o-Aminophenol:

o-Aminophenol (255.8 mg.; 0.002345 moles) was added to 20.00 ml. of twenty-four hour urine (2.6494×10^6 dpm.) obtained from rats injected with N-isopropylaniline-^{14}C hydrochloride. Concentrated hydrochloric acid (6 ml.) was added and the urine hydrolyzed for one hour (Presto pressure cooker at 15 p.s.i.). The urine was then adjusted to pH 7.0 with sodium hydroxide solution (20%) and continuously ether extracted for three hours. After removing the nitrogen on a Rinco Rotary Evaporator, 20 ml. of sodium hydroxide solution (8%) were added to the residue along with benzoyl chloride (5 ml.; 0.03 moles). The Schotten-Baumann reaction was carried out according to the procedure of Vogel (1966c). The crude residue was recrystallized from ethanol, chloroform, and benzene and counted to determine the presence of radioactivity. m.p. 182-183^{o}C.

V. CHROMATOGRAPHY:

A) Paper:

Paper chromatography was employed to determine the properties of the heretofore unidentified polar metabolite(s) of N-isopropylaniline.

Twenty-four hour urine (20.00 ml.) obtained from a rat injected with N-isopropylaniline-^{14}C hydrochloride was hydrolyzed one hour (Presto pressure cooker at 15 p.s.i.) after the addition of concentrated hydrochloric acid (6 ml.). The urine was then neutralized to pH 7.0 with sodium hydroxide solution (20%) and

continuously ether extracted for four hours. The aqueous solution remaining after ether extraction was made up to 100.0 ml. in a volumetric flask and the radioactive content determined by liquid scintillation counting. A portion of this aqueous solution (20.00 ml.) was frozen and then freeze dried. The freeze dried residue was extracted with ethanol and the volume reduced by means of a Rinco Rotary Evaporator and made up to 10.0 ml. in a volumetric flask with alcohol. Portions of this alcoholic solution (0.5 ml. equivalent to approximately 3000 dpm.) were spotted on Whatman No. 3 chromatographic paper (1.75 inch wide strips) utilizing an Agla micrometer syringe. The paper strips were then developed in two solvent systems- butanol/ammonia (7:3) and butanol/glacial acetic acid/water (8:2:2). The paper strips, after air drying, were then scanned for radioactivity.

N-phenylglycine and 2-anilinopropionic acid were also spotted on Whatman No. 3 chromatographic paper and developed in the solvent systems-butanol/ammonia (7:3) and butanol/glacial acetic acid/water (8:2:2). The detecting reagent used was freshly prepared diazotized sulphanilic acid obtained by mixing equal volumes of sulphanilic acid (1%w/v in N/1 hydrochloric acid) and aqueous sodium nitrite solution (5%w/v) followed by neutralization with an equal volume of sodium carbonate solution (10%w/v).

B) Thin Layer Chromatography:

Thin layer chromatographic plates (1.00 mm.) afforded an excellent preparative method for the separation of

the desired product from extraneous material. Thin layer plates (0.25 mm.) were used to determine the extent of reaction in the synthesis of N-isobutylaniline-^{14}C and N-isopropylaniline-^{14}C.

Thin layer plates were prepared using fluorescent silica gel (silica gel GF-254 acc. to Stahl-Merck) on a quickfit apparatus, their thickness governed and kept constant by a fixed aperture spreader. Material spotted on these plates was detected by its blockage of fluorescence as seen when the plates were subjected to short-wave ultraviolet light.

VI. DETECTION OF RADIOACTIVITY:

A) Liquid Scintillation Counting:

1) Scintillation fluid-

The scintillation fluid employed in liquid scintillation counting was comprised of the formula:

a)	POP(2,5-diphenyloxazole)	2.0 g.
b)	Dimethyl POPOP[1,4-bis-2(4-methyl-5-phenyloxazolyl)-benzene]	0.5 g.
	or	
	bis MSB[bis-(o-methylstyryl)-benzene]	0.04 g.
c)	Isopropanol (Baker Reagent)	400.0 ml.
d)	Toluene (Fisher Reagent) q.s.	1000.0 ml.

2) Internal standard-

In order to convert counts per minute (cpm.) to disintegrations per minute (dpm.) in liquid

scintillation counting, 0.1 ml. of benzoic acid-^{14}C* (approximately 3000 dpm./0.1 ml. accurately known) in toluene was added.

3) Measurement of radioactive samples-

All liquid samples to be tested for radioactivity were delivered directly into glass counting vials with an Agla micrometer syringe. All solid samples were weighed on a Cahn Gram Electrobalance and transferred directly into glass counting vials.

4) Method-

Liquid samples up to a maximum volume of 0.40 ml. were delivered into glass counting vials followed by the addition of 10.0 ml. of scintillation fluid. If the scintillation fluid was not completely miscible with the liquid samples, thixotropic gel (250 mg./vial) was added. The resulting solution or suspension was counted in a Nuclear Chicago Unilux Liquid Scintillation Counter.

To solid samples (approximately 1 mg. accurately weighed), after weighing and transferring to glass counting vials, were added Methanol (2.0 ml.) and scintillation fluid (10.0 ml.). These samples were counted as above.

All counts were converted to dpm. by the addition of internal standard.

5) Minimum radioactivity-

In this project, the minimum valid

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* ±5% accuracy (New England Nuclear Corp., Boston, Massachusetts).

radioactivity per sample in the glass counting vial was taken to be a count rate of double the background count. Since the background count averaged 35 cpm., and the efficiency of the instrument was about 70%, this would mean that the minimum valid radioactivity required would be 50 dpm. per sample in the glass counting vial. In reverse isotope dilution studies in which 1 mg. samples of the isolated derivative were counted, the minimum acceptable radioactivity was thus taken to be 50 dpm./mg. (See table V.).

B) 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 chromatograms were scanned for activity on a Nuclear Chicago Actigraph III.

The radioactivity thus detected was qualitative, and quantitative only insofar as a measure of comparison of radioactivity on the same chromatogram.

RESULTS

I. PREPARATION OF COMPOUNDS:

TABLE I: Recrystallization data on synthesized radioactive compounds:

Compound	Recrystallization Solvent	Recrystallization No.	Dpm./mg. ($x10^5$)
N-isobutylaniline ^{14}C hydrochloride	Super dry ethanol/ dry ether	6	3.79770
	"	7	3.81190
		Average	3.80480
N-isopropylaniline ^{14}C hydrochloride	Super dry ethanol/ dry ether	6	5.21073
	"	7	5.18170
	"	8	5.19517
		Average	5.19586

These results indicate that the compounds are at constant specific activity.

II. ANIMAL EXPERIMENTS:

TABLE II. N-isobutylaniline ^{14}C hydrochloride excretion data after i.p. injection in the rat:

Rat	Weight(g.)	Dose as administered dpm. x 10^6	Radioactivity excreted as % of administered dose			
			24 hr.	48 hr.	72 hr.	Total
A	345	2.47	87.7	8.8	1.7	98.2
B	325	2.47	69.5	10.5	5.6	85.6
C	320	2.47	88.3	2.5	1.3	92.1
D	360	2.42	93.2	5.8	0.9	99.9
E	310	2.42	89.9	6.7	4.9	101.5
F	335	2.42	77.1	17.0	2.4	96.5
G	380	2.51	79.0	3.6	1.5	84.1
H	390	2.51	87.8	2.2	2.1	92.1
I	380	2.51	86.4	3.6	2.1	92.1
J	325	2.51	80.7	3.2	1.6	85.5
Average excretion			84.0	6.4	2.4	92.8

From the data obtained, N-isobutylaniline is rapidly excreted in the urine after an intraperitoneal injection in the rat.

III. DISTRIBUTION STUDY:

TABLE III. Distribution of radioactivity after solvent extractions following varying conditions of hydrolysis and pH of extraction:

Sample (5.00 ml.)	Hydrolyzing Agent	Hydrolyzing Time (Hr.)	pH of Extraction	% Radioactivity extracted		
				Ether	Butanol	Total
A	Unhydrolyzed	--	Unadjusted	3.3	27.5	30.8
B	1 ml. c. HCl	1.5*	4.0	16.9	41.6	58.5
C	1 ml. c. HCl	1.5*	7.0	37.7	35.2	72.9
D	1 ml. c. HCl	1.5*	9.2	20.0	23.8	43.8
E	5 ml. c. HCl	6.0**	7.0	34.1	38.2	72.3
F	8 ml. 20% NaOH	1.0*	7.0	1.3	33.4	34.7

Note: General procedure outlined on Page 27

* Presto pressure cooker at 15 p.s.i.

** Refluxing

The data obtained indicates the majority of metabolites are extractable at pH 7 after hydrolysis. Also, the metabolites must have acidic and basic sites which result in decreased extractability at pH values other than pH 7.

IV. REVERSE ISOTOPE DILUTION STUDIES:

The reverse isotope dilution data is summarized in Table V. The theoretical quantity (Theor. qt.) referred to in the table represents the amount of isolated derivative which is formed theoretically in the conversion of the compound added to the urine, to its characterized derivative.

A) 4-Hydroxy-N-Isobutylaniline:

TABLE IV: Quantitative estimation of 4-hydroxy-N-Isobutylaniline by reverse isotope dilution:

Experiment	Final Specific Activity*(dpm./mg.)	% Radioactivity Excreted in 24 hr.	Metabolite**	
			% of 24 hr. Urine	% of Administered Dose
A	636	77.1	40.2	31.0
B	631	88.3	34.6	30.5
C	349	89.9	37.5	33.7
		Average	37.4	31.7

* 4-hydroxy-N-isobutylaniline-N,O-ditosylate

** 4-hydroxy-N-isobutylaniline

4-Hyroxy-N-isobutylaniline was determined by characterizing as the N,O-ditosylate. From table IV. it can be seen that this metabolite was excreted as 37.4% of the twenty-four hour urinary excretion products and 31.7% of the administered dose in twenty-four

TABLE V: REVERSE ISOTOPE DILUTION DATA

Metabolite	Material Added to Urine			Isolated Derivative			Dpm. in Urine Sample x 10^6	Minimum % Detectable*
	Compound	Mol. Wt.	Qt.(mg.)	Compound	Mol. Wt.	Theor.Qt.(mg.)		
			407.5	4-H-NIBA-		956.8	1.7468	2.7
4-H-NIBA	4-H-NIBAHCl	201.7	401.4	N,0-	473.6	942.5	1.4928	3.2
			398.1	DITOS		934.8	0.8699	5.4
			413.5			1141.8	0.7925	7.2
			400.7	PAP-		1106.4	0.8810	6.3
PAP	N-APAP	151.2	407.1	N,0-	417.5	1124.1	0.8668	6.5
			407.0	DITOS		1123.8	0.6479	8.7
	PAPHCl	145.6	392.0			1124.0	0.6479	8.7
N-PhG	N-PhG	151.2	502.3	N-PhGMe HCl	201.7	670.1	2.6368	1.3
2-APA	2-APA	165.2	503.0	2-APAMe HCl	215.7	656.8	2.6368	1.2
OAP	OAP	109.1	255.8	OAP-N,0-DIB	317.4	744.2	2.6494	1.4

* 50 dpm./mg. minimum as described in the section "Detection of Radioactivity".

4-H-NIBA = 4-hydroxy-N-isobutylaniline
PAP = p-aminophenol

N-PhG = N-phenylglycine
2-APA = 2-anilinopropionic acid

N-APAP = N-acetyl-p-aminophenol

4-H-NIBAHCl = 4-hydroxy-N-isobutylaniline hydrochloride
OAP = o-aminophenol

PAPHCl = p-aminophenol
4-H-NIBA-N,0-DITOS = 4-hydroxy-N-isobutyl-aniline-N,0-ditosylate
PAP-N,0-DITOS = p-aminophenol-N,0-ditosylate
N-PhGMe HCl = N-phenylglycine methyl ester hydrochloride
2-APAMe HCl = 2-anilinopropionic acid methyl ester hydrochloride

OAP-N,0-DIB = o-aminophenol-N,0-dibenzoate

hours, after injection of N-isobutylaniline-^{14}C hydrochloride (15 mg. free base/Kg.).

B) p-Aminophenol:

TABLE VI: Quantitative estimation of p-aminophenol by reverse isotope dilution:

Experiment	Final Specific Activity*(dpm./mg.)	% Radioactivity Excreted in 24 hr.	Metabolite**	
			% of 24 hr. Urine	% of Administered Dose
A	423	79.0	61.0	48.2
B	429	87.8	53.9	47.3
C	359	86.4	46.6	40.3
D***	340	80.7	59.0	47.6
E***	334	80.7	58.0	46.8
		Average	55.7	46.0

* p-aminophenol-N,O-ditosylate

** p-aminophenol

*** same twenty-four hour urine sample using N-acetyl-p-aminophenol in experiment E and p-aminophenol hydrochloride in experiment D.

p-Aminophenol was determined to be present as a metabolite by characterizing it as the N,O-ditosylate. From table VI. it can be seen that p-aminophenol was excreted as 55.7% of the twenty-four

hour urinary excretion products and 46.0% of the administered dose, after the injection of N-isobutylaniline-^{14}C hydrochloride (15 mg. free base/Kg.).

C) N-Phenylglycine:

The quantity of materials used in the determination of this metabolite by reverse isotope dilution permitted the detection of 1.3% of the metabolite (Table V.). However, after counting the recrystallized isolated derivative, radioactivity present was much below the minimum acceptable value of 50 dpm./mg. In fact, radioactivity above background was not detectable indicating that this metabolite was either absent or present to the extent of less than 1.3%. The NMR analysis of this isolated compound (Figure I.) was identical to that of synthesized N-phenylglycine methyl ester hydrochloride (Figure II.).

D) 2-Anilinopropionic Acid:

The quantity of materials used in the determination of this metabolite by reverse isotope dilution permitted the detection of 1.2% of the metabolite (Table V.). However, after counting the recrystallized isolated derivative, radioactivity present was much below the minimum acceptable value of 50 dpm./mg. In fact, radioactivity above background was not detectable indicating that this metabolite was either absent or present to the extent of much less than 1.2%. The NMR analysis of this isolated compound (Figure III.) was identical to that of synthesized 2-anilinopropionic acid methyl ester hydrochloride (Figure IV.). Although the m.p. of

the isolated compound was identical to that of N-phenylglycine methyl ester hydrochloride (m.p. 169-171°C), the NMR analysis (Figures I. to IV.) confirmed the identity of both these compounds.

E) o-Aminophenol:

The quantity of materials used in the determination of this metabolite by reverse isotope dilution permitted the detection of 1.4% of the metabolite (Table V.). However, after counting the recrystallized isolated derivative, radioactivity was below the minimum acceptable value of 50 dpm./mg. This indicated that o-aminophenol was possibly there as a metabolite but to the extent of less than 1.4%.

V. CHROMATOGRAPHY:

Paper chromatography provided a general property of the heretofore unidentified polar metabolite(s) of N-isopropylaniline. The polar metabolites were found to be acidic according to the Rf values in the solvent systems used. Scanning of the developed paper chromatograms (Figures V. and VI.) suggested the presence of more than one polar metabolite and permitted the identification of at least two major spots. The presence of various minor metabolites or decomposition products was also indicated by the scans.

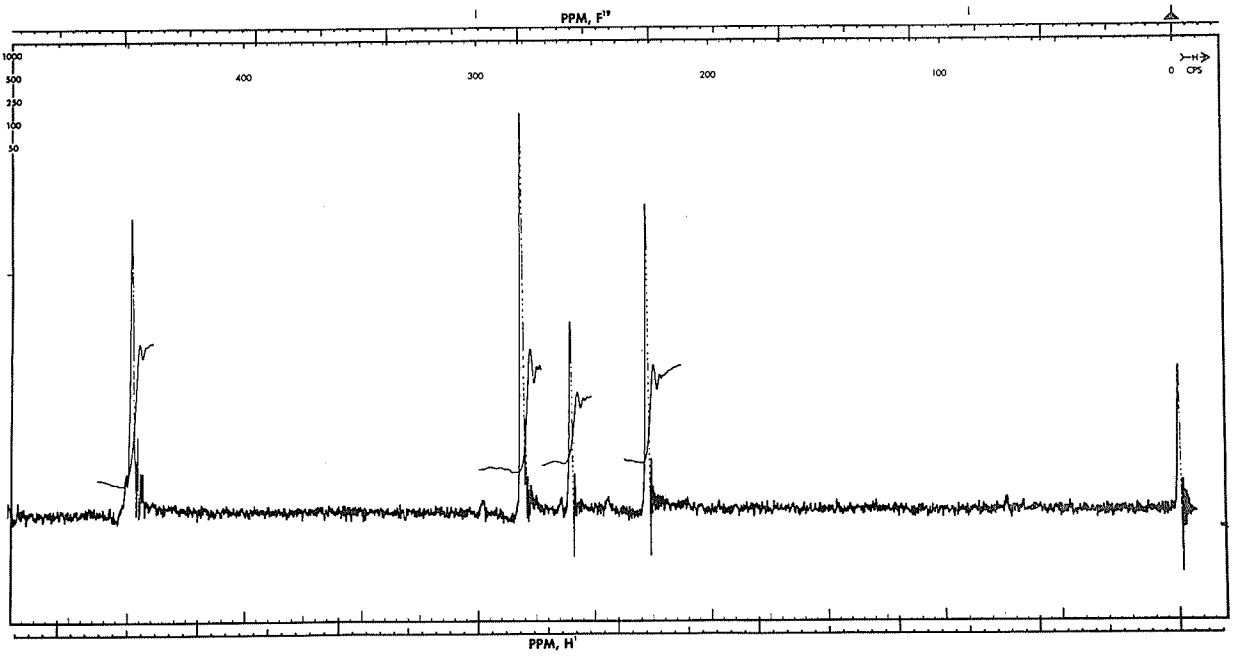


Figure I: NMR analysis of isolated N-phenylglycine methyl ester hydrochloride.

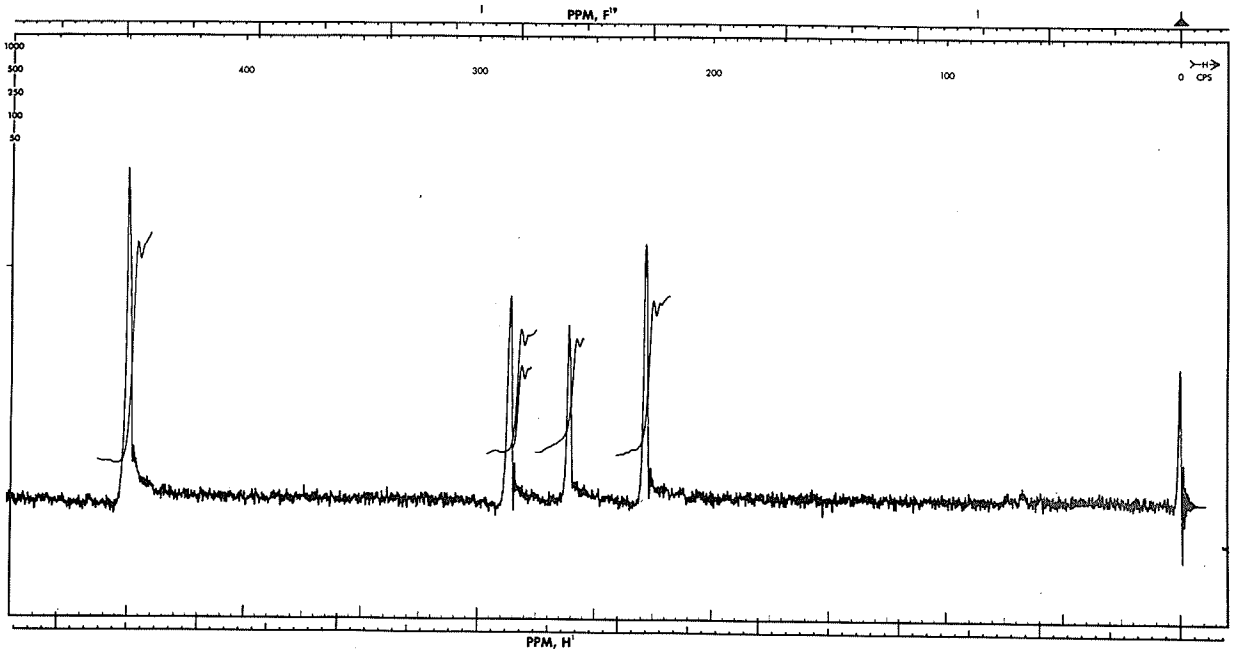


Figure II: NMR analysis of synthesized N-phenylglycine methyl ester hydrochloride.

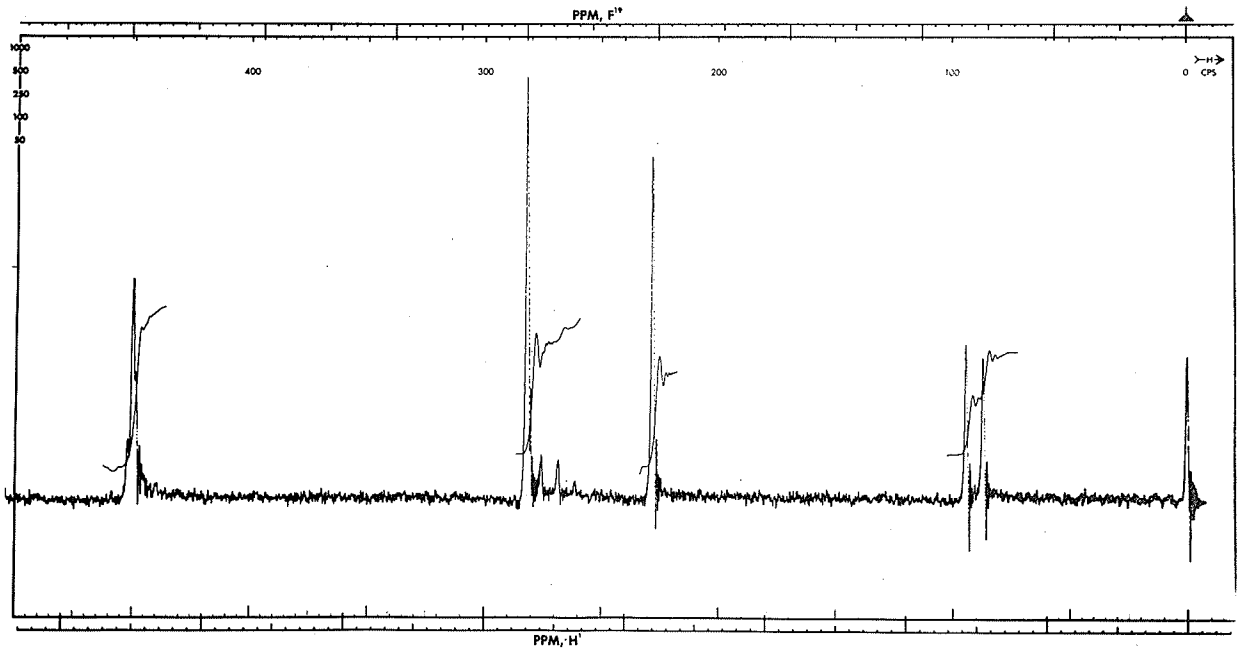


Figure III: NMR analysis of isolated 2-anilinopropionic acid methyl ester hydrochloride.

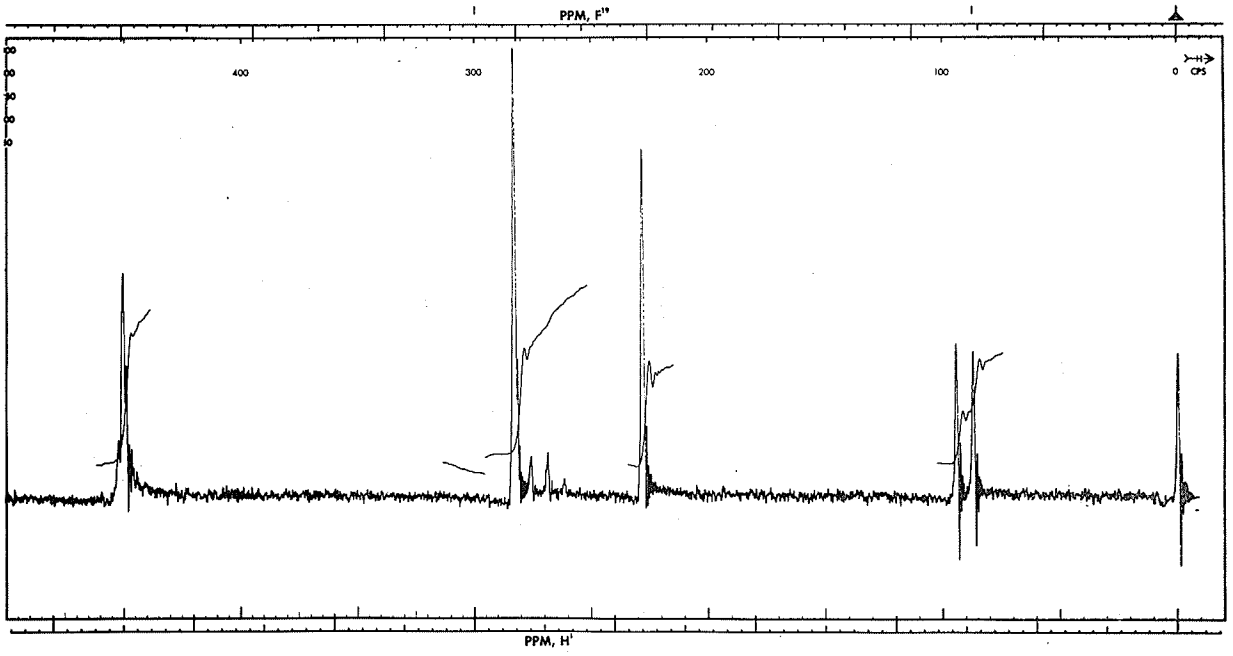


Figure IV: NMR analysis of synthesized 2-anilinopropionic acid methyl ester hydrochloride.

TABLE VII: Major polar metabolites as seen in Figures V. and VI. of N-isopropylaniline ^{14}C:

Solvent System	Spot	Rf
A	1	0.76
A	2	0.67
B	1	0.00*
B	2	0.11

A = Butanol/glacial acetic acid/water (8:2:2)

B = Butanol/ammonia (7:3)

* at origin

2-Anilinopropionic acid and N-phenylglycine were also observed by means of paper chromatography as detected with freshly prepared diazotized sulphanilic acid.

TABLE VIII: Paper chromatography of N-phenylglycine and 2-anilinopropionic acid:

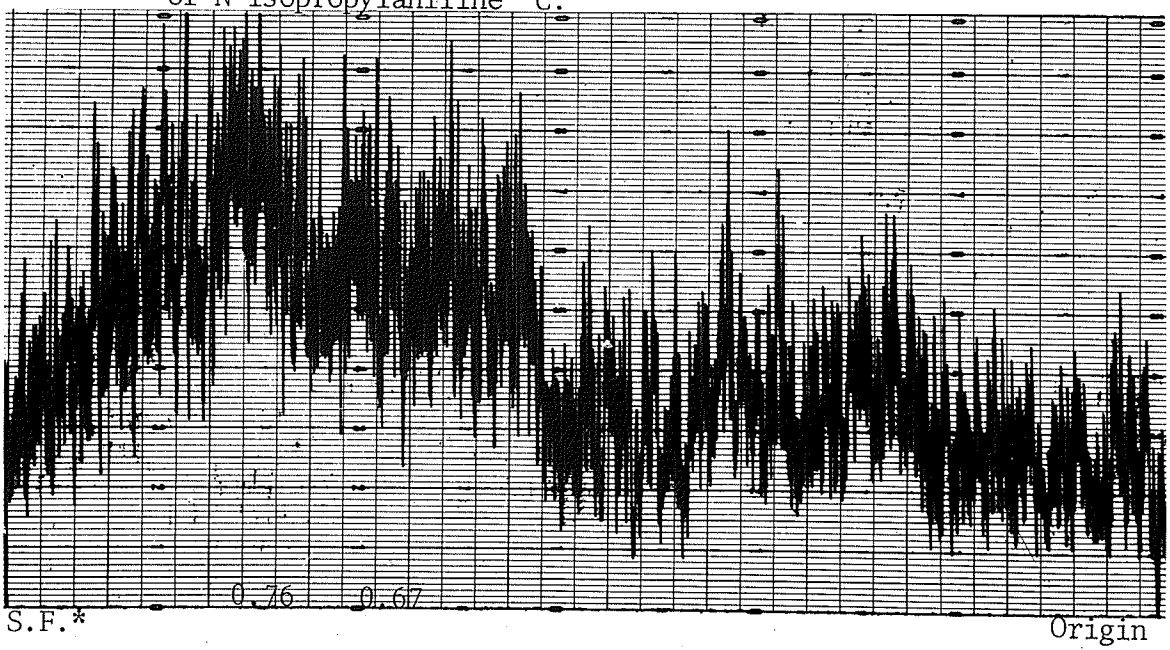
Compound	Solvent System	Rf
N-phenylglycine	A	0.85
2-anilinopropionic acid	A	0.85
N-phenylglycine	B	0.13
2-anilinopropionic acid	B	0.13

A = Butanol/glacial acetic acid/water (8:2:2)

B = Butanol/ammonia (7:3)

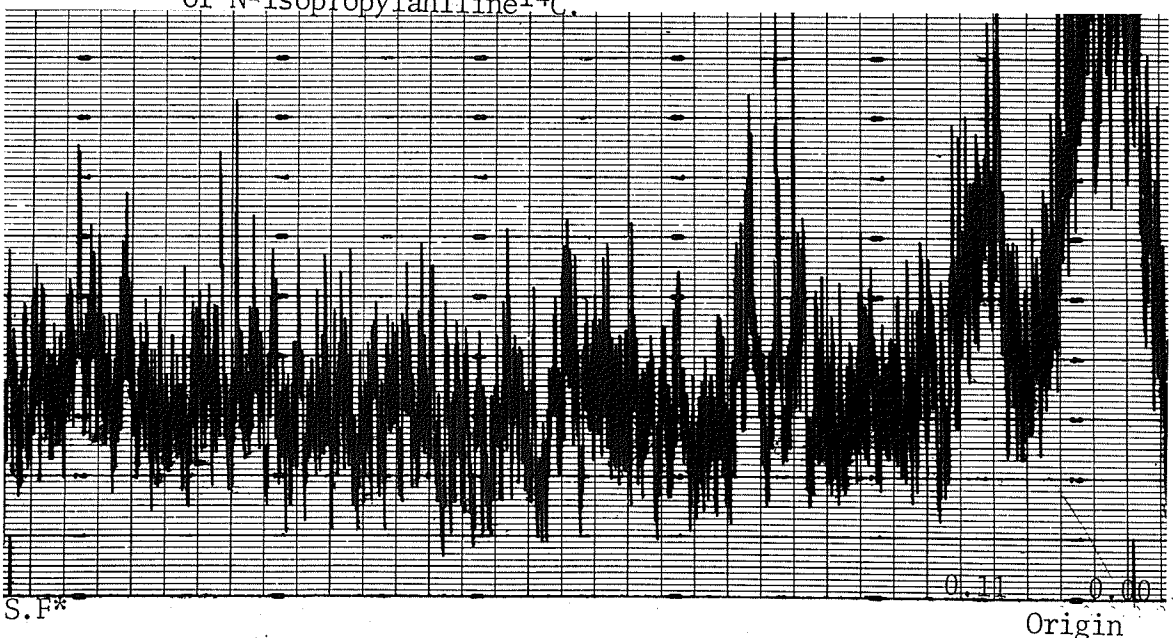
The fact that the Rf values of the two compounds in table VIII. were similar to the Rf values of the major polar metabolites (table VII.), prompted the investigation of these two compounds by reverse isotope dilution (see Page 44).

Figure V: Scanning of paper chromatogram spotted with polar metabolites of N-isopropylaniline ^{14}C.



a) about 3000 dpm. (about 0.1% of 24 hr. excreted radioactivity) spotted.
b) solvent system: butanol/glacial acetic acid/water (8:2:2)[Solvent A Page 48].

Figure VI: Scanning of paper chromatogram spotted with polar metabolites of N-isopropylaniline ^{14}C.



a) about 3000 dpm. (about 0.1% of 24 hr. excreted radioactivity) spotted.
b) solvent system: butanol/ammonia (7:3) [Solvent B Page 48].

* S.F. = solvent front.

DISCUSSION

Although the melting point of N-isobutylaniline-^{14}C hydrochloride was higher (202-203^{o}C) than the literature source (192^{o}C - Beilstein, 1950), its identity was verified by the determination of logϵ (245). Logϵ (245) was found to be 4.025 and reported to be 4.02 (Rumpf, P. and Girault, G., 1954). In addition, C, H analysis of non-radioactive N-isobutylaniline hydrochloride synthesized in identical fashion, also varified its identity. Although the melting points, methods of synthesis, and reverse isotope dilution experiments of N-phenylglycine methyl ester hydrochloride and 2-anilinopropionic acid methyl ester hydrochloride were identical, the Nuclear Magnetic Resonance studies confirmed their differences and thus their identities (Figures I. to IV.).

From the excretion data, table II., it can be seen that N-isobutylaniline is excreted rapidly in the urine after intraperitoneal injection of the hydrochloride salt in the rat. However, certain excretatory products are detectable even after seventy-two hours. The fact that very little activity (3.3%) can be extracted with ether from the unhydrolyzed urine (table III.) indicates that practically all of the injected N-isobutylaniline is transformed metabolically to water soluble metabolites, for the free base is very soluble in ether. Due to the limited total extractability of metabolites (72.9%) after hydrolysis (1.5 hours) with concentrated hydrochloric acid (1 ml.), it was thought that possibly certain

conjugated metabolites existed in the urine which had not been hydrolyzed. Therefore, more severe conditions of hydrolysis (6.0 hours) with concentrated hydrochloric acid (5 ml.) were employed. Virtually no change in total extractable metabolites (72.3%) indicated that the former hydrolysis conditions were sufficiently rigorous to hydrolyze conjugated metabolites of N-isobutylaniline. This is supported by the work of Bray et al.(1952) who reported complete hydrolysis of conjugated metabolites of similar compounds with an analogous procedure. The limited total extractability of metabolites (72-73%) may have been due to the presence of p-aminophenol (55.7%) which has a low solubility in organic solvents.

The metabolic transformation reactions, p-hydroxylation and N-dealkylation, which N-isobutylaniline undergoes in the rat are consistent with the findings of other N-alkylaromatic amines. Both p-hydroxylation and N-dealkylation have been observed in the rat with N-sec-butylaniline (Alexander et al., 1968) and N-isopropylaniline (Alexander and Sitar, 1969).

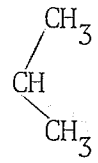
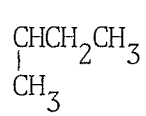
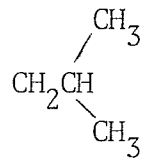
TABLE IX: Comparison of the metabolism of N-alkylanilines:

Compound	Metabolite as % of activity in 24 hr. urine			
	p-hydroxy parent cpd.	p-aminophenol	Total	Unknown metabolites
N-sec.butylaniline	10	13	23	74*
N-isopropylaniline	41.6	5.4	46.0	54.0
N-isobutylaniline	37.4	55.7	93.1	6.9

* The finding of 3-phenylaminobutyric acid (XXVI.) as 3% leaves 74% unaccounted for.

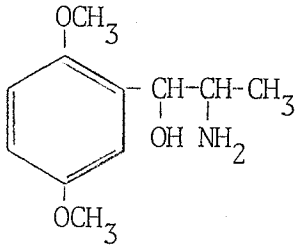
The results as compiled in table IX. are interesting from various points of view. A consistency can be observed relating to structure-activity relationship in the N-dealkylation reaction (table IX and table X).

TABLE X: Comparison of N-alkyl side chain of the N-alkylanilines listed in table IX:

Compound	N-alkyl group
N-isopropylaniline	
N-sec.butylaniline	
N-isobutylaniline	

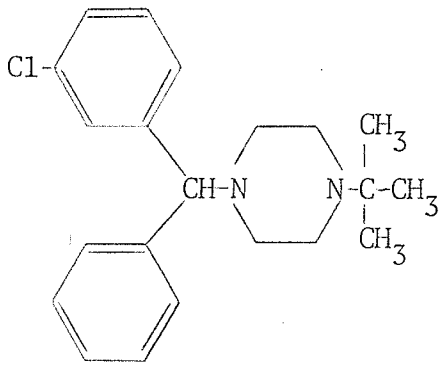
Substitution on the α-carbon of the N-alkyl group causes a marked reduction in dealkylation. When the substitution is removed to the β-carbon, dealkylation appears not to be hindered. Thus the N-dealkylation reaction is affected by steric hindrance at the α-carbon, while β-carbon substitution does not impede the enzymatic cleavage of the N-alkyl group. Substitution on the α-carbon also causes the excretion of a greater quantity of p-hydroxy parent compound. This can be seen when comparing N-isopropyl- and N-isobutylaniline and represents another possible consistency

in the metabolism of N-alkylanilines. The results for N-sec.butyl-aniline, which indicate the presence of only 10% of p-hydroxy parent compound, appear questionable and it is felt that the metabolism of this compound should be reinvestigated for the quantitative presence of metabolites. Investigations with N-tert.butylaniline might further clarify the consistencies observed with regards to N-dealkylation and formation of p-hydroxy parent compound when the α-carbon is substituted. Various investigators have examined the N-dealkylation of N-tert.butyl amines and various reports have been presented. Burns and Salvader (1967) found the N-tert.butyl analog of methoxamine (XX.) not to be dealkylated.



XX.

This finding was compatible with the mechanism of oxidative attack on the α-carbon referred to earlier (McMahon and Sullivan, 1964). However, dealkylation of an N-tert.butyl group has been observed (Kuntzman et al., 1967) with the N-tert.butyl analog (XXI.) of Chlorcyclizine.

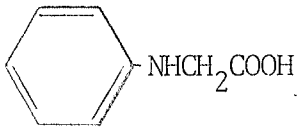


XXI.

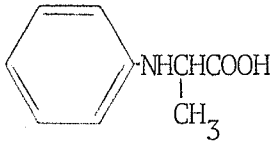
Although the results of this project shed no light on the N-dealkylation mechanism, an opinion has been reached regarding dealkylation mechanism. It is felt that dealkylation need not be explained by one or the other of the two mechanisms presented earlier. It has been shown that an enzyme or enzyme system may be induced to increase or decrease the dealkylation of a compound (McMahon, 1964; Bresnick and Stevenson, 1968). Possibly a single enzyme system exists in the appropriate cellular fraction which is capable of undergoing simple conformational changes in order to dealkylate a compound. These changes could be induced by the compound's structure. Depending on the "enzyme structure", a compound could undergo oxidative attack on the nitrogen or oxidative attack on the α-carbon. As a result, steric hindrance would not determine the mechanism but only affect the ease of a particular dealkylation process ascribed to that type of compound. A similar hypothesis has been mentioned recently in a report on cytochrome P-450 (George

and Tephly, 1968). They observed the participation of cytochrome P-450 in both the N- and O-dealkylation of morphine analogues. However varying conditions did not produce the same effects in the N- and O-dealkylation reaction. Among possibilities suggested to explain this phenomenon was one in which there were several forms of cytochrome P-450, each with different binding properties but with qualitatively similar catalytic properties.

Experiments in this project failed to detect N-phenylglycine (XXII.) and 2-anilinopropionic acid (XXIII.)

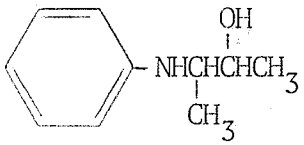


XXII.

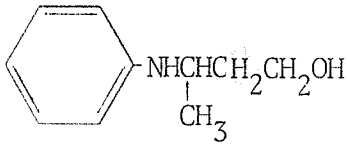


XXIII.

in the metabolism of N-isopropylaniline. Alexander et al.(1968) were unable to observe the following metabolites in the metabolism of N-sec.butylaniline: 2-anilinopropionic acid (XXIII.), 3-phenylamino-2-butanol (XXIV.) and 3-phenylamino-1-butanol (XXV.).

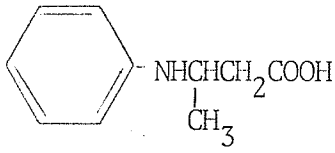


XXIV.



XXV.

However, they were able to detect 3-phenylaminobutyric acid (XXVI.) to the extent of 3% of the twenty-four hour metabolites.



XXVI.

It was this last finding along with chromatographic results (tables VII. and VIII.) which prompted the investigations with N-phenylglycine (XXII.) and 2-anilinopropionic acid (XXIII.) as the possible unknown polar metabolites of N-isopropylaniline (table IX.).

The possibility that one of the unknown polar metabolites could be o-aminophenol was also considered. It was felt though that this compound would not be present in the alcoholic solution if the procedure under the experimental section of paper chromatography were followed. Due to its greater ether solubility than p-aminophenol, it would be removed during the procedure if it were present and not constitute a part of the unknown polar metabolites. This early opinion was verified in the reverse isotope dilution study which eliminated o-aminophenol as a detectable metabolite according to the procedures employed.

Thus the majority of metabolites (93.1%) of N-isobutylaniline in twenty-four hour urine have been accounted for. The problems of unknown polar metabolites, which represent the majority of metabolites for N-isopropylaniline and N-sec.butylaniline, and

possible minor metabolites of N-isobutylaniline, still remains. However, certain possibilities as to their identity exist. According to the results in table IX., another reaction other than ring hydroxylation and dealkylation is favoured in the metabolism of N-sec.butylaniline, and is insignificant or absent in the case of N-isobutylaniline. This reaction must also be present in the metabolism of N-isopropylaniline. The only difference between N-sec.butyl-, N-isopropyl- and N-isobutylaniline is that the first two compounds have substitution on the α-carbon. Possibly N-alkylanilines with substitution on the α-carbon undergo oxidative attack on the α-carbon resulting in a stable alcohol with very little of it subsequently undergoing dealkylation. This alcohol could be conjugated and eliminated. Although α-carbon oxidation has been observed with isopropylbenzene (XIV.), it is not likely with N-alkylanilines, for the α-carbon hydroxy compounds are reputed to be unstable and do not form derivatives of alcohols or amines (Fernandez and Butler, 1963). Another possibility exists which appears more feasible. From table IX. it can be seen that all metabolites thus far identified for N-alkylanilines with the exception of 3-phenylaminobutyric acid (XXVI.) have been p-hydroxylated. The unknown polar metabolites might be p-hydroxylated analogues of the alcohols and acids resulting from side chain oxidation at a position other than the α-carbon. These compounds have thus far not been investigated as the potential polar metabolites. Nevertheless it is felt that more preliminary investigations on the

properties of these unknown polar metabolites should be carried out. From the results in this project it is known that they are acidic in nature. A procedure as outlined in the experimental section of paper chromatography would give an alcoholic solution of minimum volume for these metabolites. This solution, although very crude, could be separated on an ion-exchange column thus purifying the metabolites.

CONCLUSION

The metabolism of N-isobutylaniline-^{14}C hydrochloride has been studied in the rat. The metabolic pathways of p-hydroxylation and N-dealkylation form virtually all of the twenty-four hour metabolites (93.1%) resulting in 4-hydroxy-N-isobutylaniline (37.4%) and p-aminophenol (55.7%). N-phenylglycine, 2-anilinopropionic acid and o-aminophenol have been eliminated as the unknown polar metabolites of N-isopropylaniline, but it is known that the unknown metabolites are acidic. Three new compounds were synthesized and varified: 4-hydroxy-N-isobutylaniline hydrochloride, 4-hydroxy-N-isobutylaniline-N,O-ditosylate and N-phenylglycine methyl ester hydrochloride.

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