THE UNIVERSITY OF MANITOBA

THE METABOLISM OF N-CYCLOPENTYLANILINE-^{14}C HYDROCHLORIDE IN THE RAT

by

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ABSTRACT

The rate of urinary and fecal excretion of radioactive metabolites after the intraperitoneal injection of N-cyclopentylaniline-^{14}C hydrochloride was determined. Excretion of radioactive metabolites in the urine reached a maximum of 76% after twenty-four hours, and in the feces, 14% after forty-eight hours. A total of 77% of the administered radioactivity was accounted for in the urine, and 14% in the feces. Hydrolyses and reverse isotope dilution studies showed that p-aminophenol-^{14}C and p-hydroxy-N-cyclopentylaniline-^{14}C were excreted as conjugated metabolites, indicating that aromatic hydroxylation and dealkylation of the N-alkylaniline had occurred. The primary metabolite, p-aminophenol-^{14}C, representing 31.6% of the administered radioactivity, appeared to be excreted in the urine partially as the sulphate conjugate, but no conjugation of either of the identified metabolites with glucuronic acid could be detected. The conjugate(s) of p-hydroxy-N-cyclopentylaniline-^{14}C (8.8% of the administered dose) were not determined. No unchanged compound could be detected.

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ABBREVIATIONS USED

ADP	Adenosine Diphosphate
ATP	Adenosine Triphosphate
FAD	Flavine Adenine Dinucleotide
NADH2	Reduced Nicotinamide Adenine Dinucleotide
NADPH2	Reduced Nicotinamide Adenine Dinucleotide Phosphate
NCPA	N-Cyclopentylaniline
NCPA•HCl	N-Cyclopentylaniline Hydrochloride
NCPA•tos	N-Cyclopentylaniline Tosylate
PAP	p-Aminophenol
PAP•HCl	p-Aminophenol Hydrochloride
PAP-N,O-ditos	p-Aminophenol N,O-Ditosylate
P-OH NCPA	p-Hydroxy-N-Cyclopentylaniline
P-OH NCPA•HCl	p-Hydroxy-N-Cyclopentylaniline Hydrochloride
p-OH NCPA-N,O-ditos	p-Hydroxy-N-Cyclopentylaniline N,O-Ditosylate

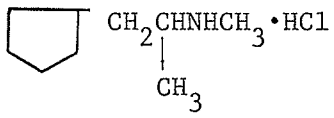
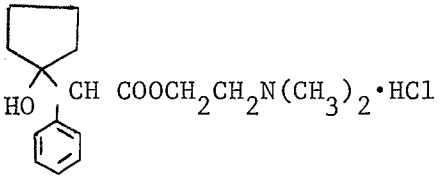
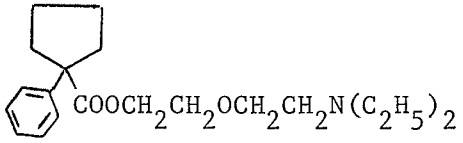
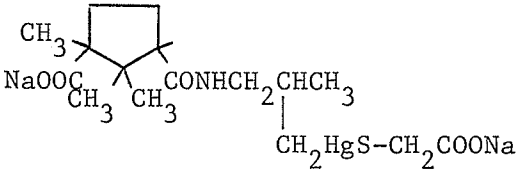
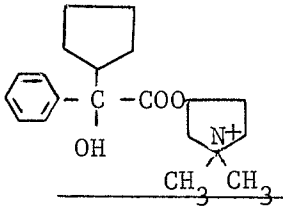
INTRODUCTION

The metabolism of N-alkyl aromatic amines has been under investigation in these laboratories for several years (1 - 8). Analyses of the results obtained from studies of straight-chain and branched-chain alkyl substituted anilines indicate an emerging structure-activity relationship with respect to the metabolism of these compounds. It appears that the extent of metabolic transformation of alkyl substituted anilines by N-dealkylation is dependent upon the chain length and degree of branching of the alkyl substituent. In order to expand this area of investigation, studies on the metabolism of an N-alkyl substituted aniline with a cyclic substituent, N-cyclopentylaniline, were undertaken. In particular, the effect of this cyclic substituent upon the metabolism of substituted anilines was compared with the previously studied straight-chain or branched-chain substituents with an identical number of carbon atoms (8).

In addition, it was hoped that these studies would add to the limited knowledge concerning the metabolism of the cyclopentyl ring, by determining whether the ring remained intact or underwent scission to produce an aliphatic N-alkyl side-chain during the metabolism of N-cyclopentylaniline.

The cyclopentyl ring forms a part of numerous drug molecules (Table I). The lack of information regarding the metabolic fate of the cyclopentyl moiety makes it desirable to learn more about the fate of this ring *in vivo*.

TABLE I: SOME CYCLOPENTYL RING-CONTAINING DRUGS

STRUCTURE	COMMON NAME	TRADE NAME	USE
	cyclopentamine hydrochloride	Clopane^{R} (Lilly)	nasal vaso-constrictor
	cyclopentolate hydrochloride	Cyclogyl^{R} (Schieffelin)	cycloplegic-mydriatic
	carbetapentane	Toclase^{R} (Pfizer)	antitussive
	mercaptomerin sodium	Thiomerin Sodium^{R} (Wyeth)	diuretic
	glycopyrrolate	Robinul^{R} (Robins)	anticholinergic

The Metabolism of Foreign Compounds

In the animal body, foreign molecules undergo a variety of metabolic transformations. These can be broadly classified into oxidations, reductions, hydrolyses and syntheses or conjugations (9). Drug metabolism *in vivo* usually consists of two phases (10). In Phase I, there occur all those reactions which may be classified as oxidations, reductions or hydrolyses. The products of Phase I may then undergo (a) excretion without further metabolism, (b) further intermediary metabolism, (c) incorporation into tissues, or (d) syntheses or conjugations to promote excretion, which constitute Phase II reactions. Most drugs are metabolized by these two phases, but some may be metabolized by only one phase, and some are not metabolized.

Oxidations comprise the most general reaction of foreign compounds *in vivo*. Included are oxidation of alcohols and aldehydes to acids, oxidation of alkyl groups and chains to alcohols and acids, hydroxylation of acyclic, aromatic and alicyclic compounds, oxidative dealkylation, deamination, dehalogenation and oxidative ring cleavage.

Many of the Phase I metabolic reactions are catalyzed by enzymes of the endoplasmic reticulum of the liver, which possesses "mixed function oxidases" which are concerned with the transformation of foreign compounds, lipids and steroids (11). Although the microsomal enzymes generally metabolize lipid-soluble foreign compounds to form more polar derivatives, polar compounds may also be metabolized by these enzymes (12).

Those metabolic reactions that apply to the N-alkyl aromatic amines will be discussed in more detail.

Metabolism of Aromatic Amines

Metabolic transformations of aromatic amines involve hydroxylation of the aromatic ring, and conjugation of the amino group (13). With N-substituted alkyl anilines, N-dealkylation or oxidation of the side-chain are possible metabolic reactions (1 - 7).

1. Aromatic Hydroxylation

Hydroxylation of foreign compounds to produce phenols has been shown to occur in mammals, frogs, fish, insects and plants (14), and with conjugation, forms the major pathway of metabolism for aromatic compounds in animals.

The reaction, $C_6H_5X \xrightarrow{[O]} HOC_6H_5X$ requires the reduced co-enzyme $NADPH_2$ and molecular oxygen (15).

The hydroxylation of all foreign compounds was originally attributed to the actions of one, non-specific enzyme system. However, differences in species and in sensitivity to enzyme inhibitors and inducers have shown that hydroxylation is carried out by numerous different enzymes (16, 17). The several microsomal enzyme systems responsible for aromatic hydroxylation appear to exhibit orientation specificity which varies with species. Parke (18) has shown that when aniline is administered to dogs, cats and other carnivores, o-aminophenol is the main product of aromatic hydroxylation, while in rabbits and other herbivores p-aminophenol is the major hydroxylated metabolite. The ratio of o- to p-aminophenol in the urine of various species of animals receiving single doses of aniline varies widely (19) as seen in Table II.

TABLE II: SPECIES DIFFERENCES IN *PARA* AND *ORTHO* HYDROXYLATION OF ANILINE

ANIMAL†	P/O RATIO*
Gerbil	15
Guinea Pig	11
Golden Hamster	10
Rabbit	7
Rat	6
Mouse	4
Ferrett	1
Cat	0.5
Dog	0.5

†para/ortho hydroxylation ratio
*dose = 160 - 250 mg./Kg. of ^{14}C-aniline

Total *o*- and *p*-aminophenols were determined by reverse isotope dilution of the forty-eight hour urine.

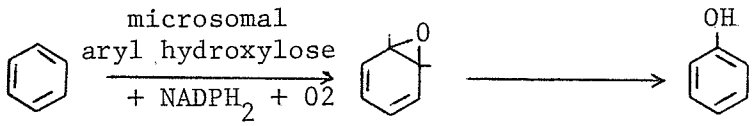
Orientation specificity is determined principally by the nature of the substituent on the aromatic ring. Since the $-NH_2$ group is ortho/para directing, hydroxylation of anilines occurs ortho and/or para to the amino group and varies with species as illustrated in Table II.

N-alkylanilines are hydroxylated almost exclusively in the *para*-position in the rat (2 - 7). No *ortho*-hydroxylated metabolites could be demonstrated with N-*iso*propylaniline nor N-*secondary*butyl aniline (2, 4). However, Horn (20) demonstrated the formation of *o*-aminophenol from N:N-dimethylaniline and N:N-dimethylaniline oxide in dogs,

and Alexander et al (21) found a trace of o-hydroxy diphenylamine in rabbits.

At least two mechanisms are responsible for the enzymic hydroxylation of aromatic compounds to form phenols (22). One mechanism produces only phenols and may involve hydroxylation by a free radical or the formation of an epoxide followed by intramolecular rearrangement to produce a phenol (Mechanism I, benzene ⟶ phenol).

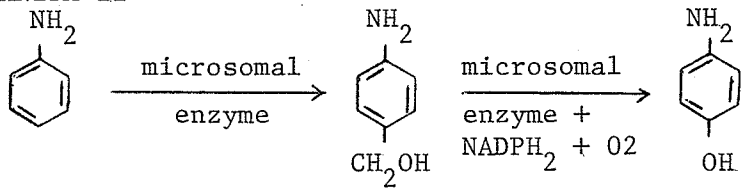
MECHANISM I



Benzene epoxide intermediate Phenol

Hydroxymethylation of the aromatic ring (Mechanism II, aniline ⟶ p-aminophenol) provides a second possible mechanism for hydroxylation producing only phenols. Phenol is formed from benzene via benzyl alcohol and p-aminophenol from aniline via p-aminobenzyl alcohol in guinea pig microsomes (23).

MECHANISM II



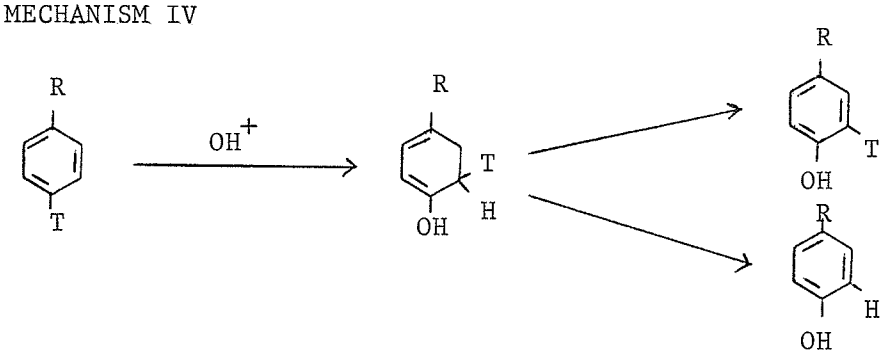
Aniline p-Aminobenzylalcohol p-Aminophenol

A more complex mechanism of hydroxylation involves the formation of 1,2 - dihydroarene - 1,2 - diols, 1,2 - dihydroarenemono-ols, and S-(1,2 - dihydrohydroxylaryl) cysteines which probably have an

arene - 1,2 - epoxide as a common precursor (Mechanism III, naphthalene). Phenols, catechols, hydrocarbons and mercapturic acids comprise the metabolites of this hydroxylation reaction.

A fourth, more recent explanation of aromatic hydroxylation, offered by Guroff et al (24) involves the phenomenon of hydroxylation-induced intramolecular shift, named the "NIH" Shift (Mechanism IV). These workers have shown that a frequent consequence of hydroxylation in aromatic systems is an intramolecular migration of the group displaced by the hydroxyl (usually an hydrogen atom) to an adjacent position on the aromatic ring. This mechanism contradicts the concept of direct substitution as a mechanism for hydroxylation.

MECHANISM IV

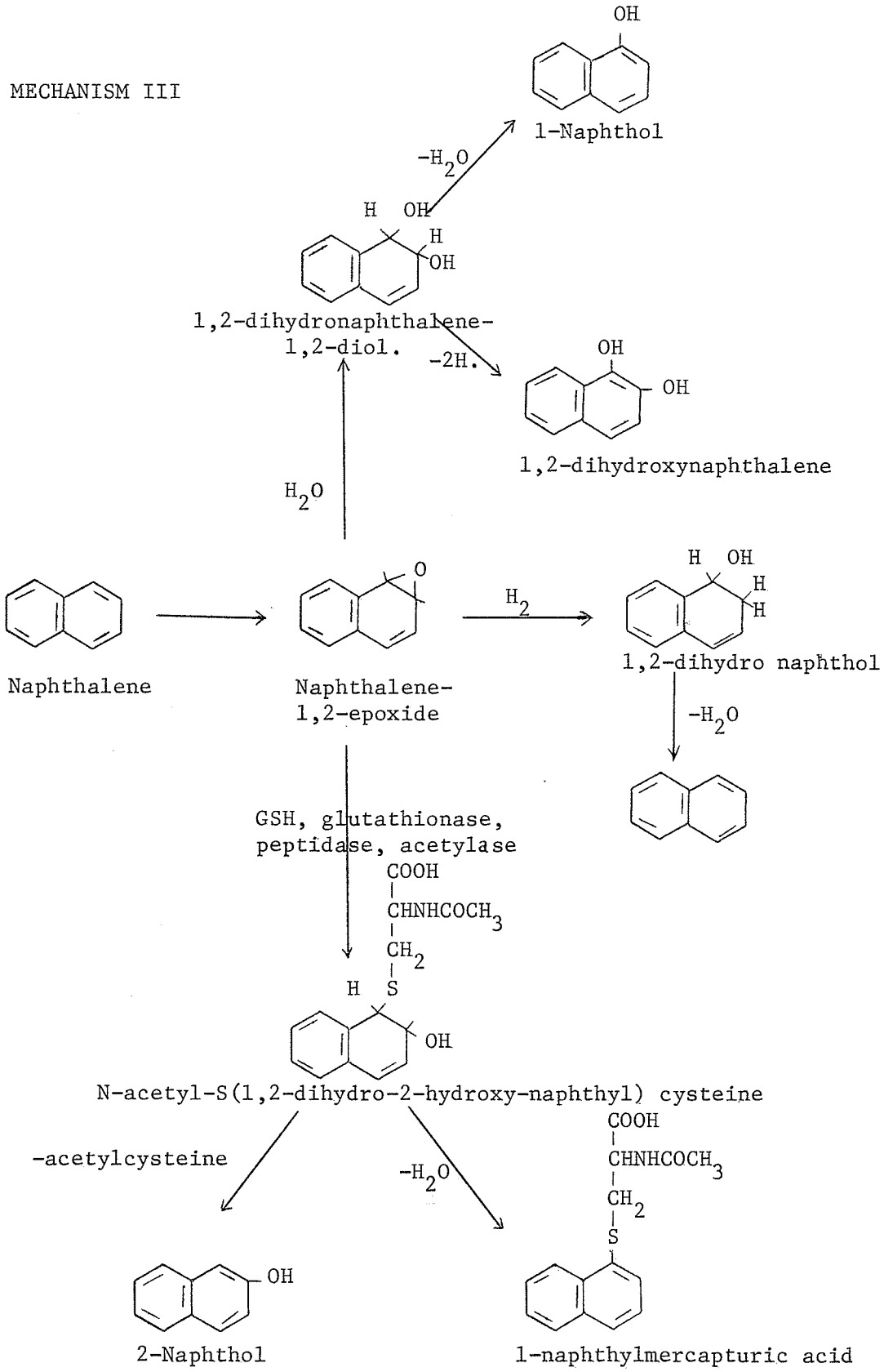


T = tritiated hydrogen

2. N-Dealkylation

The oxidative removal of O-, N-, or S-alkyl groups to produce the corresponding phenol, amine or thiol is a common metabolic reaction. Dealkylation has been shown to occur in mammals, birds and amphibia (14). The enzyme systems which catalyze these reactions are located in the microsomal fraction of the liver and require

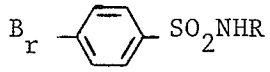
MECHANISM III



$NADPH_2$ and molecular oxygen.

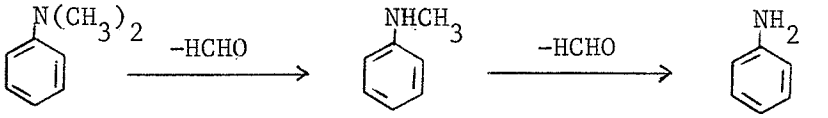
Smith et al (25) studying the anticonvulsant activity of N-alkyl substituted 4-bromobenzenesulphonamides (I) found the extent of dealkylation of the amides in the mouse to occur as follows:

$$R = CH_3 > C_2H_5 > CH_2CH = CH_2 > CH(CH_3)_2 > CH_2CH_2CH_3 > CH_2CH_2CH_2CH_3$$



I

Secondary and tertiary amines undergo N-dealkylation to form primary amines and an aldehyde as follows:

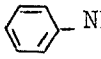


N:N-dimethylaniline N-methylaniline Aniline

N-dealkylation of higher alkylamines, for example, butyl-4-aminopyrine (26), occurs, but at a much slower rate. The in vivo dealkylation of an homologous series of monosubstituted N-alkylanilines has been studied by measuring spectro-photometrically the amount of free p-aminophenol produced (8) and a relationship was observed between the length and structure of the side-chain and the extent of dealkylation (Table III). For straight-chain N-alkyl substituents, N-dealkylation decreased rapidly as the length of the side-chain increased, with maximum dealkylation occurring with N-ethylaniline. When the N-alkyl substituent was a branched chain, dealkylation was affected by the position of the branching. Branching on the *α-carbon con-

*α carbon atom adjacent to nitrogen

TABLE III: IN VIVO DEALKYLATION OF MONOALKYLANILINES IN THE RAT

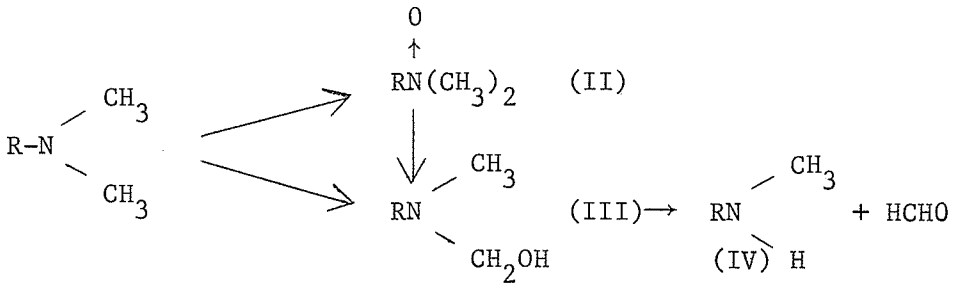
N-ALKYL SUBSTITUENT	STRUCTURE  NHR	% p-AMINOPHENOL EXCRETED IN 24 HR. URINE (AVERAGE OF 3 RATS)
Straight-chain		
methyl	- CH_3	75
ethyl	- C_2H_5	86
n-propyl	- C_3H_7	52
n-butyl	- C_4H_9	49
n-pentyl	- C_5H_{11}	21
n-hexyl	- C_6H_{13}	37
n-heptyl	- C_7H_{15}	15
Branched-chain		
iso-propyl	- $CH(CH_3)_2$	15
sec-butyl	- $CH(CH_3)CH_2CH_3$	15
1-methylbutyl	- $CH(CH_3)CH_2CH_2CH_3$	11
iso-butyl	- $CH_2CH(CH_3)_2$	40
2-methylbutyl	- $CH_2CH(CH_3)CH_2CH_3$	26
iso-pentyl	- $CH_2CH_2CH(CH_3)_2$	19
1-ethylpropyl	- $CH(CH_2CH_3)_2$	b
tert-butyl	- $C(CH_3)_3$	b
neo-pentyl	- $CH_2C(CH_3)_3$	21
Cyclic substituents		
cyclobutyl	- C_4H_7	77
cyclopentyl	- C_5H_9	34
cyclohexyl	- C_6H_{11}	b
cycloheptyl	- C_7H_{13}	b

b = if dealkylation occurs, it is below the sensitivity of the method.

dose rate = 92 μmoles/Kg.

siderably lowered the extent of dealkylation, while branching at the **β-carbon had less effect on dealkylation and branching on the ***γ-carbon had almost no effect on the extent of dealkylation when compared to the straight-chain compounds. With the cyclic substituents, N-dealkylation decreased as the size of the ring increased.

The mechanism of dealkylation involves the formation of an N-oxide (II) or hydroxyalkyl (III) intermediate which spontaneously undergoes molecular rearrangement to produce an amine (IV) and an aldehyde. The N-hydroxyalkyl intermediates are sometimes sufficiently stable to form glucuronide conjugates which are excreted in the urine, as are the N-hydroxymethyl metabolites of the herbicide diphenamid (27).



3. N-Oxidation

N-oxides can be intermediates in N-dealkylation, formed from N-alkyl amines by the action of an N-oxide synthetase requiring FAD, $NADPH_2$ or $NADH_2$ and molecular oxygen.

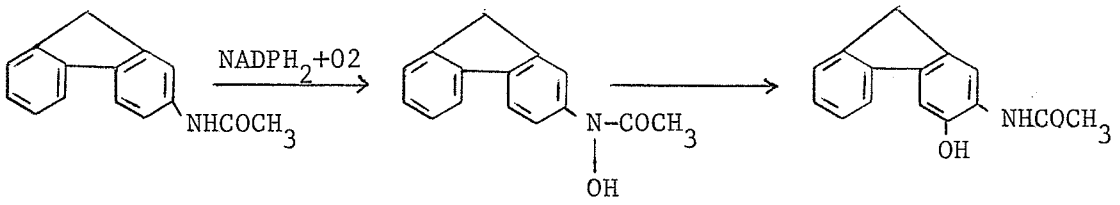
4. N-Hydroxylation

Aromatic amines may undergo biological hydroxylation of the amino group to form hydroxylamino compounds. Hydroxylation of aniline

**β carbon atom one removed from nitrogen
***γ carbon atom two removed from nitrogen

produces phenylhydroxylamine, and many other amines including N-methyl- and N-ethylanilines, toluidines, 2-naphthylamine, and 2-N-acetamido-fluorene are oxidized to the corresponding hydroxylamino derivatives. The N-hydroxylamino metabolites of 2-N-acetamido-fluorene are considered to be active carcinogens (28).

The N-hydroxylating system requires $NADPH_2$ and molecular oxygen, and occurs in the microsomal fraction of the liver, lungs and bladder mucosa (29). N-hydroxylation is usually only a minor pathway in the metabolism of aromatic amines. The hydroxylamines formed may be isomerized into o-aminophenols, and it has been suggested that hydroxylamines are intermediates in ortho-hydroxylation, although it is unlikely that this is the main pathway for ortho-hydroxylation. The N-hydroxylation of 2-N-acetamidofluorene (V) and the subsequent intramolecular rearrangement of the intermediate (VI) to form the o-hydroxy derivative (VII) is illustrated:



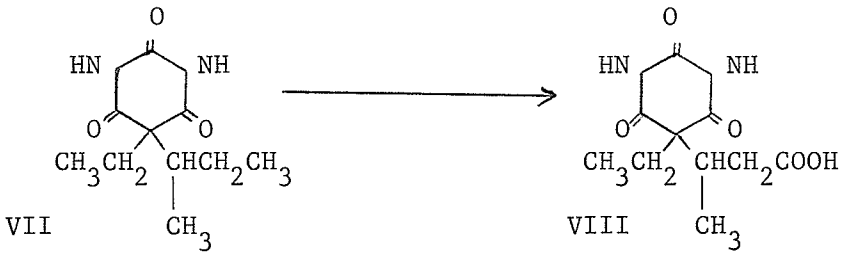
2-N-acetamidofluorene — 3-hydroxy-2-acetamidofluorene

V — VI — VII

5. Side-chain Oxidation

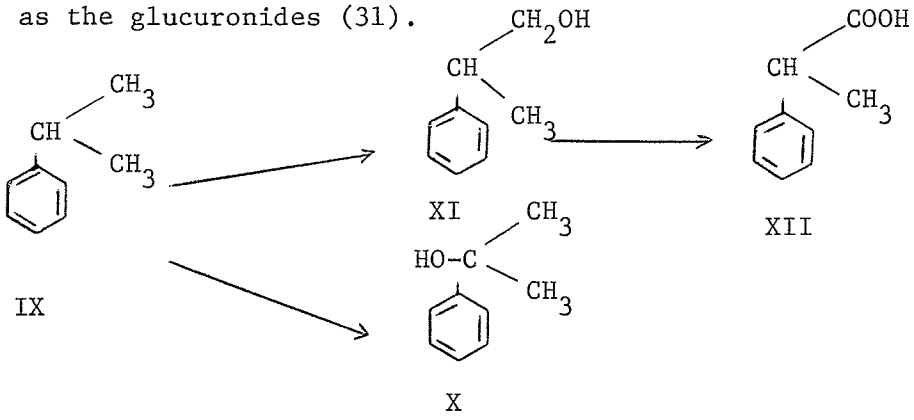
Oxidation of alkyl side-chains has been observed in the metabolism of numerous compounds. Maynert and Losen (30) reported the presence of the metabolite, 5-ethyl-5(1-methyl-2-carboxyethyl) bar-

bituric acid (VIII) after the metabolism of butabarbital (VII), indicating the occurrence of side-chain oxidation.



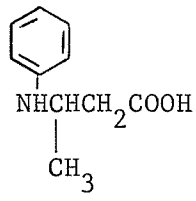
VII VIII

Isopropylbenzene (IX) is metabolized by oxidation of the side-chain to produce the three metabolites, 2-phenylpropan-2-ol (X), hydrotropyl alcohol (XI), and hydrotropic acid (XII) which are excreted as the glucuronides (31).

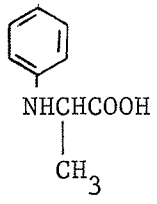


IX XI XII X

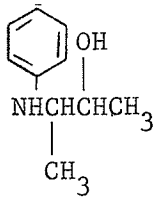
ω-oxidation of side-chains has been observed in previous studies of N-alkylanilines. With N-sec.butylaniline, 3-phenylaminobutyric acid (XIII) was detected to the extent of 3% of the twenty-four hour metabolites, but no 2-anilinopropionic acid (XIV), 3-phenylamino-2-butanol (XV), nor 3-phenylamino-1-butanol (XVI) could be detected (3).



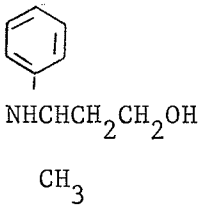
XIII



XIV

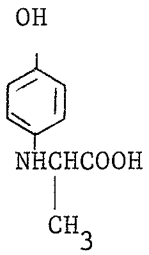


XV



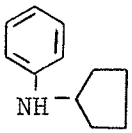
XVI

N-isopropylaniline produced the metabolite, 2-(4-hydroxyphenyl-amino)-propionic acid (XVII) by oxidation of the side-chain (6).



XVII

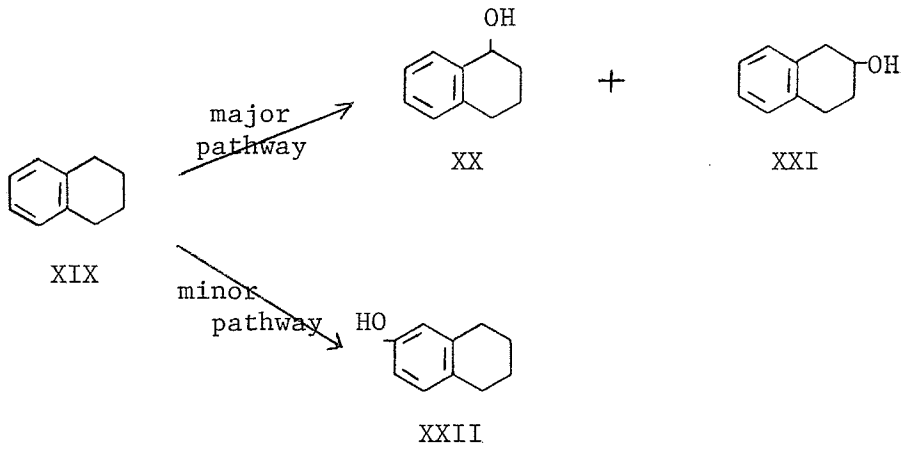
The compound under study in these experiments, N-cyclopentyl-aniline (XVIII), has a cyclic side-chain, and oxidation of this could take the form of alicyclic hydroxylation, or oxidative ring scission. The latter is unlikely, since it occurs most readily with heterocyclic and aromatic rings. However, β-oxidation of an aliphatic side-chain attached to the alicyclic ring could result in ring scission.



XVIII

Alicyclic rings are more readily hydroxylated than aromatic rings (32), so that hydroxylation of N-cyclopentylaniline should favour attack on the cyclopentyl ring. The metabolism of tetralin

(XIX) (1, 2, 3, 4 - tetrahydronaphthalene) provides an example since in the rabbit the major metabolites are conjugates of 1-tetralol (XX) and 2-tetralol (XXI), and only a trace of a phenol, 5, 6, 7, 8 - tetrahydro-2-naphthol (XXII) is formed (33).



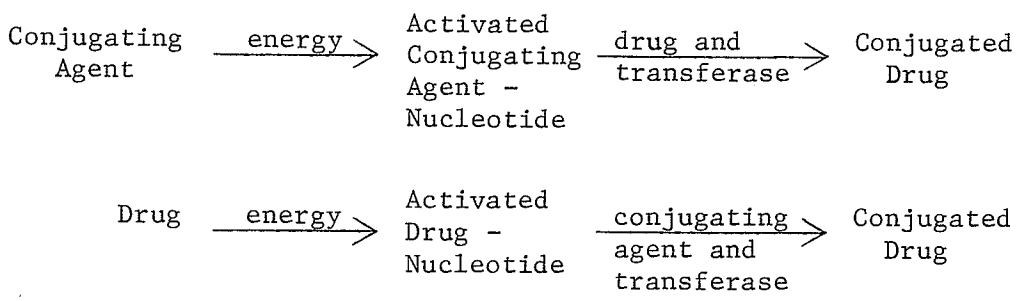
6. Conjugation or Synthesis

Conjugations are biosyntheses in which foreign compounds or their metabolites combine with endogenous substrates to form conjugates. Conjugation takes place by addition to a functional group of the foreign molecule and generally results in the compound becoming more polar, less lipid-soluble and therefore readily excreted from the body.

Unlike Phase I reactions, conjugations show discernible species patterns and can be correlated with phylogenetic development. Conjugation with sugars to form glucosides occurs in most plants, bacteria, shellfish and insects, whereas most vertebrates utilize glucuronic acid for conjugations. Peptide conjugations also show phylo-

genetic development. Conjugation with glycine occurs in all terrestrial animals. Arthopods form conjugates with arginine, reptiles and most birds with ornithine, and man and the chimpanzee with glutamine.

A conjugation reaction requires an "active" intermediate compound, usually a nucleotide, and a transferring enzyme. There are two types of conjugating mechanisms (14); one in which the conjugating agent forms part of the active intermediate and the other in which the drug or its Phase I metabolite forms part of the active intermediate, viz.:

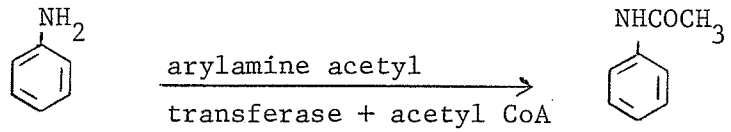


Conjugation of aromatic amines may take several forms. The amino group may be subjected to acetylation or conjugation with glucuronic acid, sulphuric acid or other conjugating agents. Aromatic hydroxylation of the amine provides a possible second centre for conjugation.

a) Acetylation

Acetylation of -OH, -SH and $-NH_2$ groups can occur *in vivo*, but only the latter occurs frequently. N-acetylation is a general pathway of metabolism for sulphonamides, aliphatic amines, some foreign

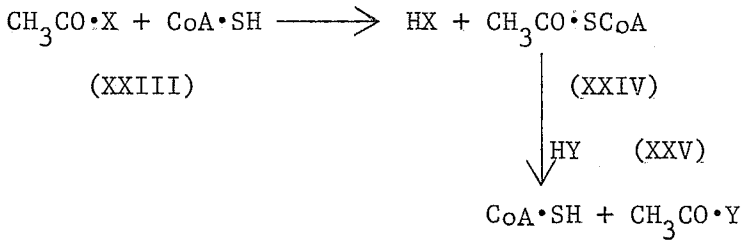
amino acids, hydrazines and aromatic amines (13) as follows:



Aniline Acetanilide

Deacetylation of the conjugate may occur in certain species, so that the extent of excretion of acetylated amines may depend upon a competition between hepatic acetylation and renal deacetylation (34).

The active intermediate in biological acetylation is a derivative of coenzyme A (XXIII), acetyl CoA (XXIV) which condenses with a suitable accepting agent (XXV). The general reaction for acetylation can be represented as follows:



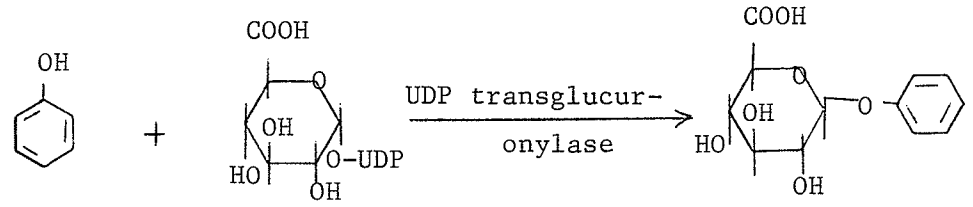
where $CH_3CO{\cdot}X$ = source of acetyl groups

$CH_3CO{\cdot}Y$ = acetylated drug.

b) Glucuronic Acid Conjugation

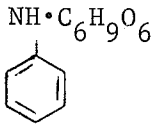
O-glucuronides are formed by the conjugation of glucuronic acid with alcohols or acids to produce respectively, ether-type or ester-type glucuronides.

Phenols and aminophenols generally are excreted as ether-type O-glucuronides (or ethereal sulphates) as illustrated:



Phenol glucuronic acid intermediate Phenyl-β-D-glucuronide

N-glucuronides may also be formed, and the nitrogen atom to which the glucuronyl moiety is attached may be in an aromatic amino group, a sulphonamide group, a carbamyl group or in a heterocyclic nitrogen compound. The aliphatic and aromatic N-glucuronides are labile, while amide glucuronides are more stable. Aniline glucuronide (XXVI) and other aromatic and aliphatic N-glucuronides appear to be artifacts formed spontaneously from glucuronic acid and the free amine (35).



XXVI

Glucuronide formation is a two-step process involving the biosynthesis of the coenzyme donor, uridine diphosphate glucuronic acid (XXVII), and the transfer of the glucuronyl moiety to the acceptor (XXVIII) by UDP transglucuronylases as follows:

$$\text{Glucose-1-phosphate} + \text{UTP} \xrightarrow[\text{ferase}]{\text{trans}} \text{UDPG} + P_2O_7^{----}$$

$$\text{UDPG} + 2\text{NAD} \xrightarrow{\text{dehydrogenase}} \text{UDPGA} + 2\text{NADH}_2$$

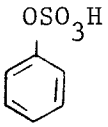
$$\underset{\text{(XXVII)}}{\text{UDPGA}} + \text{ROH} \xrightarrow{\text{transglucuronylase}} \underset{\text{(XXVIII)}}{\text{RO}\cdot C_6H_9O_6} + \text{UDP}$$

where UTP = uridine triphosphate

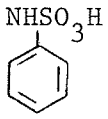
UDPG = uridine diphosphate-α,D-glucose

c) Sulphate Conjugation

Another common class of conjugates is the sulphate esters or "ethereal sulphates". Aryl sulphates (XXIX), alkyl sulphates and sulphamates (XXX) are the three most common types of sulphate conjugates.



XXIX



XXX

Sulphate esters are biosynthesized by the transfer of sulphate from adenosine-3'-phosphate-5'-phosphosulphate (XXXI) to a phenol, alcohol or amine by sulphate-transferring enzymes, viz.:

$$ATP + SO_4^{--} \xrightleftharpoons{\text{transferase}} APS + P_2O_7^{----}$$

$$APS + ATP \xrightleftharpoons{\text{transferase}} PAPS + ADP$$

$$PAPS + ROH \xrightleftharpoons{\text{transferase}} RO{\cdot}SO_3H + ADP$$

where APS = adenosine=5'-phosphosulphate

$RO{\cdot}SO_3H$ = conjugate drug (sulphate ester).

d) Other Conjugations

i) Methylation is a common biochemical reaction, and involves the transfer of a methyl group from the coenzyme, S-adenosylmethionine to amines, phenols and thiols. Primary, secondary and tertiary heterocyclic amines may undergo N-methylation.

ii) N-formylation is a method of conjugation which occurs infrequently. The metabolite, N-formyl-2-aminophenol was recently isolated from a culture of χanthocillin (χanthocillinum DAB-7)(36).

iii) Conjugation as a phosphate ester is a rare mode of conjugation. One of the few known examples is the conjugation of 2-naphthylamine as bis(2-amino-1-naphthyl) phosphate detected in the urine of dogs.

When a molecule has two or more functional groups, it is usual for only one to become conjugated. However, double conjugates may be formed when conjugation at one functional group does not render the molecule sufficiently polar to permit rapid excretion. Acetamidophenols are examples of conjugated compounds which require further conjugation. They are present in the urine as sulphates or glucuronides (37). The major excretory product of aminophenols in the rabbit is N-acetyl-p-aminophenylglucuronide (38). Some N-acetyl-p-aminophenylsulphate was isolated from human urine as a metabolite of acetanilide in 1889 (39), but most was excreted conjugated as the glucuronide.

The modes of conjugation discussed in this section have been those which might possibly occur with an N-alkyl aromatic amine. No conjugates of these compounds have as yet been identified.

Metabolism of the Cyclopentyl Ring

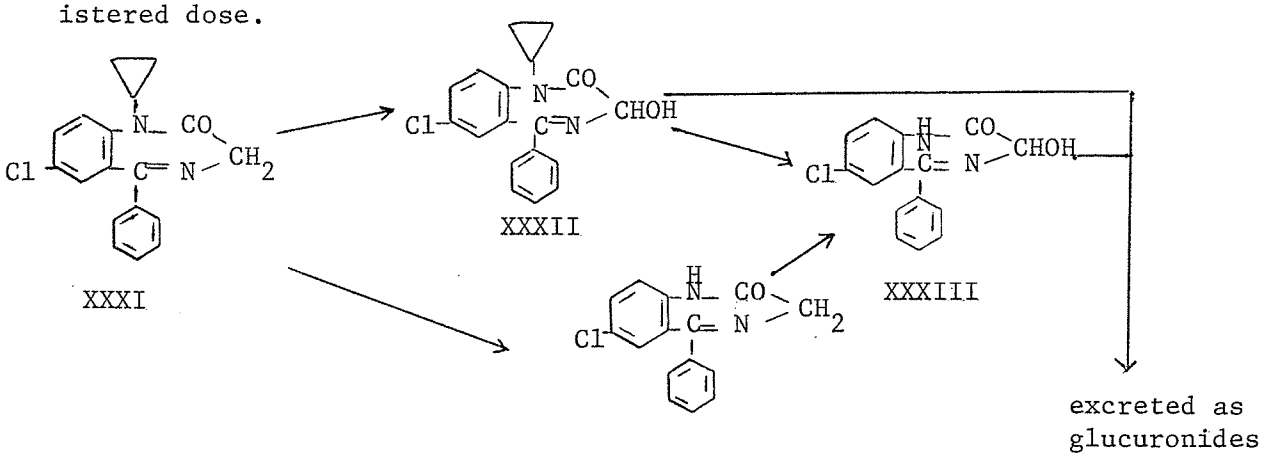
In the animal body, saturated cyclic compounds may be metabolized by one of several routes. Some alicyclic rings may undergo

aromatization, some may undergo scission to produce aliphatic chains, and others may be dehydrogenated, a process, which in the case of sterols and sex hormones, may produce carcinogenic compounds (40). The fate of the cyclopentyl ring *in vivo* remains to be clearly defined, but some idea as to its metabolism may be provided by reviewing the known metabolic reactions of the cyclopropyl and cyclohexyl, as well as the cyclopentyl moiety.

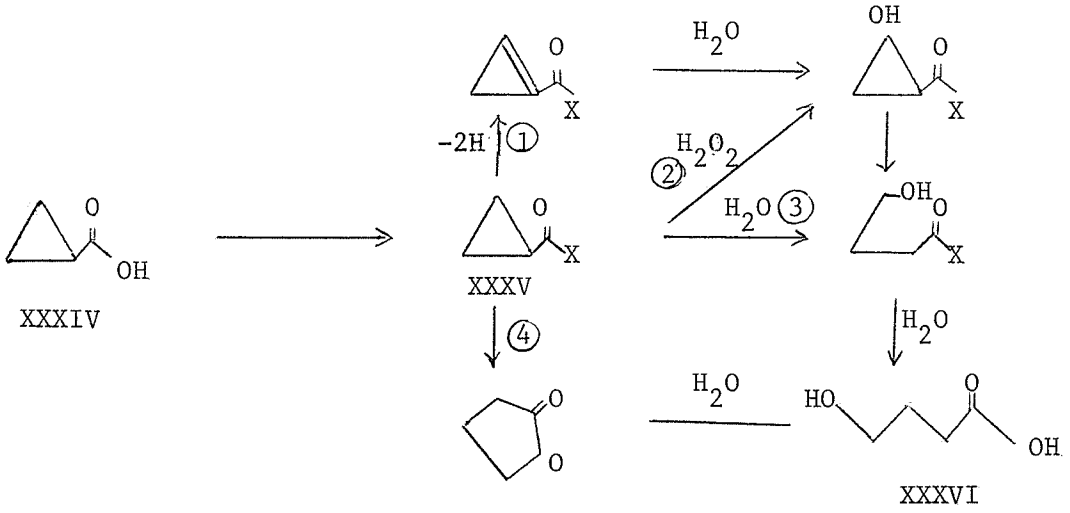
a) Cyclopropyl Derivatives

The simplest of the alicyclic ring systems, cyclopropane gas, undergoes no metabolic change in the organism and is almost entirely eliminated by its route of entry, the lungs. Its ethers, cyclopropylmethyl-, cyclopropylethyl-, and cyclopropylvinyl-ether are also eliminated unchanged (41).

Wood and Reiser (42) and Chung (43) have indicated that the cyclopropyl ring is not metabolized by whole rats or rat liver mitochondria. A study of the metabolism of the tranquilizer, prazepam (XXXI) in man (44) suggests that the cyclopropyl group is either removed unaltered by dealkylation to produce oxazepam (XXXIII), accounting for up to 46% of the dose or is eliminated unchanged as 3-hydroxyprazepam (XXXII) which represents up to 32% of the administered dose.



Recently, Kaneshiro and Thomas (45) have indicated that when Agrobacterium tumefaciens cells were grown under special conditions, the cyclopropane fatty acids (cis-11,12-methylene-octadecanoate and cis-9,10-methylenehexanedecanoate) found in the phospholipids of these micro-organisms could serve as precursors for the corresponding methyl branched-chain fatty acids. Another report concerning cleavage of the cyclopropyl ring involves the fungus Fusarium oxysporum Schlectendahl, which has been shown capable of degrading the cyclopropyl ring of cyclopropanecarboxylic acid-1-^{14}C to fulfill its metabolic needs for carbon and energy (46). Schiller and Chung have indicated that the sequence of reactions involved in the degradation of the cyclopropyl ring included an activation of cyclopropanecarboxylic acid (XXXIV) to the ester, cyclopropanecarboxylic acid-X (XXXV), followed by the addition of water across the ring and loss of the activating group as ring scission occurred, with the resultant formation of γ-hydroxybutyric acid (XXXVI) and its further metabolism to polar compounds. Four hypothetical pathways for the degradation of the cyclopropyl ring are presented as follows:



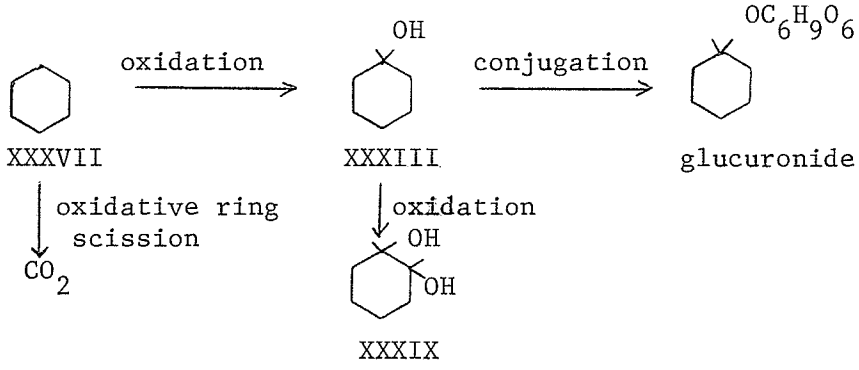
Pathway 1 requires an α,β-dehydrogenation similar to that found in fatty acid oxidation (47), followed by a Michael-type addition of water (48) across the unsaturated bond to yield β-hydroxycyclopropanecarboxylic acid-X, which is analogous to the enoyl hydrase reaction encountered in fatty acid oxidation. The next step consists of cleavage of the carbon-carbon single bond by the addition of two hydrogen atoms, a reaction for which no biochemical precedent is available. The final step is hydrolysis of γ-hydroxybutyric acid-X to γ-hydroxybutyric acid. Pathway 2 requires the participation of molecular oxygen to degrade the cyclopropyl ring by an oxidation of the saturated carbon atom, a reaction which can be observed in the α-hydroxylation (49) and ω-hydroxylation (47) of fatty acids. Pathway 3 involves the addition of water to break a carbon-carbon single bond, a reaction which could be justifiably expected because cyclopropanecarboxylic esters behave like α,β-unsaturated esters when attacked by nucleophiles (50). The remaining pathway, 4, requires that the cyclopropanecarbonyl moiety be linked to -X by an oxygen ester linkage, which might allow an internal rearrangement to form γ-butyrolactone, which upon hydrolysis would produce free γ-hydroxybutyric acid.

b) Cyclohexyl Derivatives

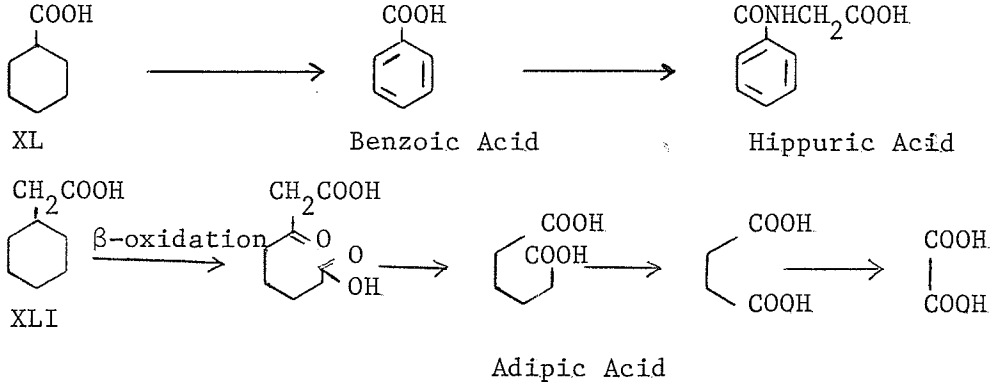
When cyclohexane-^{14}C (XXXVII) was fed to rabbits, studies showed that the cyclohexyl ring remained intact in most of the metabolites. Cyclohexanol (XXXVIII) (38% of dose) and (±)trans-cyclohexane-1,2-diol (XXXIX) (7% of dose) were excreted in the urine as glucuronides.

A substantial portion (30% of dose) was exhaled unchanged in the breath, and 9% was excreted as respiratory $^{14}CO_2$. No phenol, adipic acid, nor other carboxylic acids were detected (51).

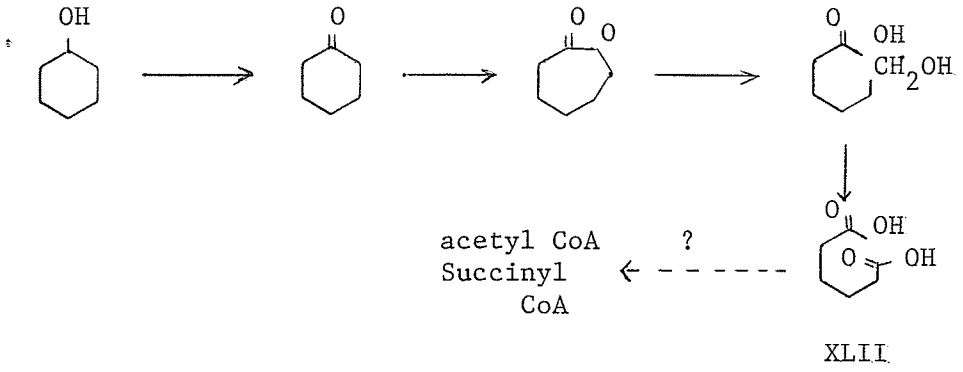
In rabbits, cyclohexanol was excreted mainly as the glucuronide (60%), but a small amount was further oxidized to trans-cyclohexane-1,2-diol (6%). Cyclohexanone metabolism involved reduction to cyclohexanol and excretion as the glucuronide.



Cyclohexanecarboxylic acids were metabolized either by aromatization or oxidative ring scission. Cyclohexanecarboxylic acid (XL) and compounds which can be oxidized to it, e.g. cyclohexyl fatty acids with the formula $C_6H_{11}(CH_2)n\ COOH$ where n is an even number, were oxidized to benzoic acid and excreted as its conjugate hippuric acid. However, cyclohexylacetic acid (XLI) and cyclohexyl fatty acids where n is an odd number, were metabolized by oxidative scission of the cyclohexyl ring and complete oxidation (52).

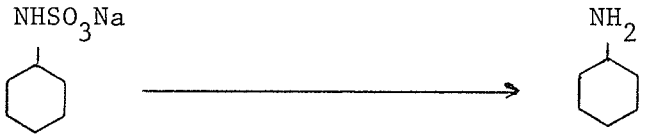


Ring scission of the cyclohexyl ring of cyclohexanol to produce adipic acid (XLII) has been reported to occur in Nocardia globerula CL1 (53).



XLII

In man , the cyclohexyl ring of cyclamate (XLIII) remained intact throughout metabolic transformation to cyclohexylamine (XLIV) (54).

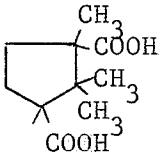


XLIII XLIV

Studies of cyclohexylamine-^{14}C in rats, dogs and humans (55) showed this compound to be primarily excreted unchanged.

c) Cyclopentyl Derivatives

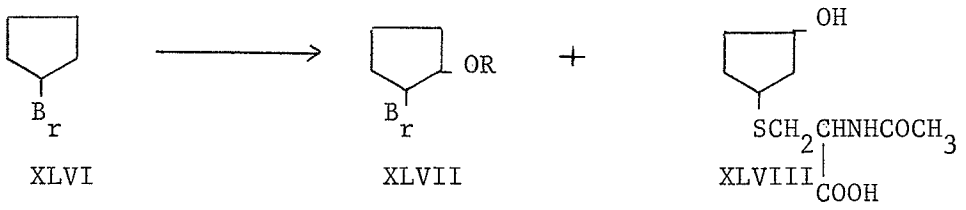
The cyclopentyl ring as found in the derivative, camphoric acid (XLV) (1:2:2 - trimethyl-cyclopentane-1,2-dicarboxylic acid) is excreted unchanged in the urine of dogs (56).



XLV

Similarly, the cyclopentyl ring remained intact when bromo-

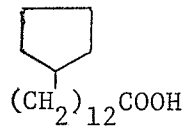
cyclopentane (XLVI) was fed to rabbits and rats (57, 58). The metabolites were found to consist primarily of conjugated derivatives of the hydroxylated ring, and included 2-hydroxybromocyclopentyl-glucuronide or sulphate (XLVII) (57), and 3-hydroxycyclopentylmercapturic acid (XLVIII) (58).



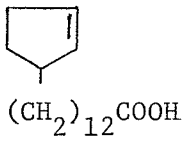
XLVI XLVII XLVIII

R = glucuronide or sulphate

There are several reports of the metabolic degration of the cyclopentyl ring. Chaulmoogric acid (XLIX) and its dihydro derivative, ω-cyclopentyltridecoic acid (L) appeared to be completely metabolized, since neither the original compound nor any metabolite could be detected in the urine (59) of dogs.

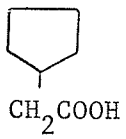


L

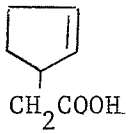


XLIX

Similarly, cyclopentylacetic acid (LI) and cyclopentyleneacetic acid (LII) appeared to be completely metabolized after injection into dogs.



LI



LII

These acids are probably metabolized by β-oxidation to carbon dioxide and water, the oxidation proceeding from the side-chain into the ring. In the case of cyclopentylacetic acid, the first oxidation would be in the ring.

In summary, it appears that alicyclic rings may undergo metabolic changes in which either the integrity of the ring remains intact, or the ring undergoes scission and the compound is completely oxidized. In the case of the cyclopropyl, cyclopentyl, and cyclohexyl rings, a side-chain containing a carboxyl group seems to be necessary for ring cleavage, since oxidation proceeds through the side-chain into the ring. With the exception of the reported scission of the cyclohexyl ring of cyclohexanol by Nocardia globerula CLI, no alicyclic ring has been observed to undergo scission except through the carboxyl side-chain by β-oxidation.

It seems unlikely, therefore, that the cyclopentyl ring of N-cyclopentylaniline would undergo scission before being removed by dealkylation. What happens to the cyclopentyl ring after it is removed would be difficult to observe in this study, since only the benzene ring of N-cyclopentylaniline was radioactively labelled.

MATERIALS AND METHODS

I. MATERIALS

Melting points were obtained using a Hoover Unimelt capillary melting point apparatus and were uncorrected. Elemental analyses were carried out by Pascher and Pascher Microanalytisches Laboratorium, Bonn, Germany. Mass spectra were performed in a Finnigan 1015 mass spectrometer in the Department of Chemistry, University of Manitoba. Infra-red spectra were obtained from Perkin-Elmer 700 and Beckman IR8 infra-red spectrophotometers.

Aniline-^{14}C hydrochloride (uniformly labelled; specific activity, 5.0 mc/mM.) used in the synthesis of radioactive N-cyclopentylaniline hydrochloride was purchased from New England Nuclear Corporation, Boston, Massachusetts.

Cyclopentanone was freshly distilled from a technical grade, supplied by the Aldrich Chemical Company, Milwaukee, Wisconsin.

N-acetyl-*p*-aminophenol was purchased as Baker grade 4-hydroxyacetanilide from the J. T. Baker Chemical Company, Phillipsburg, New Jersey.

p-aminophenyl sulphate was synthesized according to the method of Burkhardt and Wood (60).

Dry hydrogen chloride gas was generated by the dropwise addition of concentrated sulphuric acid onto sodium chloride. The gas was passed through a sulphuric acid drying trap placed between the generating flask and the outlet tube.

Gibb's reagent, 2,6-dichloroquinone-4-chloroimide (Aldrich Chemical Company), 1% w/v in alcohol, was employed as a chromatographic spray reagent.

Freshly prepared diazotized sulphanilic acid used as a detecting reagent was prepared by mixing cold 5% aqueous sodium nitrite solution, cold 1% sulphanilic acid solution in N/1 hydrochloric acid, and cold 10% aqueous sodium bicarbonate solution (in that order) in a ratio of 1:1:2.

p-dimethylaminobenzaldehyde (Grade B, Calbiochem Laboratories, Los Angeles, California), 1% w/v solution in N/1 hydrochloric acid, was used as a chromatographic spray reagent.

The pH 6.8 phosphate buffer (61) employed in the incubation of urine aliquots with β-glucuronidase (bacterial) was prepared by mixing 37.8 ml. of 0.5 molar potassium acid phosphate with 20.8 ml. of 0.5 molar sodium phosphate, and diluting with distilled water to one litre.

The pH 5.0 acetate buffer (62) employed in the incubation of urine aliquots with sulphatase and with β-glucuronidase (bovine liver) was prepared by mixing 73.8 ml. of molar acetic acid with 50.0 ml. of molar sodium hydroxide and diluting with distilled water to one litre.

β-glucuronidase (Type II, bacterial) with an activity of 56,800 Units/Gm. was supplied by the Sigma Chemical Company, St. Louis, Missouri. β-glucuronidase (Type B-I, bovine liver) with an activity of 600,000 Units/Gm. was also supplied by the Sigma Chemical Company.

The enzyme was dissolved in water to give a solution containing 55 Units/ml. To verify its activity, two solutions were prepared, each containing phenolphthalein glucuronic acid (2 mg.) (Sigma Chemical Company) and the appropriate buffer (1 ml.). β-glucuronidase solution (1 ml.) was added to one solution and both were incubated at 37°C. for three hours on a metabolic shaker. Three drops of ammonia were added to each solution. The solution containing the enzyme turned bright pink, while the blank remained colourless, indicating that the β-glucuronidase had cleaved the conjugate and released free phenolphthalein.

Sulphatase (Type II from Limpets) with an activity of 6.5 Units/mg. was supplied by the Sigma Chemical Company. The enzyme was dissolved in water to give an activity of 50 Units/ml. of solution. In order to verify its activity, two solutions were prepared, each containing p-aminophenyl sulphate (2 mg.) and pH 5.0 acetate buffer (3 ml.). Sulphatase solution (1 ml.) was added to one flask and both were incubated on a metabolic shaker for four hours at 37°C. Upon introduction of phenol reagent (3 ml., see below) to the solutions, the solution containing the enzyme produced a distinct blue colour, while the blank showed no colour change, indicating that the conjugate had been cleaved by the enzyme to release p-aminophenol.

The above phenol reagent was prepared by mixing 0.2 N sodium hydroxide solution (8 parts), phenol solution (1 Gm. phenol in 88 ml. water, 2 parts) and bromine solution (100 ml. N sodium carbonate and 15 ml. of 10% w/v aqueous bromine solution, 2 parts). This reagent

produces a blue colour by coupling with free p-aminophenol to produce an indolephenol dye when utilized according to the procedures of Brodie and Axelrod (63), and Welch and Conney (64).

The following solvent systems were utilized for thin layer chromatography:

Solvent 1: water

Solvent 2: isopropanol/benzene (5:95)

Solvent 3: benzene/methanol/glacial acetic acid (90:16:8)

Solvent 4: 95% ethanol/chloroform (1:1)

Solvent 5: n-butanol/glacial acetic acid/water (4:1:1)

Solvent 6: chloroform/isopropanol/methanol/ammonia (10:10:5:2)

II. RADIOANALYTICAL METHODS

A. Liquid Scintillation Counting

1. Scintillation Fluids

(a) The scintillation fluid used in liquid scintillation counting of all samples except those referred to in (b) was prepared according to the formula:

POP (2,5-diphenyloxazole)	2.00 Gm.
bis-MSB |bis-(o-methylstyryl)-benzene|	0.04 Gm.
isopropanol	400.00 ml.
toluene, to produce	1000.00 ml.

(b) Aliquots of the non-aqueous solutions used in combustion of the feces were counted in a scintillation fluid consisting of

the following:

POP (2,5-diphenyloxazole)	4.00 Gm.
dimethyl POPOP |1,4-bis-2(4-methyl 5-phenyloxazolyl)-benzene|	0.10 Gm.
toluene, to produce	1000.00 ml.

2. Method

All liquid samples were delivered into 20 ml.-capacity glass counting vials by means of an Agla micrometer syringe, an Eppendorf pipette (100 or 200 microlitre capacity), or a 1.0 ml. delivery pipette. The appropriate scintillation fluid (10 ml.) was added, and where necessary to produce complete suspension or solubilization, Cab-O-Sil (Thixotropic Gel Powder, 200 mg.) or 0.3 N NCS Solubilizer (1 ml.) was added.

Solid samples were weighed on a Cahn Gram Electrobalance and transferred in weighing boats to glass counting vials. Scintillation fluid (10 ml.) was added, and thixotropic gel or solubilizer included when necessary.

All solutions or suspensions prepared as described above were counted in a Nuclear Chicago Unilux Liquid Scintillation Counter, Model No. 6850.

3. Internal Standard

In order to determine the efficiency of counting for each sample and thus provide a means of converting counts per minute (cpm.) to disintegrations per minute (dpm.), an internal standard was employed. After the initial counting, each sample was re-counted fol-

lowing the addition of 0.1 ml. of benzoic acid-^{14}C. solution in toluene (about 3000 dpm/0.1 ml. accurately determined).

4. Minimum Radioactivity

The minimum valid radioactivity was chosen as a figure equal to twice the background count, which averaged 32 cpm. Under the conditions used, the counting efficiency was approximately 66%, so that in order to be considered valid, the minimum detectable radioactivity would have to be at least 50 dpm. above background.

B. Detection of Radioactivity on Chromatograms

Paper and thin layer chromatograms were scanned for radioactivity with a Nucleur Chicago Actigraph III Instrument. The relative proportions of radioactivity in each peak were determined by the use of a digital integrator attachment, Nuclear Chicago Model No. 8735.

The following instrumental settings were maintained for all scans:

High Voltage	- 1050 volts
Time Constant	- 10 seconds
Collimator Slit Width	- 12 mm.
Helium-Butane Pressure	- 7 p.s.i.
Count Rate	- variable, generally 150 cpm. for paper chromatograms and 100 cpm. for thin layer chromatograms
Scan Speed	- 60 cm/hr. for paper chromatograms; 120 cm/hr. for thin layer chromatograms
Gate Response	- medium

Gate Level	- 85% of 30
Analog	- 10^2
Program	- differential/integral
Print Sequence	- T-C
Sampling Period	- one minute

EXPERIMENTAL

I. PREPARATION OF COMPOUNDS

A. N-Cyclopentylaniline-^{14}C Hydrochloride

N-cyclopentylaniline-^{14}C hydrochloride was synthesized by a modification of the procedure of Schellenberg (65). Aniline-^{14}C hydrochloride (6.45 mg.; 0.25 mc.) and aniline hydrochloride (499.2 mg.; 0.0039 moles total) were dissolved in water (30 ml.). The solution was made just alkaline to litmus using sodium hydroxide solution, then 95% ethanol (16 ml.), glacial acetic acid (4.5 ml.) and anhydrous sodium acetate (2.17 Gm.) were added. The solution was cooled to 0^0C., then redistilled cyclopentanone (1.01 Gm.; 0.0120 moles) was added. With constant stirring, sodium borohydride (1.90 Gm.; 0.050 moles) was added in thirty milligram portions over a period of forty-five minutes, maintaining the temperature below 10^0C. The mixture was stirred an additional ten minutes, then was made alkaline with ammonia, and extracted with ether (4 x 5 ml. portions). The etheral extract was dried over anhydrous sodium sulphate, then filtered and evaporated to a small volume under a slow stream of nitrogen. The liquid residue was spotted on thick layer (1 mm.) chromatographic plates of Silica Gel GF-254, and allowed to develop in a solvent of petroleum ether (boiling range 30^0-60^0C.)/benzene (2:3). The plates were air-dried and the band corresponding to N-cyclopentylaniline (Rf=0.36) located under short-wave ultra-violet light and scraped from the plates. The silica gel scrapings were packed in a

glass column (15 cm. x 2.5 cm.) and eluted with dry ether. Dry hydrogen chloride gas was passed through the etheral eluate, and the crude product (0.5258 Gm.; 68.06% yield) was filtered off, dried over sulphuric acid and recrystallized to constant specific activity (6.85 x 10^5 dpm./mg.) from anhydrous ethanol/ethyl acetate (m/p. 152 - 153^{0}C.).

Absorptivity of the purified product was measured in equal parts of 95% ethanol and 0.008 N sodium hydroxide solution on a Beckman model DU spectrophotometer, E(245 nm)=10,844 (logE=4.04).

Thin layer chromatography of the purified product by converting it to the free base showed only one radioactive spot (Figure 1)* corresponding to N-cyclopentylaniline (Rf=0.36) developed in petroleum ether (30-60)/benzene (2:3).

Non-radioactive N-cyclopentylaniline hydrochloride was similarly prepared and analyzed. |m.p.=152-153^{0}C.; E(245nm)=10,671, logE=4.04|

calculated for $C_{11}H_{16}NCl$:	C, 66.82%	H, 8.15%
found for $C_{11}H_{16}NCl$:	C, 66.86%	H, 8.24%

The mass spectrum (Figure 1 in Appendix I) of the purified product revealed a spike at a m/e of 161, which corresponded to the calculated molecular weight (161.24) of the free base.

The infra-red spectrum (Figure 1 in Appendix II) of the product was performed in nujol.

An unsuccessful attempt was made to prepare larger quantities of non-radioactive N-cyclopentylaniline hydrochloride from nitrobenzene and cyclopentanone using the method of Emerson and Uraneck (66). Analyses of the resulting product indicated that aniline hydrochlor-

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*Figures and Tables are to be found in "Results and Discussion".

ide was the major compound formed.

B. p-Hydroxy-N-Cyclopentylaniline Hydrochloride

p-hydroxy-N-cyclopentylaniline hydrochloride was synthesized by a modification of the method of Emerson and Uraneck (66). p-nitrophenol (13.9 Gm.; 0.10 moles) and redistilled cyclopentanone (8.4 Gm.; 0.10 moles) were placed in a Parr pressure reaction apparatus with glacial acetic acid (10 ml.), 95% ethanol (150 ml.) and platinum oxide (0.10 Gm.). The mixture was subjected to hydrogen gas at 700 psi for four hours with constant shaking. The mixture was filtered and the alcohol removed in vacuo. Dilute sulphuric acid (30 ml.) was added to the remaining liquid residue and the acidic solution washed with ether and neutralized with sodium hydroxide solution. The resulting precipitate was collected by filtration, dried over sulphuric acid in a vacuum dessicator and dissolved in anhydrous ether. The ethereal solution was dried over anhydrous sodium sulphate overnight and filtered. Dry hydrogen chloride gas was passed through the solution to produce the crude product (10.4 Gm.; 48.8% yield) which was recrystallized from anhydrous ethanol/ethyl acetate. (m.p. 179.5 - 180.5^{0}C.)

calculated for $C_{11}H_{16}NOCl$:	C, 61.82%	H, 7.55%
found for $C_{11}H_{16}NOCl$:	C, 61.48%	H, 7.79%

The mass spectrum of the compound (Figure 2 in Appendix I) showed a spike at m/e of 177, corresponding with the calculated molecular weight (177.24) of the free base.

The infra-red spectrum of the product (Figure 2 in Appendix II)

in nujol showed an absorption at approximately 3200 $cm.^{-1}$, owing to the O-H stretching vibrations of the phenolic hydroxyl group.

C. p-Aminophenol Hydrochloride

p-aminophenol hydrochloride was prepared in these laboratories (67) by dissolving a maximum amount of p-aminophenol in hot, concentrated hydrochloric acid and allowing the solution to cool. The crude product was recrystallized from hot, concentrated hydrochloric acid. m.p. 304 - 305°C., reported 306°C. (68)

calculated for C_6H_8NOCl:	C, 49.50%	H, 5.54%
found for C_6H_8NOCl:	C, 48.90%	H, 5.60%

The mass spectrum of the purified product (Figure 3 in Appendix I) had a spike at m/e of 109, which corresponded to the calculated molecular weight (109.13) of the free base.

The infra-red spectrum of this compound in nujol (Figure 3 in Appendix II) showed a broad absorption band at approximately 3200 $cm.^{-1}$ due to the O-H and N-H stretching vibrations of the phenolic hydroxyl and the amino groups.

D. N-Cyclopentylaniline Tosylate

This derivative was prepared from p-toluenesulphonyl chloride and N-cyclopentylaniline hydrochloride according to the method of Vogel (69), and was recrystallized from ethyl acetate/petroleum ether (30 - 60). m.p. 115.5 - 116.5°C.

calculated for $C_{18}H_{21}NSO_2$:	C, 68.54%	H, 6.71%
found for $C_{18}H_{21}NSO_2$:	C, 68.51%	H, 6.85%

The mass spectrum of this compound (Figure 4 in Appendix I)

revealed a spike at m/e of 315, which confirmed that the product was a monotosylate derivative of N-cyclopentylaniline with a calculated molecular weight of 315.42.

The infra-red spectrum of this compound in nujol (Figure 4 in Appendix II) was performed.

E. p-hydroxy-N-cyclopentylaniline-N,O-ditosylate

The procedure of Vogel (70) was employed in the preparation of this compound, using p-hydroxy-N-cyclopentylaniline hydrochloride and p-toluenesulphonyl chloride. The crude product was recrystallized from ethyl acetate/petroleum ether (30 - 60). m.p. 131.5^{0}C.

calculated for $C_{25}H_{27}NS_2O_5$: C, 61.83% H, 5.60%

found for $C_{25}H_{27}NS_2O_5$: C, 61.33% H, 5.51%

The mass spectrum of the product (Figure 5 in Appendix I) revealed a spike at m/e of 485, corresponding to the calculated molecular weight of 485.63.

The infra-red spectrum of the compound in nujol (Figure 5 in Appendix II) showed that the N,O-ditosylate derivative had been formed. Had the monotosylate derivative been formed, absorption at approximately 3300 cm.$^{-1}$ would have been present due to the N-H or O-H stretching vibrations of a free amino or hydroxyl group.

F. p-aminophenol-N,O-ditosylate

p-aminophenol hydrochloride and p-toluenesulphonyl chloride were reacted according to the method of Vogel (70) to prepare the ditosylate of p-aminophenol. The crude product was recrystallized from ethyl acetate/petroleum ether (30 - 60). m.p. 170 - 171^{0}C.,

reported in Beilstein, 169°C. from benzene.

calculated for $C_{20}H_{19}NS_2O_5$:	C, 57.54%	H, 4.59%
found for $C_{20}H_{19}NS_2O_5$:	C, 57.36%	H, 4.61%

The mass spectrum of the product (Figure 6 in Appendix I) revealed a spike at m/e of 417, which corresponded to the calculated molecular weight of 417.48.

The infra-red spectrum of this compound in nujol (Figure 6 in Appendix II) showed that the ditosylate, not the monotosylate, derivative had been formed.

II. ANIMAL EXPERIMENTS

A. Collection of Urine and Feces

Male Sprague-Dawley rats (375 - 425 Gm.) were each injected intraperitoneally with an aqueous solution of N-cyclopentylaniline-^{14}C hydrochloride (15 mg. free base/Kg.). The rats were isolated in individual metabolism cages (Acme) designed to separate the urine and feces. Urine was collected at intervals of twenty-four, forty-eight, seventy-two, ninety-six and one-hundred-sixty-eight hours after injection. The urine samples were centrifuged, and the centrifugation pellets washed and re-centrifuged. The supernatant urine and the washings were combined and diluted volumetrically to 25.0 ml. with water. Aliquots (0.1 ml.) were analyzed for radioactivity and the remainder of each solution frozen.

Feces were collected at twenty-four, forty-eight and seventy-two hours after injection and frozen until required for analysis.

B. Analysis of Urine

1. Determination of Urinary Excretion Rate

Aliquots of each urine sample were accurately measured using an Agla micrometer syringe, and scintillation fluid (10 ml.) was added to each. The radioactivity present in each sample was determined by liquid scintillation counting.

2. Chromatography

Aliquots of the twenty-four hour urine (0.1 ml.) and the forty-eight hour urine (1.0 ml.) were streaked over a one-inch strip of Whatman No. 3 MM. chromatographic paper and subjected to ascending chromatography for twenty-four hours in a solvent system consisting of n-butanol/acetone/water (3:0.6:1). The air-dried strips were scanned to determine the number of radioactive peaks present, and the proportion of radioactivity present in each peak was determined by the use of the digital integrator.

Larger quantities of each radioactive metabolite were obtained by streaking Whatman 3 MM. chromatographic papers (eighteen inches wide) with twenty-four hour urine (23 ml.) using a Desaga Automatic sample applicator. The chromatograms were developed for twenty-four hours in n-butanol/acetone/water (3:0.6:1) and portions of each chromatogram were scanned for radioactivity. The areas corresponding to the radioactive metabolites were eluted from the papers with water (200 ml.) and the solutions were frozen and lyophilized. The freeze-dried residues from each radioactive peak were stored in a sulphuric acid dessicator until required for individual analysis of each metabolite.

Portions of each lyophilized residue were spotted on thin layer Silica Gel plates and developed in solvent systems 1, 5 and 6 in order to determine how many radioactive compounds comprised each peak. The air-dried plates were scanned for radioactivity.

3. Reverse Isotope Dilution Studies

i) Whole Urine

Accurately weighed quantities of N-cyclopentylaniline hydrochloride, p-hydroxy-N-cyclopentylaniline hydrochloride and p-aminophenol hydrochloride corresponding to 400 mg. free base in Experiment 1 and 500 mg. free base in Experiment 2 were added to a 20.0 ml. aliquot of twenty-four hour urine of known radioactivity. Concentrated hydrochloric acid (5 ml.) was added and the urine was hydrolyzed for one hour in a Presto pressure cooker at 15 psi. The hydrolyzed urine was adjusted to pH 7.8 with ammonia, then continuously extracted with ether for three hours. The ether layer was dried over anhydrous sodium sulphate, then filtered.

Experiment 1:- The ether extract was evaporated to dryness using a stream of nitrogen and the residue was re-dissolved in a small quantity of absolute alcohol. The alcoholic solution was streaked onto thick layer (1 mm.) chromatographic plates of Silica Gel GF-254 and allowed to develop in a solvent of 5% isopropanol/benzene. The plates were air-dried and the bands corresponding to the free bases N-cyclopentylaniline (Rf=0.78), p-hydroxy-N-cyclopentylaniline (Rf=0.30) and p-aminophenol (Rf=0.10) located under short wavelength (254 nm.) ultra-violet light and scraped off into three

columns. Each free base was eluted from its column with dry ether, and the ethereal solutions were evaporated to dryness using a stream of nitrogen.

Experiment 2:- To the ether extract, a small quantity of Silica Gel for Chromatographic Absorption (British Drug Houses) was added and the ether was removed *in vacuo*. The remaining solid was scraped onto the top of a ten-centimeter column of Silica Gel as above, which was previously activated by heating overnight at 100^0C. The free bases were eluted from the column in an ethanol/benzene mixture in which the percentage of ethanol present was gradually increased from 0% to 15%. The presence of the bases in the eluate was determined by the use of diazotized sulphanilic acid reagent for N-cyclopentylaniline and *p*-hydroxy-N-cyclopentylaniline, and p-dimethylaminobenzaldehyde solution for *p*-aminophenol. Each fraction was reduced to dryness *in vacuo*.

To each of the isolated free bases obtained from Experiment 1 or 2, *p*-toluenesulphonyl chloride (5.0 Gm.; 0.026 moles) and pyridine (10 ml.) were added and the resulting solutions refluxed for one hour then poured over ice-water. The water-mixture was extracted with ether (3 x 10 ml.) and the ether extracts were subsequently washed with dilute hydrochloric acid (2 x 10 ml. portions), 10% w/v sodium carbonate solution (2 x 10 ml. portions) and distilled water (2 x 10 ml. portions). The ether layers were dried over anhydrous sodium sulphate overnight, filtered and the ether removed with a stream of nitrogen. The resulting crude products were recrystallized

to constant specific activity (Tables X and XI) from ethyl acetate/ petroleum ether (30 - 60).

ii) Individual Metabolites

Reverse isotope dilution studies were carried out on the major metabolite (Peak IV as shown in Figure 2). A portion of the lyophilized residue from this peak was dissolved in a known volume of water (10.0 ml.), and an aliquot (0.1 ml.) counted to determine the number of dpm./ml. present in the solution. Another aliquot (5.0 ml., 1.84×10^5 dpm.) was pipetted into a beaker and p-aminophenol hydrochloride (534 mg.) and concentrated hydrochloric acid (5 ml.) were added. The solution was boiled for one hour in a Presto pressure cooker at 15 psi. The hydrolysate was adjusted to pH 6.85 with ammonia and continuously extracted with ether for three hours. The ether layer was dried over anhydrous sodium sulphate, filtered and the ether removed with a stream of nitrogen. The N,O-ditosylate of p-aminophenol was prepared from the free base as described previously and was recrystallized to constant specific activity (Table XIV) from ethyl acetate/petroleum ether.

4. Hydrolysis Studies of Urine

i) Acid Hydrolysis

(a) Whole Urine:- An aliquot of twenty-four urine (1.0 ml.) was hydrolyzed under acidic conditions (five drops of concentrated hydrochloric acid) on a water bath for one hour. The hydrolysate was made alkaline with ammonia, and extracted with ether (5 x 5 ml. portions). The ether extract was reduced to a small volume

using a stream of nitrogen, spotted on thin layer Silica Gel plates and developed in Solvent Systems 1, 2 and 3.

N -cyclopentylaniline and p-hydroxy-N-cyclopentylaniline were simultaneously chromatographed with the ether extract and p-aminophenol was co-chromatographed with a portion of the ether extract. The air-dried plates were scanned for radioactivity and the standards located under ultra-violet light.

The remainder of the ether extract was spotted on Whatman 3 MM. chromatographic paper and co-chromatographed with N-cyclopentylaniline, p-hydroxy-N-cyclopentylaniline and p-aminophenol in a solvent system consisting of n-butanol/acetone/water (3:0.6:1) for twenty-four hours. The air-dried chromatogram was scanned for radioactivity and the standards located by spraying with Gibb's reagent.

An aliquot (0.1 ml.) of the aqueous extract of the hydrolyzed urine was pipetted into a glass counting vial with scintillation fluid (10 ml.) and 0.3 N NCS Solubilizer and counted to determine whether any radioactivity remained in the aqueous phase.

(b) Isolated Metabolites:- The three major metabolites (Peaks III, IV and V, Figure 2) were individually subjected to acid hydrolysis. The lyophilized residue of Peaks III, IV and V were each dissolved in a small volume of water and hydrochloric acid (five drops) was added to each. The solutions were heated on a water bath for one hour, made alkaline with ammonia and ether extracted (3 x 2 ml. portions). The ether extracts were concentrated using a stream of nitrogen and spotted on thin layer Silica Gel plates

with the standards, N-cyclopentylaniline, p-hydroxy-N-cyclopentylaniline, and p-aminophenol. The plates were developed in Solvent Systems 1, 2 and 3, air-dried and scanned for radioactivity. The standards were located under ultra-violet light.

A portion of the ether extract from the Peak IV acid hydrolysate was purified by thin layer chromatography in benzene/methanol/glacial acetic acid (90:16:8). The radioactive peak was located by scanning and was scraped off and eluted from the Silica Gel with ether. This purified ether extract was analyzed by gas-liquid chromatography (GLC) with a Beckman GC-5 gas chromatograph, employing the following conditions: column packing 0.5% amine 220 on 100 - 120 mesh chromasorb G at 165^{0}C., helium as the carrier gas, and a hydrogen flame detector (Figure 8).

ii) β-Glucuronidase Hydrolysis

(a) Whole Urine:- A twenty-four hour urine sample (0.5 ml.) was incubated in pH 6.8 phosphate buffer (1 ml.) with β-glucuronidase solution (1 ml.) on a metabolic shaker for sixteen hours at 37^{0}C. Aliquots (0.4 ml.) of the hydrolyzed urine were streaked over one inch on Whatman 3 mm. chromatographic paper and developed ascendingly in n-butanol/acetone/water (3:0.6:1) for twenty-four hours. The chromatogram was air-dried and scanned for radioactivity.

The remainder of the hydrolysate was made alkaline with ammonia and ether extracted (5 x 5 ml. aliquots). The ether extract was concentrated using a stream of nitrogen, spotted on thin layer Sil-

ica Gel plates and developed in Solvent Systems 1, 2 and 3. N-cyclopentylaniline, p-hydroxy-N-cyclopentylaniline and p-aminophenol were simultaneously chromatographed. The air-dried plates were scanned for radioactivity and the standards located under ultra-violet light.

(b) Isolated Metabolites:- A portion of the lyophilized residue representing the major metabolite (Peak IV, Figure 2) was dissolved in water (10 ml.) and 1.0 ml. aliquots used for the enzyme hydrolysis studies. β-glucuronidase solution (1 ml.) and the appropriate buffer (1 ml.) were added to the aliquot, and the solution was incubated for sixteen hours at 37^0C. on a metabolic shaker.

Hydrolysis of Peak IV was attempted using both bacterial and bovine types of β-glucuronidase in separate incubations. The former enzyme required the use of pH 6.8 phosphate buffer, and the latter, pH 5.0 acetate buffer to ensure optimum activity.

Following incubation, aliquots of the solution were spotted on thin layer Silica Gel plates and developed in Solvent Systems 4, 5 and 6. An aliquot of the solution containing unhydrolyzed Peak IV, and p-aminophenol were spotted as standards. All plates were air-dried and scanned for radioactivity. Ultra-violet light was used to detect the position of p-aminophenol.

The remainder of the solution was made alkaline with ammonia and ether extracted (3 x 3 ml. portions). The ethereal extract was concentrated using a stream of nitrogen, spotted on thin layer Silica

Gel plates, and developed in Solvent Systems 1, 2 and 3. p-aminophenol was chromatographed simultaneously as a standard. The air-dried plates were scanned for radioactivity and p-aminophenol located under ultra-violet light.

iii) Sulphatase Hydrolysis

(a) Whole Urine:- A twenty-four hour urine sample (0.5 ml.) was incubated in pH 5.0 acetate buffer (2 ml.) with sulphatase solution (2 ml.) on a metabolic shaker at 37^0C. for sixteen hours. After incubation an aliquot (0.4 ml.) was spotted over one inch on Whatman 3 mm. chromatographic paper and developed for twenty-four hours in n-butanol/acetone/water (3:0.6:1). The air-dried chromatogram was scanned for radioactivity.

(b) Isolated Metabolites:- An aliquot (1 ml.) of the 10 ml. solution of the lyophilized residue of Peak IV (Figure 2) in water was incubated in pH 5.0 acetate buffer (2 ml.) with sulphatase solution (2 ml.) on a metabolic shaker for sixteen hours at 37^0C. The solution was made just alkaline and aliquots were spotted on thin layer Silica Gel plates and co-chromatographed with the standards p-aminophenol and N-acetyl-p-aminophenol. The plates were developed in Solvent Systems 3, 4, 5 and 6, air-dried and scanned for radioactivity. The standards were located under ultra-violet light.

An aliquot (0.4 ml.) of the hydrolysate was co-chromatographed with N-acetyl-p-aminophenol on Whatman 3 mm. paper for twenty-four hours in n-butanol/acetone/water (3:0.6:1). The air-dried chromatogram was scanned for radioactivity and the N-acetyl-p-aminophenol lo-

cated under ultra-violet light.

A portion of the hydrolysate was chromatographed simultaneously with N-acetyl-p-aminophenol on thick layer (1 mm.) plates of Silica Gel GF-254 plates in benzene/methanol/glacial acetic acid (90:16:8). The plates were air-dried and the band having the same Rf value as N-acetyl-p-aminophenol was located under short-wave ultra-violet light and scraped off. The Silica Gel was extracted with anhydrous ether then removed by filtration, and the ether reduced to a small volume under a stream of nitrogen. This purified extract of the compound released by sulphatase hydrolysis was analyzed by gas-liquid chromatography with a Beckman GC-5 gas chromatograph on a temperature-programmed OV25 column. N-acetyl-p-aminophenol, p-aminophenol and p-aminophenyl sulphate were co-chromatographed as reference standards.

An apparatus was devised to collect the volatile components from the major peaks from the GLC and to determine whether these peaks were radioactive. Preliminary experiments showed that most of the radioactivity was present as a condensate in the collection tubing; therefore a collection apparatus was devised which included a large W tube immersed in ice-water to facilitate complete condensation of the gases from the GLC column. The condensate was washed from the collection tubing with toluene (about 5 ml.) and counted in scintillation fluid (10 ml.) to determine the presence of any radioactive compounds.

C. Analysis of Feces

The feces collected at twenty-four, forty-eight, and seventy-two hours after injection were washed with water, and large food

particles were removed with forceps. The feces were freeze-dried or dried over sulphuric acid, then powdered with a mortar and pestle. Portions of approximately 50 mg. of dried feces were accurately weighed on a Cahn Gram Electrobalance and combusted at 750°C. in the combustion train of a Coleman Carbon-Hydrogen Analyzer. The carbon dioxide produced was passed through a sulphuric acid trap (5 ml.) then collected in the first of two ethanolamine (freshly distilled)/methanol (1:4; 11 ml.) collection tubes. A second combustion was performed on each sample to assure complete combustion.

The sulphuric acid trap was diluted volumetrically to 100 ml. with water and aliquots (0.2 ml.) were added to scintillation fluid (10 ml.) and counted by liquid scintillation to determine whether any of the radioactivity possibly present in the feces was retained in the sulphuric acid moisture trap.

The ethanolamine/methanol traps were diluted volumetrically to 25 ml. with methanol, and aliquots (1 ml.) removed for liquid scintillation counting.

Portions of benzoic acid ^{-14}C of known radioactivity were weighed on the Cahn Gram Electrobalance, combusted at 750°C. and counted in a similar manner to determine the efficiency of recovery of the carbon dioxide ^{-14}C (Table in Appendix III) which was 99.30 $\pm$ 2.06%.

RESULTS AND DISCUSSION

I. PREPARATION OF COMPOUNDS

N -cyclopentylaniline-^{14}C hydrochloride was synthesized as described in "Methods", and was recrystallized to constant specific activity as shown in Table IV.

TABLE IV: RECRYSTALLIZATION DATA FOR N-CYCLOPENTYLANILINE-^{14}C HYDROCHLORIDE

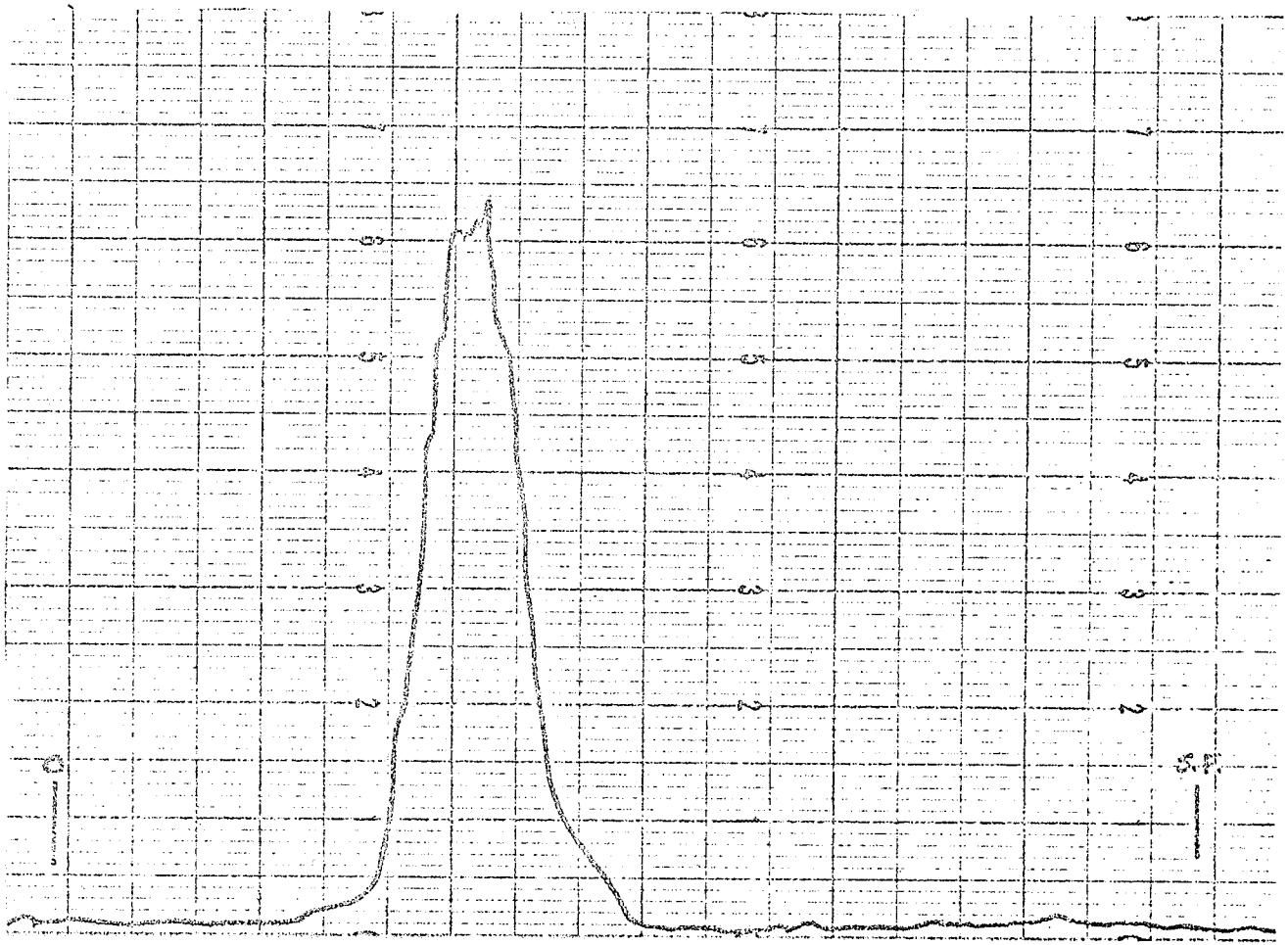
Recrystallization Number	Recrystallization Solvent	Specific Activity (dpm.x10^5/mg.)
2	anhydrous ethanol/ethyl acetate	6.907
3	" " " "	6.887
4	" " " "	6.797
5	" " " "	6.779

The radioactive product was co-chromatographed, as the free base in petroleum ether (30 - 60)/benzene (2:3) with N-cyclopentylaniline (Rf = 0.36, Figure 1).

II. ANIMAL EXPERIMENTS

The details of the administration of N-cyclopentylaniline-^{14}C for these metabolic studies are presented in Table V. All excretion

FIGURE 1: THIN LAYER RADIOCHROMATOGRAPHIC SCAN OF N-CYCLOPENTYLANILINE-^{14}C.



Solvent: petroleum ether (30 - 60)/benzene (2:3). Count rate = 1000 cpm. Scan speed = 60 cm./hr.

data and other results were determined from the amount of radioactivity administered to each rat as indicated below.

TABLE V: ADMINISTRATION OF N-CYCLOPENTYLANILINE-^{14}C HYDROCHLORIDE TO RATS

Rat	1	2	3	4	5	6
Weight (Gm.)	375	390	380	420	425	420
Dose (μm/Kg.)	93.86	94.10	93.95	93.82	93.78	*64.79
Dose (mg. free base)	5.67	5.90	5.75	6.35	6.43	* 4.39
Radioactivity injected (dpm. x 10^6)	4.71	4.92	4.79	5.28	5.34	3.65

*Rat 6 did not receive a full dose of 15 mg./Kg.

A. Analysis of Urine

1. Rate of Urinary Excretion

The rate of excretion of radioactive metabolites in the urine for rats 1, 2 and 3 is presented in Table VI, and for rats 4, 5 and 6 in Table VII. The latter is presented separately since these rats were much older and the results obtained from them rather inconsistent.

TABLE VI: URINARY EXCRETION DATA - RATS 1, 2 AND 3*

Time Interval	% Excretion of Radioactivity in Rat			Average (%)
	1	2	3	
0 - 24 hours	75.94	75.78	74.19	73.30
24 - 48 hours	1.03	1.23	2.17	1.48
48 - 72 hours	0.22	0.51	0.30	0.34
72 - 96 hours	0.13	0.17	0.11	0.14
96 - 168 hours	0.18	**	0.07	0.13
Total Excretion in one week	77.50	77.69	76.84	77.39

*Expressed as a percentage of the administered dose.

**No urine was produced.

The results included in Table VI indicate that urinary excretion of radioactive components was at a maximum in the first twenty-four hours after injection of N-cyclopentylaniline-^{14}C hydrochloride. An average of 75.30% of the administered dose was accounted for in this interval, and 2.09% more was recovered during the remainder of the week. The amount of radioactivity recovered decreased over each time interval; however, it was still possible to detect radioactivity in the urine after one week. An average of 22.61% of the administered radioactivity was not accounted for in the urine.

TABLE VII: URINARY EXCRETION DATA - RATS 4, 5 AND 6*

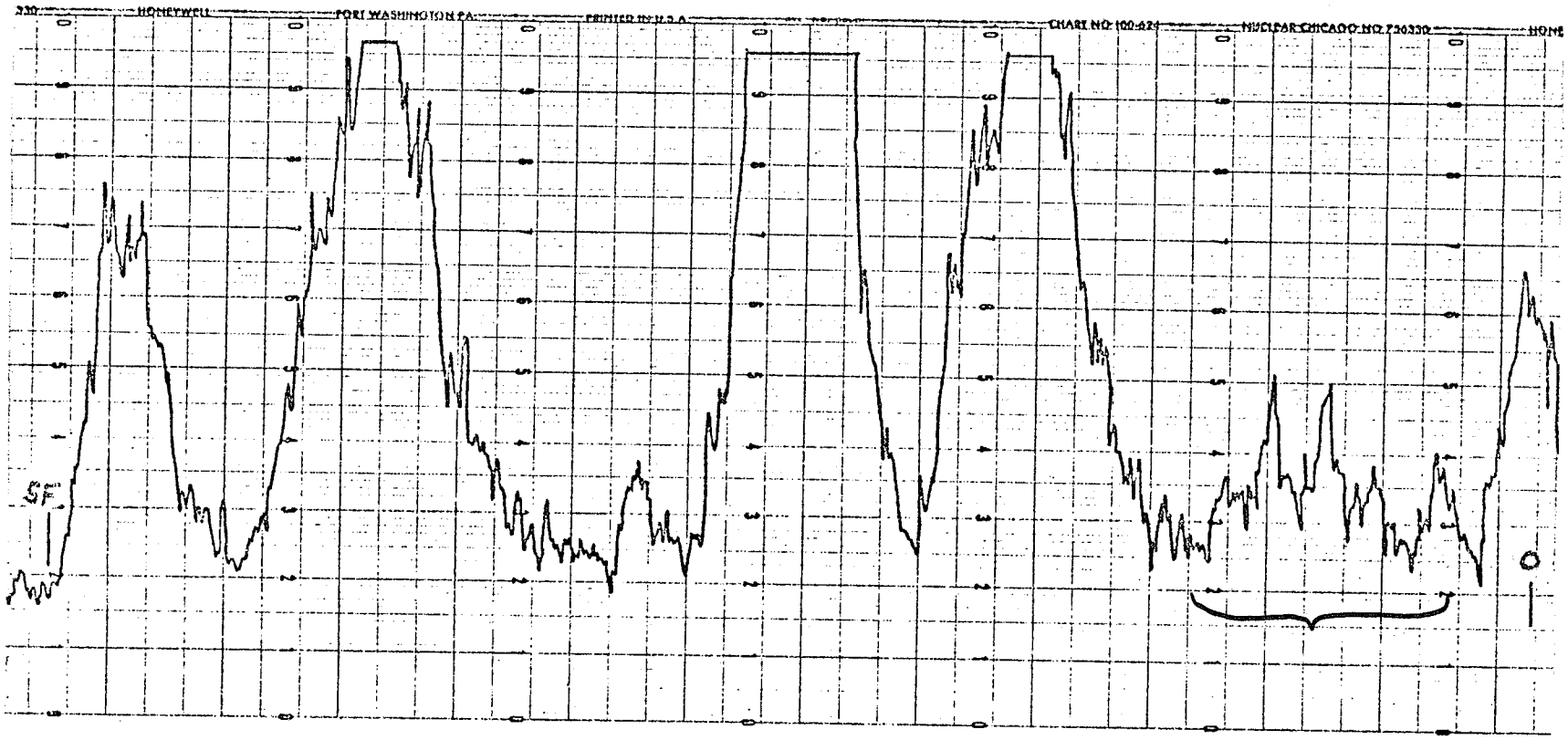
Time Interval	% Excretion of Radioactivity in Rats		
	4	5	6
0 - 24 hours	71.50	60.60	52.80
24 - 48 hours	1.70	7.90	5.40
Total Excretion in 48 hours	73.20	68.50	58.20

*Expressed as a percentage of administered dose.

2. Chromatography

A typical radiochromatographic scan of an aliquot of twenty-four hour urine from a rat dosed with N-cyclopentylaniline-^{14}C is shown in Figure 2. Six areas of radioactivity were evident in the scans of twenty-four hour urine, and these areas were designated as Peaks I through VI. For rats 1, 2 and 3, the relative proportions of radioactivity in each peak were consistent (Table VIII). Peaks III, IV and V together accounted for almost 80% (78.4%) of the radioactivity

FIGURE 2: A TYPICAL RADIOCHROMATOGRAPHIC SCAN OF TWENTY-FOUR HOUR URINE FROM A RAT DOSED WITH N-CYCLOPENTYLANILINE $^{-14}$C. HCl



Peak No.	VI	V	IV	III	II	I
Rfx100	94	78	49	34	17	0
*%	5.7	16.1	43.5	18.8	9.5	5.9

This scan was obtained by chromatographing a 0.1 ml. aliquot of twenty-four hour urine from rat 1. Scan speed = 60 cm./hr. Count rate = 150 cpm. *Average of values from rats 1, 2 and 3. Solvent = n=butanol/acetone/water (13:0.6:1).

in the twenty-four hour urine. Peak IV alone constituted almost half of the radioactivity (43.5%) present in twenty-four hour urine.

The radiochromatographic scans obtained from rats 4, 5 and 6 showed little evidence of Peak VI, but whether this difference was due to the difference in age of the two groups of rats was not determined.

A scan of an aliquot of forty-eight urine (Figure 3) revealed that the major metabolite found in twenty-four hour urine (Peak IV) comprised most of the activity in the forty-eight hour urine. Peaks I, III and VI found in twenty-four hour urine were negligible in the forty-eight hour urine, while there still remained a trace of Peak V and Peak II appeared to have increased.

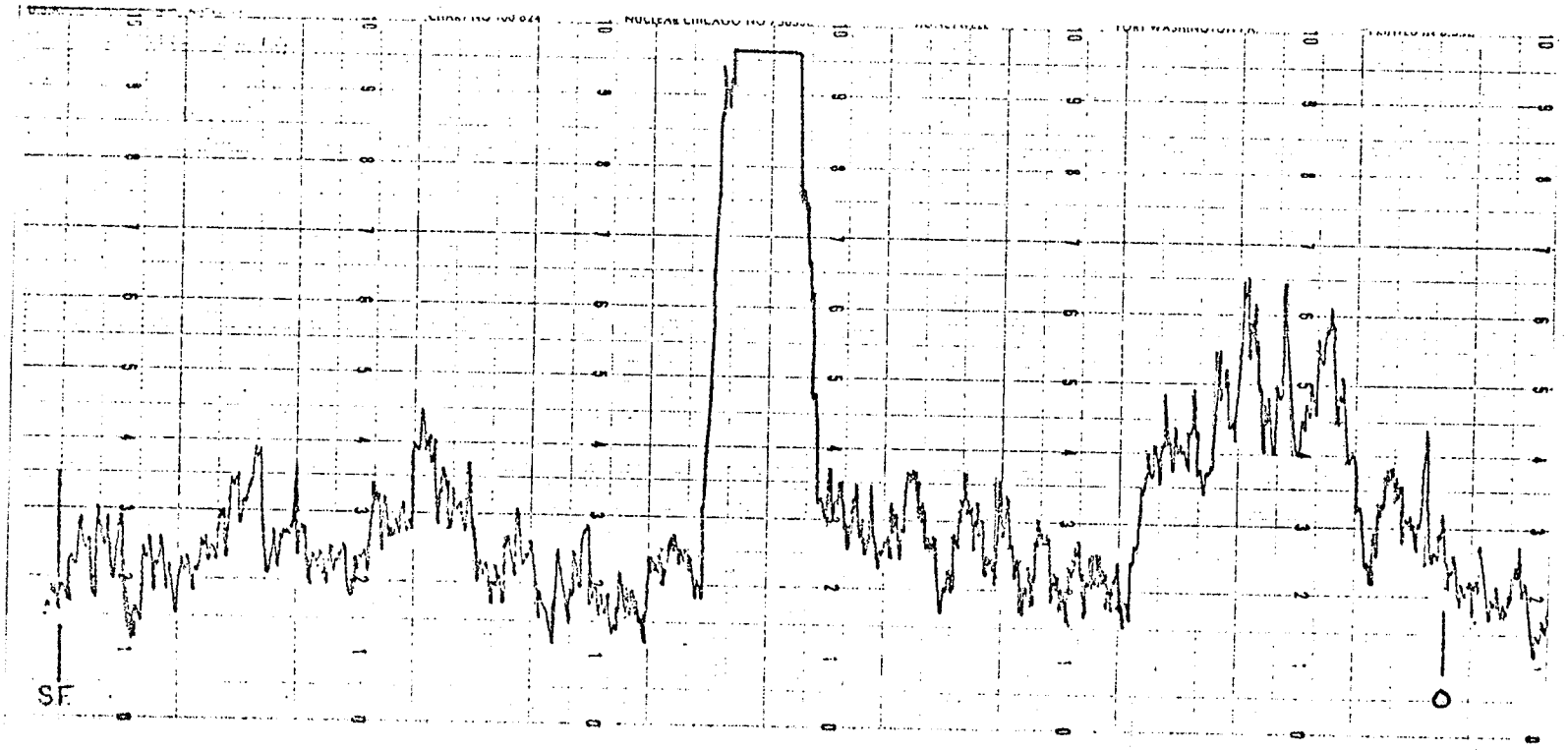
TABLE VIII: DISTRIBUTION OF RADIOACTIVITY IN TWENTY-FOUR HOUR URINE

Peak No.	% of 24-hour Activity in Each Peak*			Average
	Rat 1	Rat 2	Rat 3	
I	4.3	6.4	7.1	5.9
II	8.9	9.6	10.1	9.5
III	17.7	17.8	20.9	18.8
IV	44.5	44.7	41.3	43.5
V	17.9	15.1	15.4	16.1
VI	6.6	6.3	4.2	5.7
Total	99.9	99.9	99.0	99.5

*Determined by integration of radiochromatographic scans using digital integrator.

Thin layer chromatography of portions of the lyophilized residue from each individual peak revealed that the six peaks represented at

FIGURE 3: A RADIOCHROMALOGRAPHIC SCAN OF FORTY-EIGHT HOUR URINE FROM A RAT DOSED WITH N-CYCLOPENTYLANILINE-^{14}C HCl



Peak No.	V	IV	II
Rfx100	72	48	17
*%	4.8	51.6	43.7

This scan was obtained by chromatographing a 1.0 ml. aliquot of forty-eight hour urine rat 3. Scan speed = 60 cm./hr. Count rate = 100 cpm. Solvent = n-butanol/acetone/water (3:0.6:1).

least ten different radioactive metabolites. Peaks I, II, III and IV each appeared to be composed of at least two metabolites. Peak IV appeared to contain only one radioactive metabolite, since it produced a single, sharp peak in all solvent systems. Peak VI could not be detected after chromatography due to the small quantities available for spotting.

3. Reverse Isotope Dilution Studies

The data for reverse isotope dilution studies using urine is summarized in Table IX.

a) N-Cyclopentylaniline:- This suspected metabolite was isolated as the monotosylate derivative, which yielded only background radioactivity when counted, indicating that if any parent compound was excreted in the twenty-four hour urine it was present in quantities too small to be detected by this method.

b) p-Hydroxy-N-Cyclopentylaniline:- The presence of p-hydroxy-N-cyclopentylaniline as a metabolite was confirmed by isolating the radioactive ditosylate derivative. This metabolite accounted for 14.89% of the radioactive excretion products in the twenty-four hour urine, and 8.80% of the administered radioactivity (Table X) when calculated as an average of two trials.

TABLE X: RECRYSTALLIZATION DATA FOR p-HYDROXY-N-CYCLOPENTYLANILINE-N,O-DITOSYLATE

Experiment Number	Specific activity (dpm./mg.) after Recrystallization						Dpm. in Metabolite	% of Activity Administered
	2	3	4	5	6	7		
1	385	379	383			382	418,655	8.60
2			315	304	311	310	426,222	9.00

Average 8.80%

TABLE IX: REVERSE ISOTOPE-DILUTION FOR METABOLITES OF N-CYCLOPENTYLANILINE-^{14}C IN THE TWENTY-FOUR HOUR URINE OF RATS

Suspected Metabolite	Experiment Number	Material Added to Urine		Isolated Derivative		Dpm. x 10^6 in		% Radioactivity in Metabolite	
		Cmpd.	Qtty. (mg.)	Cmpd.	dpm./mg.	dose adm.	24-hr. Urine	% dose adm.	% 24-hour Urine
NCPA	1	NCPA·HCl	590.4	NCPA-tos	B.G*	4.92	2.91	N.D.**	N.D.**
p-OH NCPA	1	p-OH NCPA·HCl	482.3	p-OH NCPA N,O-ditos	382	4.92	2.91	8.60	14.41
	2	p-OH NCPA·HCl	605.0	p-OH NCPA N,O-ditos	310	4.71	2.79	9.00	15.27
PAP	1	PAP·HCl	533.6	PAP-N,O-ditos	974	4.92	2.91	30.20	51.31
	2	PAP·HCl	665.9	PAP-N,O-ditos	815	4.71	2.79	33.00	55.89

*B.G. - background counts only

**N.D. - below the level of detection

c) p-Aminophenol:- The metabolite, p-aminophenol, was determined to be present by isolating the radioactive ditosylate derivative. p-Aminophenol comprised 53.5% of the twenty-four hour urinary metabolites of N-cyclopentylaniline-^{14}C, and 31.60% of the dose administered (Table XI).

TABLE XI: RECRYSTALLIZATION DATA FOR p-AMINOPHENOL-N,O-DITOSYLATE

Experiment Number	Specific Activity (dpm./mg.) after Recrystallization					Dpm. in Metabolite	% of Activity Administered
	2	3	4	5	Aver.		
1		960	990	973	974	1,490,540	30.20
2	*815					1,556,315	33.01
						Average	31.60%

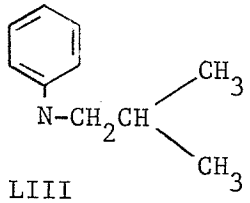
*Only two recrystallizations could be performed due to lack of sufficient compound.

The presence of p-hydroxy-N-cyclopentylaniline and p-aminophenol as urinary metabolites of N-cyclopentylaniline indicates that this compound has undergone aromatic hydroxylation and N-dealkylation during its metabolic transformation, with N-dealkylation representing the major pathway.

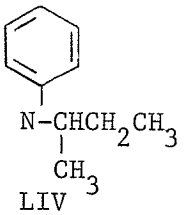
The high degree of N-dealkylation occurring during the metabolism of this compound is unexpected, since N-dealkylation of substituted anilines seems to be inversely related to intramolecular hindrance at the point of dealkylation. That is, side-chains with branching at the *α-carbon hinder dealkylation to a greater extent than straight side-chains and those with branching at the **β-carbon atom. Metabolic studies of N-isobutylaniline (5), N-secondarybutylaniline (3)

*carbon atom next to nitrogen
**carbon atom one removed from nitrogen

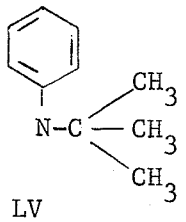
and N-tertiarybutylaniline (7) illustrate this relationship. Dealkylation to produce p-aminophenol occurs to the extent of 55.7% of the twenty-four hour metabolites with N-isobutylaniline (LIII), where branching is on the β-carbon atom.



LIII



LIV

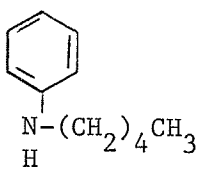


LV

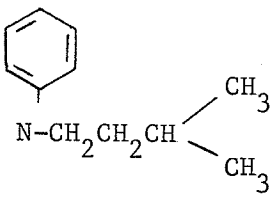
Dealkylation is reduced to 13% of twenty-four hour metabolites with N-secondarybutylaniline (LIV) where branching occurs on the α-carbon, and is almost eliminated (1.9%) with N-tertiarybutylaniline (LV) where there is extensive branching from the α-carbon atom.

The cyclopentyl ring of N-cyclopentylaniline embodies a type of α-branching similar to that present in N-secondarybutylaniline with the exception that the two branches are joined to produce a planar, five-membered ring. One would expect N-dealkylation to represent only a minor metabolic pathway, yet it accounts for over 50% of the twenty-four hour metabolites.

For anilines substituted with side-chains possessing the same number of carbon atoms as N-cyclopentylaniline, e.g. n-pentylaniline (LVI) and N-isopentylaniline (LVII, N-dealkylation occurs to a lesser extent (21% and 19% respectively) due to the length of the side-chain (8).



LVI



LVII

N-cyclopentylaniline does not conform to this pattern. A possible explanation for the high degree of dealkylation of an α-branched side-chain, as exhibited by N-cyclopentylaniline, is based on the principle of steric hindrance. Unlike the lengthy straight-chain or α-branched chain substituents which are free to rotate about the nitrogen atom and obstruct dealkylation processes, the five-membered ring of N-cyclopentylaniline is bound in a rigid structure which is planar, and therefore would offer less resistance to attack by dealkylating enzymes. The low extent of dealkylation observed with N-cyclohexylaniline (71), due possibly to the blocking of dealkylation by the non-planar cyclohexyl ring offers further proof for this theory.

4. Hydrolysis Studies

a) Acid Hydrolysis Studies:- Thin layer chromatography of the ether extract of the basified hydrolysate obtained from acid-hydrolyzed urine resolved two ether-soluble metabolites (Figure 4). The major metabolite co-chromatographed with p-aminophenol in three solvent systems (Table XII), and the smaller radioactive peak had the same Rf value as p-hydroxy-N-cyclopentylaniline in three solvent systems.

TABLE XII: THIN LAYER CHROMATOGRAPHIC RESULTS OF ETHER-EXTRACTABLE ACID HYDROLYZED URINE

Component	Rfx100 in solvents			Colour Reaction
	1	2	3	
p-aminophenol	74	12	17	yellow with pdab*
N-cyclopentylaniline	origin	80	97	blue with Gibbs
p-hydroxy-N-cyclo-pentylaniline	18	40	36	brown with Gibbs
Ether-extractable radioactivity** a)	74	12	17	yellow with pdab
b)	18	39	35	not detectable with Gibbs

*pdab - p-dimethylaminobenzaldehyde reagent
**determined by scanning

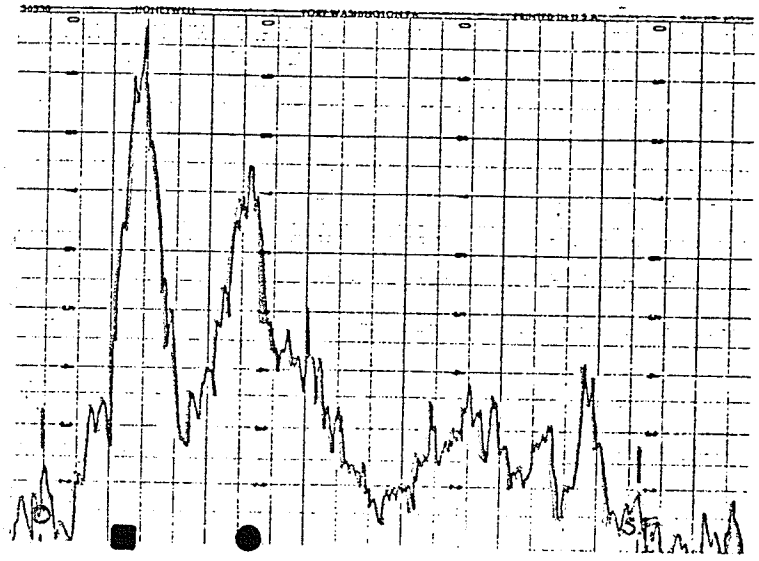


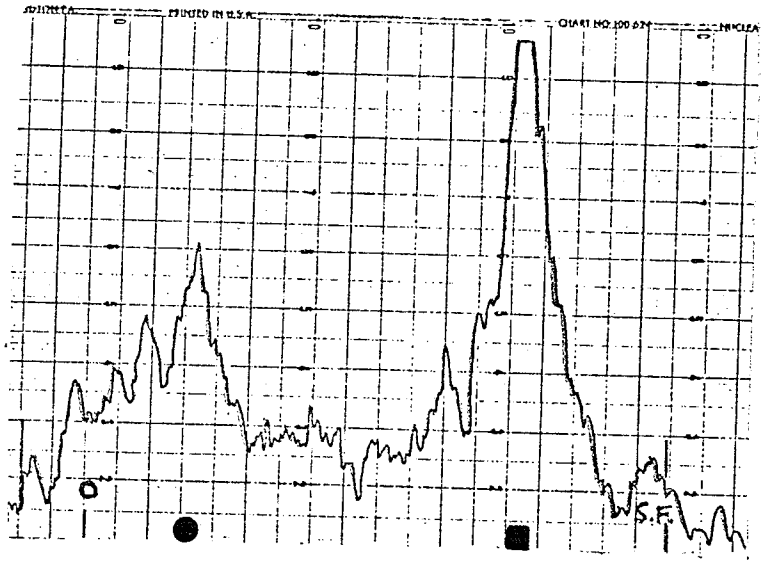
FIGURE 4:

Thin Layer Chromatography of Ether Extract after Acid Hydrolysis

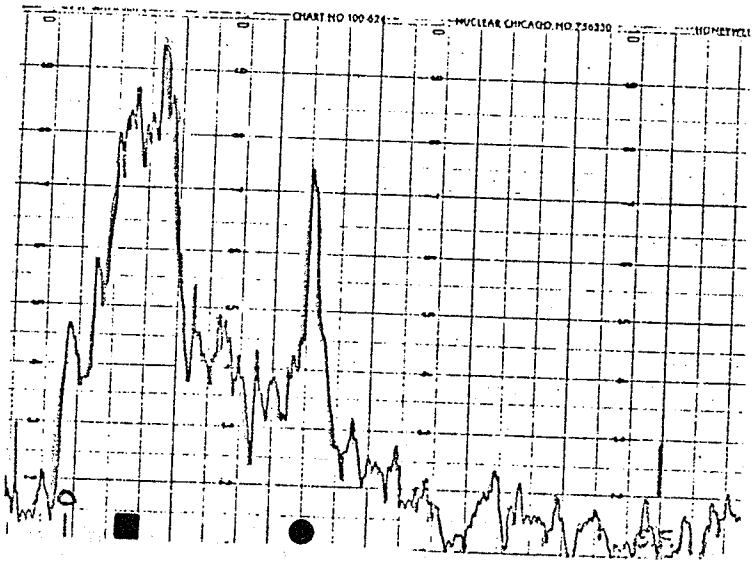
■ PAP reference

● p-OH NCPA reference

Solvent #3.
120 cm./hr. 100 cpm.



Solvent #1
120 cm./hr. 100 cpm.



Solvent #2
120 cm./hr. 100 cpm.

Paper chromatography of the ether extract in n-butanol/acetone/water (3:0.6:1) revealed a possibility of three ether-extractable metabolites, two of which co-chromatographed with the standards p-aminophenol and p-hydroxy-N-cyclopentylaniline (Table XIII).

TABLE XIII: PAPER CHROMATOGRAPHIC RESULTS OF ETHER-EXTRACTABLE ACID HYDROLYZED URINE

Component	Rfx100	Colour Reaction with Gibbs
p-aminophenol	80	purple-brown
N-cyclopentylaniline	93	blue
p-hydroxy-N-cyclopentylaniline	89	brown
Ether-extractable radioactivity* a)	80	purple-brown
b)	72	not detectable
c)	89	not detectable

*determined by scanning

Acid hydrolysis of twenty-four hour urine confirmed, by thin layer and paper chromatography, the presence of p-aminophenol and p-hydroxy-N-cyclopentylaniline as metabolites.

Paper chromatography revealed that the major urinary metabolites were acid-hydrolyzed conjugates, since after acid hydrolysis all radioactivity was located near the solvent front, and the normal pattern of radioactivity in twenty-four hour urine was abolished.

Liquid scintillation counting of an aliquot of the aqueous extract, obtained after ether extraction of the basified acid hydrolysate, revealed the presence of radioactivity in the aqueous fraction. However, no reproducible results could be obtained due to the rapid dedetioration of the hydrolysate extract in scintillation fluid, resulting in widely fluctuating counting data.

b) β-Glucuronidase Hydrolysis Studies:- Incubation of an aliquot of urine with β-glucuronidase produced no noticeable change in the distribution of radioactivity after paper chromatography* (Figure 5 showing urine before incubation with β-glucuronidase and Figure 6 showing urine after incubation with the enzyme).

Thin layer chromatography of the ether extract of the basified hydrolysate obtained from β-glucuronidase-incubated urine revealed no ether-extractable radioactivity when the plates were scanned.

These results suggest that none of the six peaks representing metabolites of N-cyclopentylaniline were present in the urine as glucuronic acid conjugates, since no free bases were detected after incubation and the normal distribution of radioactivity in the urine remained the same.

c) Sulphatase Hydrolysis Studies:- The normal distribution of radioactivity in twenty-four hour urine was altered during incubation with the enzyme sulphatase (Figure 5 showing urine before incubation with sulphatase and Figure 7 showing urine after incubation with sulphatase). Most of the peaks became more diffuse, probably because of the viscous nature of the urine solution after incubation with sulphatase. Peak III underwent a very noticeable reduction in radioactivity. These results seemed to indicate that some of the urinary metabolites of N-cyclopentylaniline were present as sulphates.

The sulphatase enzyme used was an aryl sulphatase, capable of readily hydrolysing ethereal sulphate conjugates, but its action upon sulphamate conjugates, where the sulphate group links to the molecule

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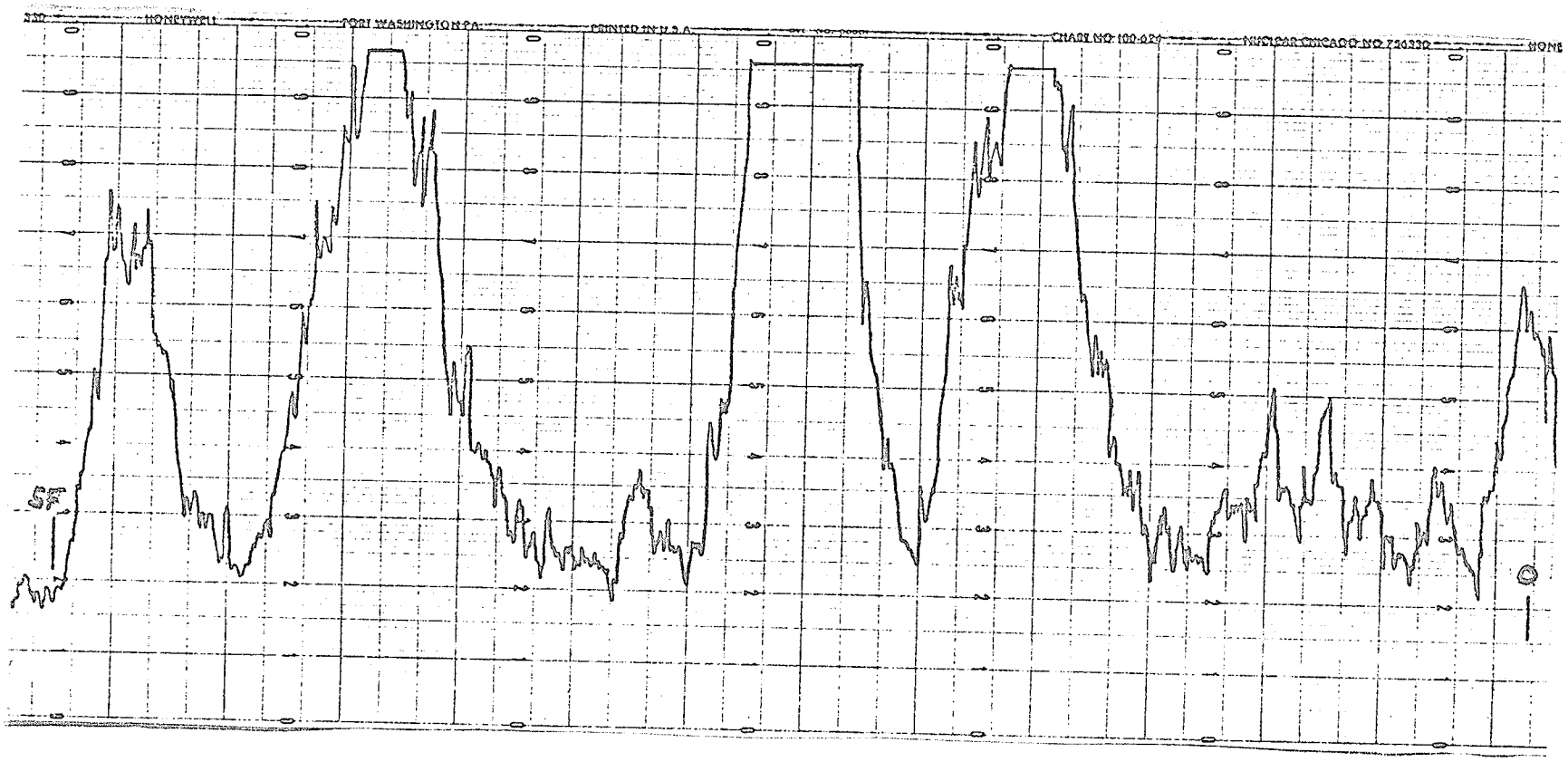
*see footnote to Figure 6.

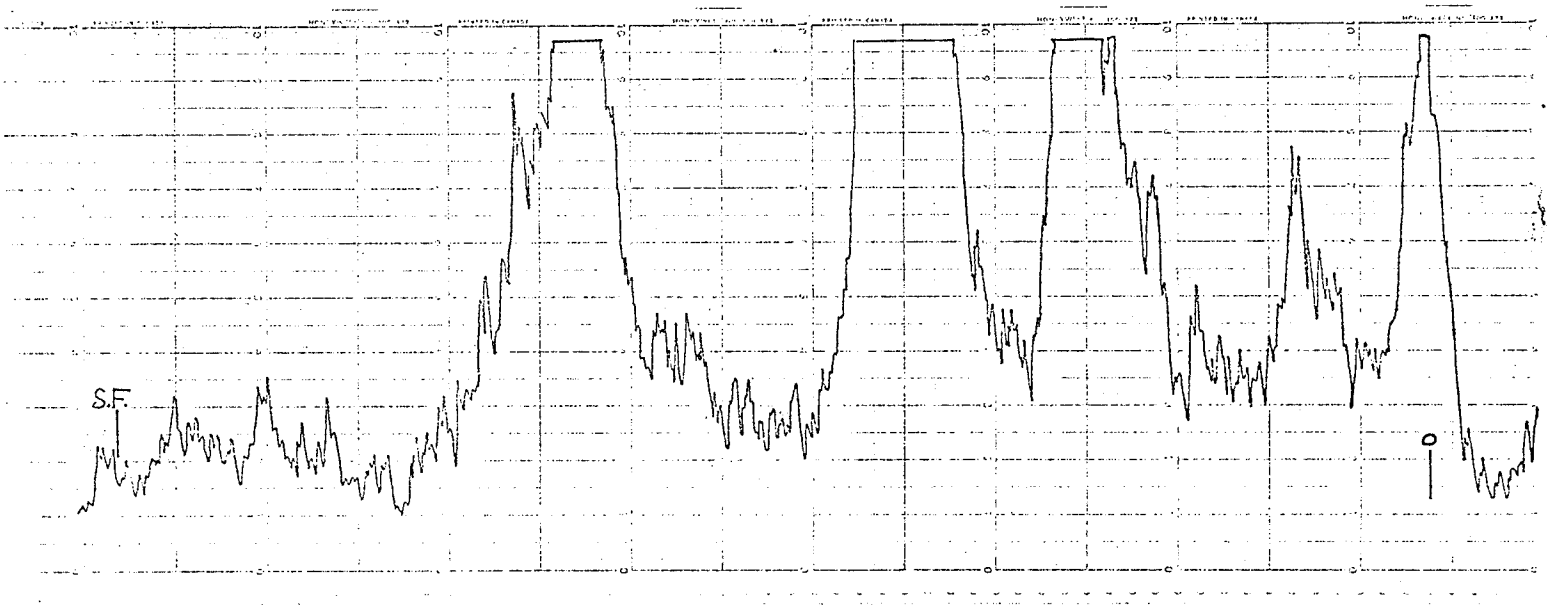
FIGURE 5: RADIOCHROMATOGRAPHIC SCAN OF TWENTY-FOUR HOUR URINE BEFORE HYDROLYSIS



Peak No.	IV	V	IV	III	II	I
Rf x 100	94	78	49	34	17	0
*%	5.7	16.1	43.5	18.8	9.5	5.9

Scan speed = 60 cm/hr. Count rate = 150 cpm. *Average of values from rats 1, 2 and 3. Solvent = n-butanol/acetone/water (3:0.6:1).

FIGURE 6: RADIOCHROMATOGRAPHIC SCAN OF TWENTY-FOUR HOUR URINE AFTER INCUBATION WITH B-GLUCURONIDASE

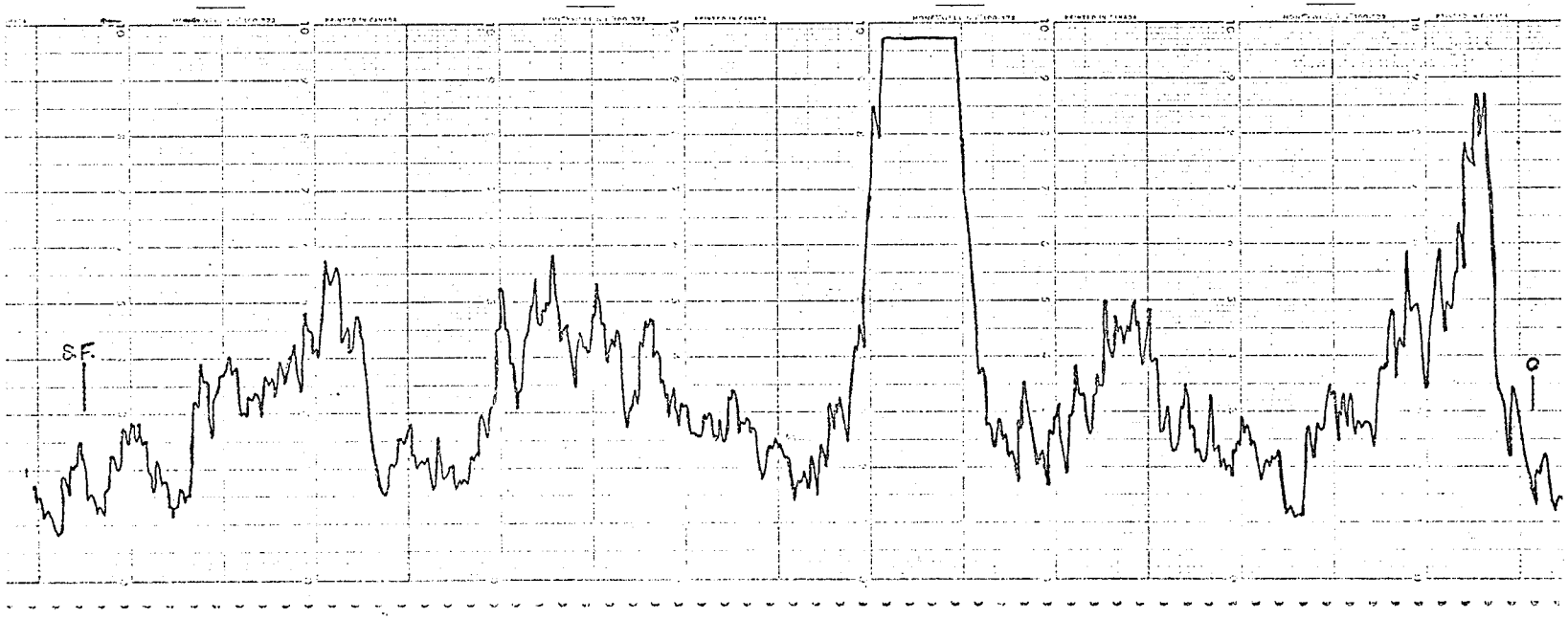


Peak No.	V	IV	III	II	I
Rf	64	43	26	10	origin
%	24.0	36.5	19.6	10.1	9.5

Scan speed = 60 cm./hr. Count rate = 100 cpm. Solvent = n-butanol/acetone/water (3:0.6:1).

The absence of Peak VI could not be definitely attributed to the action of the enzyme, since its presence in urine samples was variable.

FIGURE 7: RADIOCHROMATOGRAPHIC SCAN OF TWENTY-FOUR HOUR URINE AFTER INCUBATION WITH SULPHATASE



Peak No.	VI	V	IV	III	I
Rfx100	89	70	45	30	6
%	9.4	23.0	42.7	9.1	16.6

Scan speed = 60 cm./hr. Count rate = 100 cpm. Solvent = n-butanol/acetone/water (3:0.6:1).

via a nitrogen atom, is weak and hydrolysis occurs only very slowly. Any sulphamate conjugates present in the urine might therefore remain undetected.

B. Partial Analysis of Peaks III and V

a) Acid Hydrolysis Studies:- After acid hydrolysis, Peak III produced one large radioactive peak and a smaller peak, when chromatographed on thin layer Silica Gel plates. Neither peak co-chromatographed with the standards N-cyclopentylaniline, p-hydroxy-N-cyclopentylaniline nor p-aminophenol.

Peak V after acid hydrolysis appeared to be composed of possibly two radioactive metabolites, neither of which co-chromatographed with the standards N-cyclopentylaniline, p-hydroxy-N-cyclopentylaniline, nor p-aminophenol.

Peaks III and V, which together total an average of 34.9% of the activity in twenty-four hour urine (Table VIII) appeared to be composed of some unidentified metabolite(s). Since the metabolites p-aminophenol and p-hydroxy-N-cyclopentylaniline together account for 68.4% of the twenty-four hour urine as determined by reverse isotope dilution studies, peaks III and V appear to account for the one-third of the radioactive metabolites remaining to be identified.

C. Analysis of Peak IV

Since Peak IV represented the major metabolite in both twenty-four hour and forty-eight hour urine, further attempts were made to identify the metabolite by reverse isotope dilution, acid hydrolysis and enzyme hydrolysis of the lyophilized residue of the isolated peak.

a) Reverse Isotope Dilution

Reverse isotope dilution of Peak IV with p-aminophenol indicated that 84.4% of this peak was comprised of the radioactive metabolite p-aminophenol (Table XIV).

TABLE XIV: REVERSE ISOTOPE DILUTION OF PEAK IV FOR p-AMINOPHENOL

Experiment Number	Specific Activity (dpm./mg.) of Recrystallization Number			Dpm. in Metabolite	% of Peak IV as PAP
	2	3	4		
1	101	100	101	155,633	84.4%

b) Acid Hydrolysis

The ether extract of the basified acid hydrolysate of Peak IV produced a single, distinct radioactive peak which co-chromatographed with p-aminophenol in three solvent systems (Table XV).

Gas chromatography of the ether extract showed that the radioactive metabolite co-chromatographed with p-aminophenol with a retention time of 5'12" (Table XVI and Figure 8).

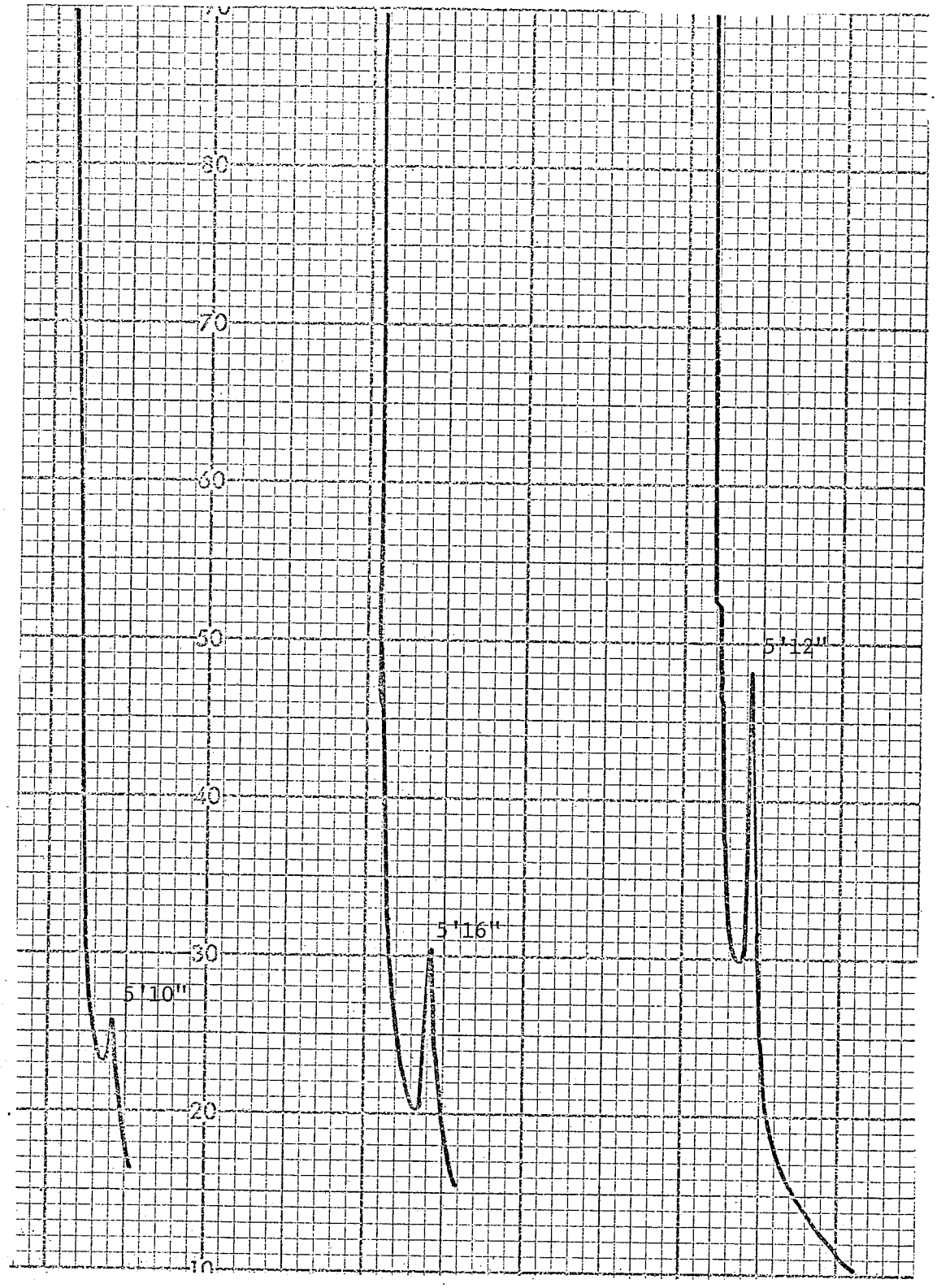
These results concur with the results of reverse isotope dilution which indicate that p-aminophenol constitutes almost all of Peak IV.

TABLE XV: THIN LAYER CHROMATOGRAPHIC RESULTS OF ETHER EXTRACT OF ACID HYDROLYZED PEAK IV

Component	Rfx100 in solvents		
	1	2	3
p-aminophenol	72	8	14
N-cyclopentylaniline	origin	77	96
p-hydroxy-N-cyclopentylaniline	18	38	34
Peak IV*	72	8	14

*determined by scanning

FIGURE 8: GAS CHROMATOGRAPHIC SCAN OF ETHER-EXTRACT OF ACID HYDROLYZED PEAK IV AND p-AMINOPHENOL STANDARD



a) hydrolyzed Peak IV ether extract

b) p-aminophenol

a) and b)

TABLE XVI: GAS CHROMATOGRAPHIC RESULTS OF ETHER EXTRACT OF ACID HYDROLYZED PEAK IV

Component	Retention Time
p-aminophenol	5'16"
Peak IV	5'10"
p-aminophenol & Peak IV	5'12"

c) β-Glucuronidase Hydrolysis

Incubation of Peak IV with both bacterial and bovine β-glucuronidase caused no hydrolysis of the metabolite present, as indicated by thin layer chromatographic results (Table XVII). Thin layer chromatography of the ether extract showed that no free base was released during incubation with enzyme. These results concur with the negative results obtained from incubation of the whole urine with β-glucuronidase, both of which reveal that Peak IV does not appear to be a glucuronic acid conjugate.

TABLE XVII: THIN LAYER CHROMATOGRAPHIC RESULTS AFTER β-GLUCURONIDASE INCUBATION OF PEAK IV

Component	Rf x 100 in Solvent		
	4	5	6
p-aminophenol	92	60	88
Peak IV before incubation*	34	54	55
Radioactive component after incubation*	35	51	57

*determined by scanning

d) Sulphatase Hydrolysis

Incubation of Peak IV with the enzyme sulphatase produced two radioactive peaks of equal size after thin layer chromatography (Table XVIII). One radioactive peak co-chromatographed with N-acetyl-p-aminophenol in four solvent systems, and the second peak chromatographed simultaneously with unhydrolyzed Peak IV. Chromatography of the same solution several weeks later (stored frozen) showed that the radioactivity was found almost exclusively in the peak that co-chromatographed with N-acetyl-p-aminophenol, while the conjugated metabolite was no longer present, indicating that perhaps the conjugate was not completely hydrolyzed by overnight incubation.

TABLE XVIII: THIN LAYER CHROMATOGRAPHIC RESULTS AFTER SULPHATASE INCUBATION OF PEAK IV

Component	Rfx100 in Solvent			
	3	4	5	6
p-aminophenol	26	94	60	88
N-acetyl-p-aminophenol	48	94	89	90
Peak IV before incubation	15	33	56	54
Radioactive Components*				
a)	48	94	89	90
b)	14	34	59	51

*determined by scanning

Paper chromatography of the hydrolysate produced similar results. N-acetyl-p-aminophenol co-chromatographed with the radioactive peak having an Rf = 0.83, and the second peak had an Rf value the same as that of unhydrolyzed Peak IV (Rf = 0.49) in n-butanol/acetone/water (3:0.6:1).

The results of thin layer and paper chromatography suggested that N-acetyl-p-aminophenyl sulphate was the major metabolite of N-cyclopentylaniline. Acid hydrolysis results concurred with this, since acetyl and sulphate conjugates would be removed under conditions of acid hydrolysis to produce the free p-aminophenol that was detected by thin layer chromatography.

N-acetyl-p-aminophenol present as a double conjugate would be a likely metabolite of p-aminophenol, since this compound is known to undergo N-acetylation as a part of its metabolism (37), and the compound N-acetyl-p-aminophenol is not polar enough to be excreted in the urine without further conjugation (37).

The presence of the metabolite N-acetyl-p-aminophenol would most probably be due to N-acetylation of the compound after hydroxylation and dealkylation had occurred. Another possible mechanism for the formation of N-acetyl-p-aminophenol would involve scission of the cyclopentyl ring and removal of three carbon atoms to produce the acetyl moiety; however, this is not a likely reaction since carbon atoms are usually removed in pairs by β-oxidation. Such a reaction could be confirmed or eliminated by labelling the carbon atoms of the cyclopentyl ring with radioactivity and performing similar metabolic studies.

Gas chromatography of the purified extract of Peak IV after sulphatase hydrolysis provided results that conflicted with those obtained in thin layer and paper systems. The compound released by sulphatase hydrolysis produced two peaks which when collected and counted, proved to be radioactive. Neither peak co-chromatographed with N-

acetyl-p-aminophenol. The peaks instead had the same retention times, and co-chromatographed with p-aminophenol and p-aminophenyl sulphate (Table XIX). Unless by some method the metabolite is de-acetylated upon injection into the gas chromatograph due to some element in the hydrolysate, it appears that N-acetyl-p-aminophenyl sulphate is not a metabolite of N-cyclopentylaniline, in spite of the convincing thin layer and paper chromatographic results.

TABLE XIX: GAS CHROMATOGRAPHIC ANALYSES OF PEAK IV HYDROLYSATE

Component	Retention Time
N-acetyl-p-aminophenol	6'42"
p-aminophenol	2'22"
p-aminophenyl sulphate	8'49"
*Radioactive Extract	
a)	2'07"
b)	8'54"

*Peaks collected, counted and determined to be radioactive

The Peak IV metabolite may be some p-aminophenol derivative closely related to the N-acetyl derivative. Incubation with the enzyme sulphatase has shown that it is a sulphate conjugate.

D. Analysis of Feces

Radioactivity in the feces reached a maximum in the interval between twenty-four and forty-eight hours after the intra-peritoneal injection of N-cyclopentylaniline-^{14}C hydrochloride (Table XX).

All of the radioactivity collected was found to be present in the first ethanolamine/methanol collection tube. Neither the sulphuric acid trap nor the second ethanolamine/methanol collection tube

trapped radioactive $^{14}CO_2$.

Combustion of known quantities of benzoic acid-^{14}C indicated that the method used for collection and counting of the $^{14}CO_2$ resulted in a recovery of 99.03± 2.06% of the activity combusted. (Appendix III) All values shown in Table XX are as observed and are not corrected to 100% efficiency.

TABLE XX: FECAL EXCRETION OF RADIOACTIVITY AFTER INJECTION OF N-CYCLOPENTYLANILINE-^{14}C

Trial No.	% of Dose Administered								
	Rat 1			Rat 2			Rat 3		
	0-24 hr.	24-48 hr.	48-72 hr.	0-24 hr.	24-48 hr.	48-72 hr.	0-24 hr.	24-48 hr.	48-72 hr.
1	3.1	6.9	0.40	2.0	12.0	0.30	no feces	14.0	0.10
2	2.8	6.5	0.50	2.1	14.3	0.30		13.3	0.15
3	1.5	6.5	---	---	8.7	---		13.4	---
4	---	6.5	---	---	9.3	---		---	---
Average	2.3	6.6	0.45	2.1	11.1	0.30	0	13.6	0.13
Total Excretion/Rat	9.35			13.5			13.7		
Average Excretion	12.2%								

Dose injected: Rat 1 4.71×10^6 dpm.
Rat 2 4.92×10^6 dpm.
Rat 3 4.79×10^6 dpm.

CONCLUSION

An average of 77.39% of the radioactivity administered was accounted for in urinary excretion, and 12.2% in fecal excretion, accounting for a total of 89.59% of the administered radioactivity. The total of urinary and fecal excretion for each rat is shown in Table XXI.

The results obtained from these metabolic studies suggest that the following metabolic pathway (Figure 9) may account for the metabolic degradation of N-cyclopentylaniline-^{14}C (LVIII).

Aromatic hydroxylation of the aniline nucleus has been demonstrated by the presence of conjugates of p-hydroxy-N-cyclopentylaniline (LIX) in the urine, accounting for 8.80% of the administered radioactivity. The major metabolite, p-aminophenol (LX) (31.60% of the administered dose), was formed by N-dealkylation of the hydroxylated derivative of N-cyclopentylaniline, and appeared as a conjugate or double conjugate in the urine. Conjugation of any of the metabolites with glucuronic acid could not be demonstrated, but conjugation with sulphuric acid was observed. Qualitative analyses of the feces were not performed.

FIGURE 9:

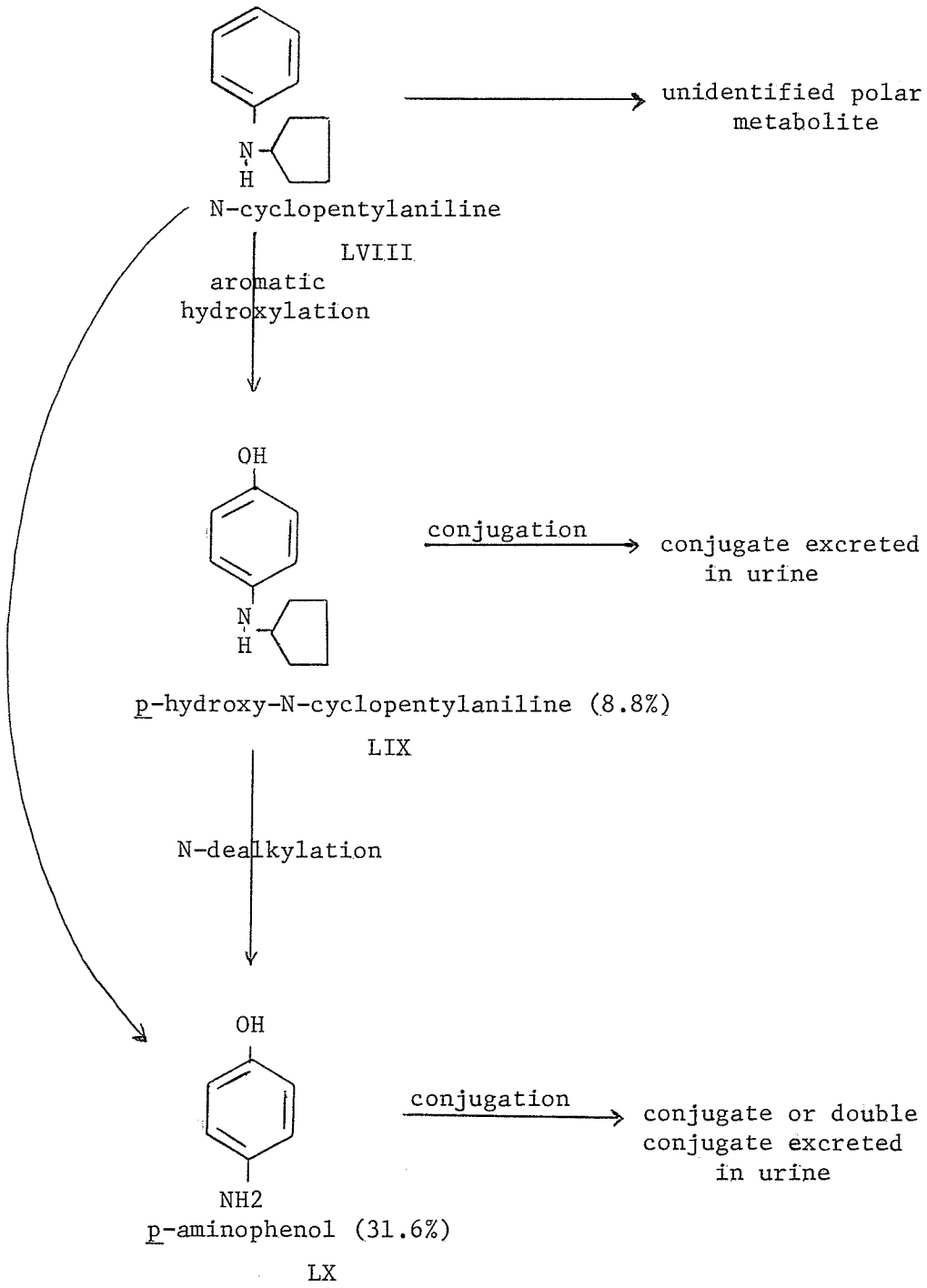

unidentified polar metabolite
N-cyclopentylaniline
LVIII
aromatic hydroxylation
conjugation
conjugate excreted in urine
p-hydroxy-N-cyclopentylaniline (8.8%)
LIX
N-dealkylation
conjugation
conjugate or double conjugate excreted in urine
NH2
p-aminophenol (31.6%)
LX

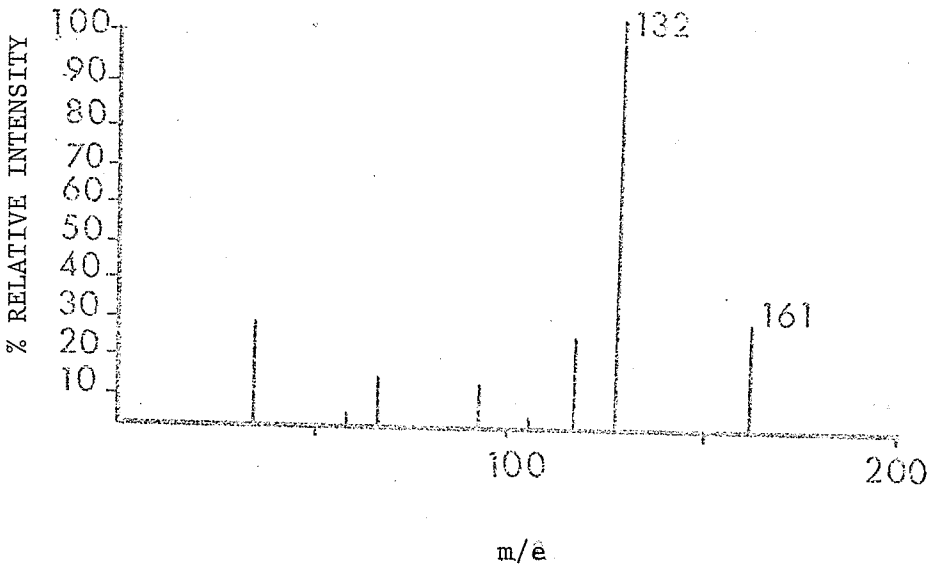
TABLE XXI: SUMMARY OF EXCRETION DATA*

Time Interval	Rat 1			Rat 2			Rat 3		
	Urine	Feces	Total	Urine	Feces	Total	Urine	Feces	Total
0-24 hr.	75.94	2.3	78.24	75.78	2.1	77.88	74.19	0.0	74.19
24-48 hr.	1.03	6.6	7.63	1.23	11.1	12.33	2.17	13.6	15.77
48-72 hr.	0.22	0.45	0.67	0.51	0.30	0.81	0.30	0.13	0.43
72-96 hr.	0.13	-	0.13	0.17	-	0.17	0.11	-	0.11
96-168 hr.	0.18	-	0.18	0.0	-	0.0	0.07	-	0.07
Total/rat	77.50	9.35	86.85	77.69	13.5	91.19	76.84	13.7	90.54

*% of dose administered

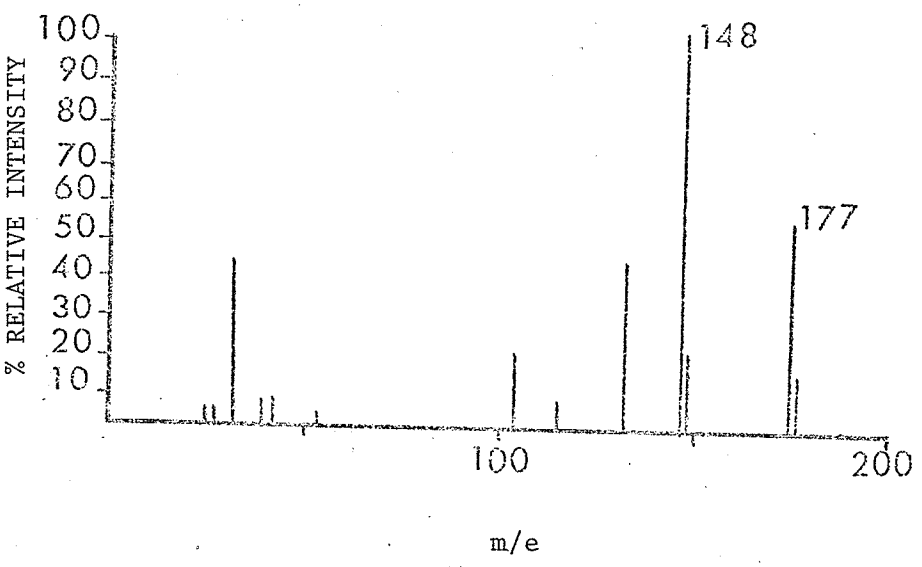
APPENDIX I

FIGURE 1: MASS SPECTRUM OF N-CYCLOPENTYLANILINE HYDROCHLORIDE



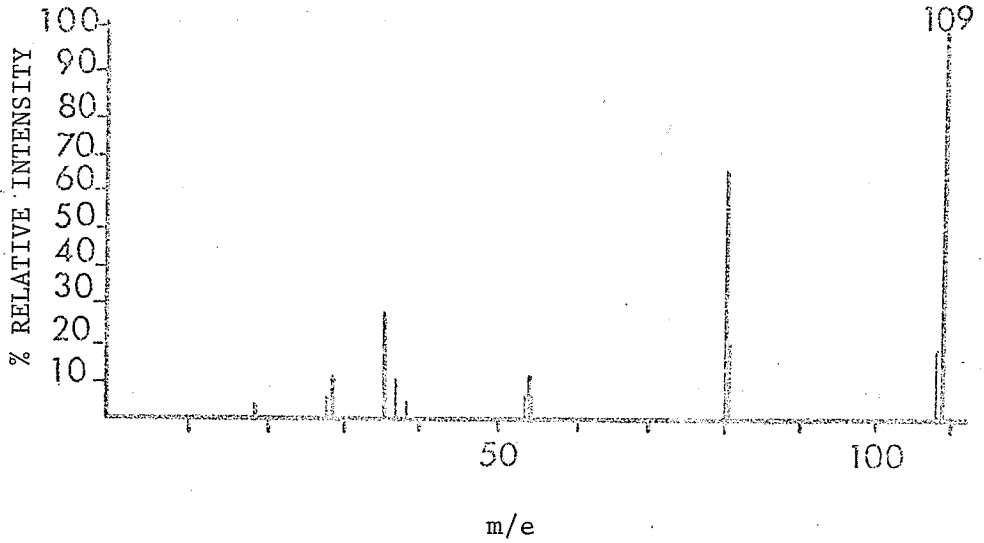
Scan = 10 - 230 amu., sample temp. = 35°C., 100 μA at 70V.

FIGURE 2: MASS SPECTRUM OF p-HYDROXY-N-CYCLOPENTYLANILINE HYDROCHLORIDE



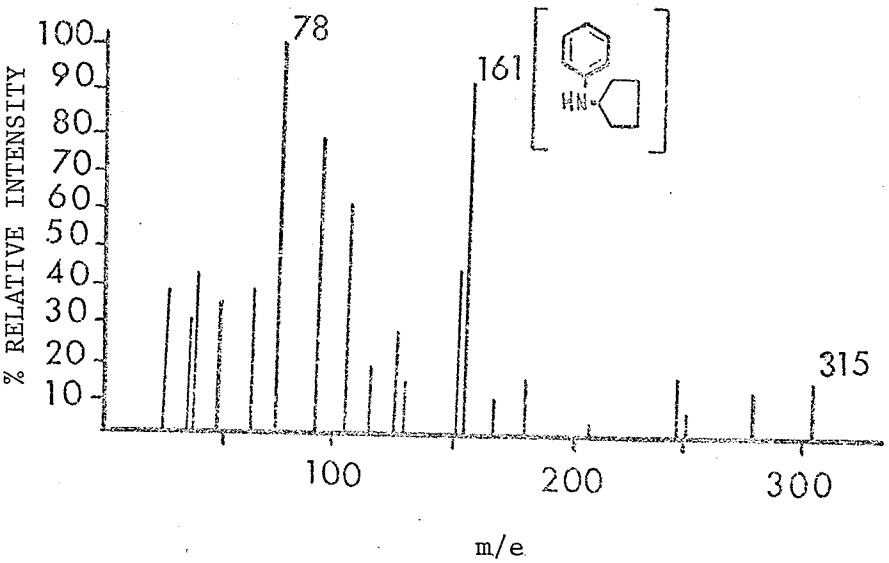
Scan = 10 - 230 amu., sample temp. = 35°C., 200 μA at 70V.

FIGURE 3: MASS SPECTRUM OF p-AMINOPHENOL HYDROCHLORIDE



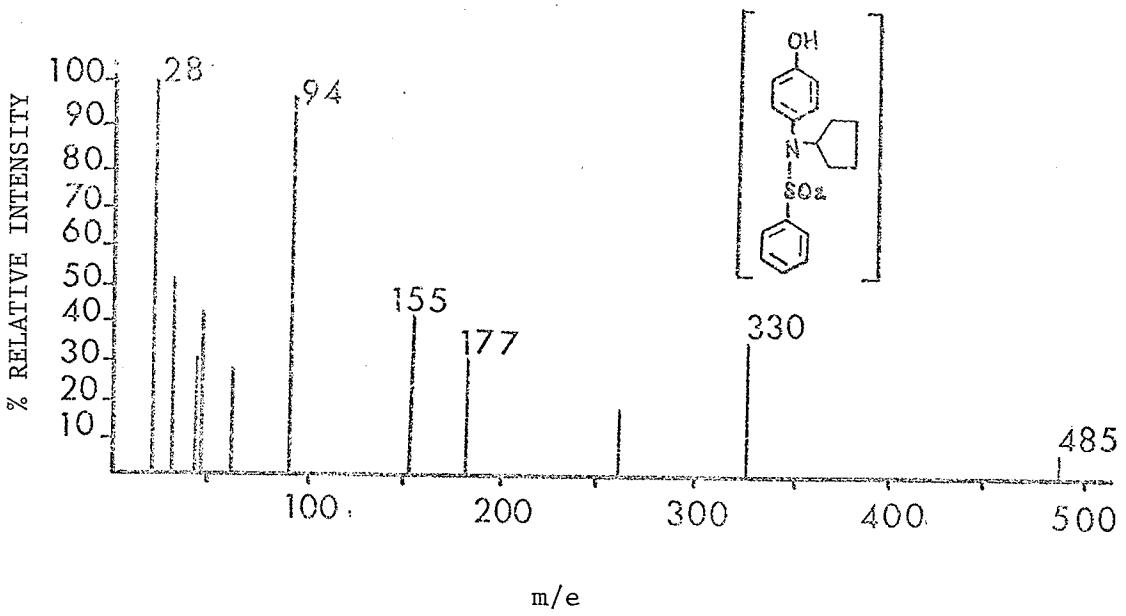
Scan = 10 - 150 amu., sample temp. = 40°C., 200 μA at 80V.

FIGURE 4: MASS SPECTRUM OF N-CYCLOPENTYLANILINE TOSYLATE



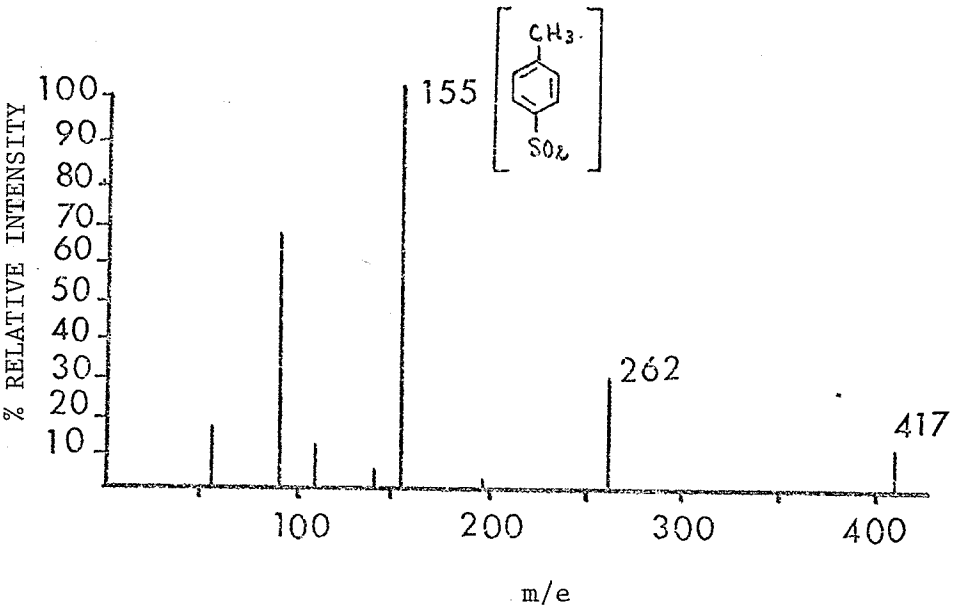
Scan = 10 - 330 amu., sample temp. = 40°C., 25 μA at 70V.

FIGURE 5: MASS SPECTRUM OF p-HYDROXY-N-CYCLOPENTYLANILINE-N,O-DITOSYLATE



Scan = 10 - 500 amu., sample temp. = 100°C., 500 μA at 90V.

FIGURE 6: MASS SPECTRUM OF p-AMINOPHENOL-N,O-DITOSYLATE



Scan = 10 - 450 amu., sample temp. = 70°C., 500 μA at 80V.

APPENDIX II

FIGURE 1: INFRA-RED SCAN OF N-CYCLOPENTYLANILINE HYDROCHLORIDE IN NUJOL

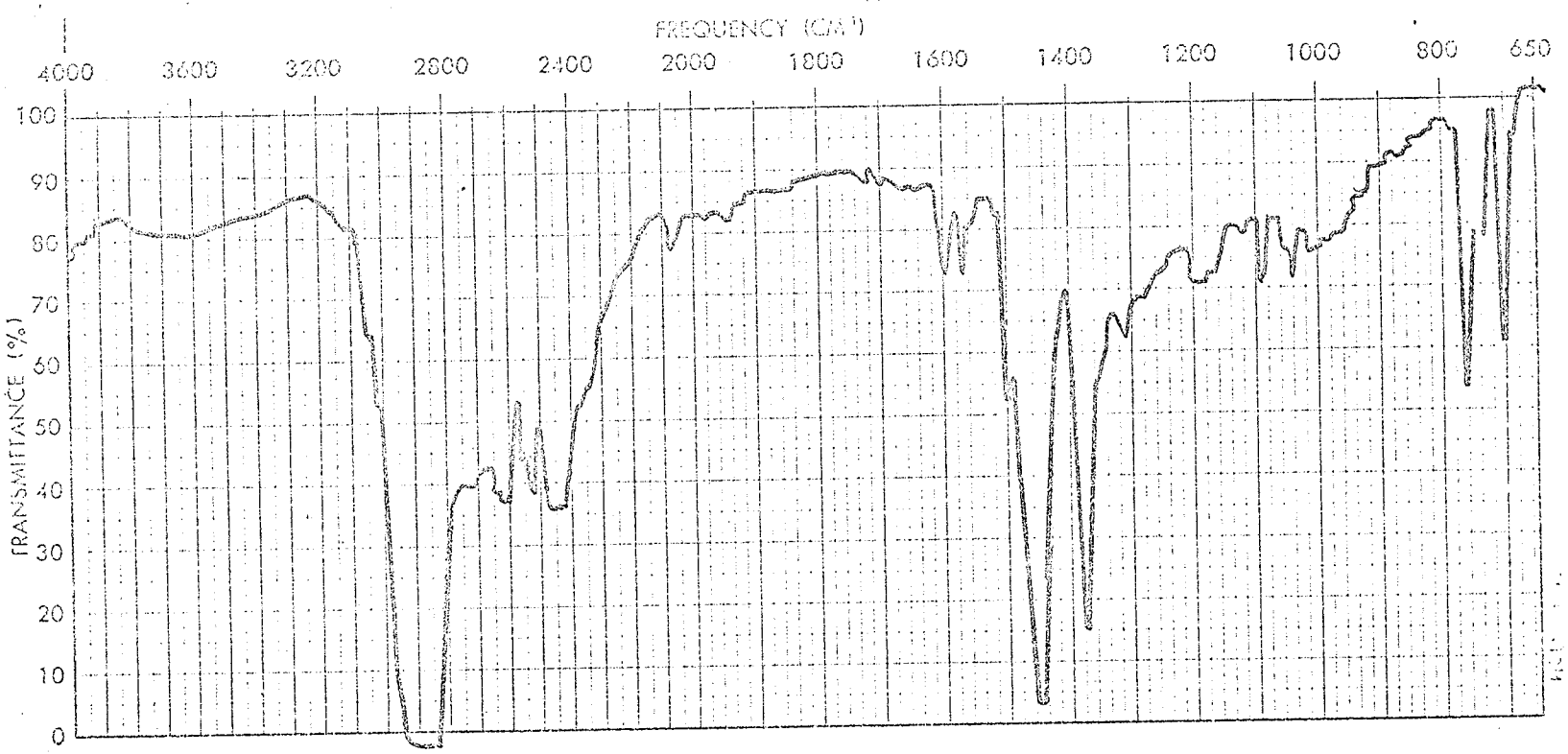


FIGURE 2: INFRA-RED SCAN OF p-HYDROXY-N-CYCLOPENTYLANILINE HYDROCHLORIDE IN NUJOL

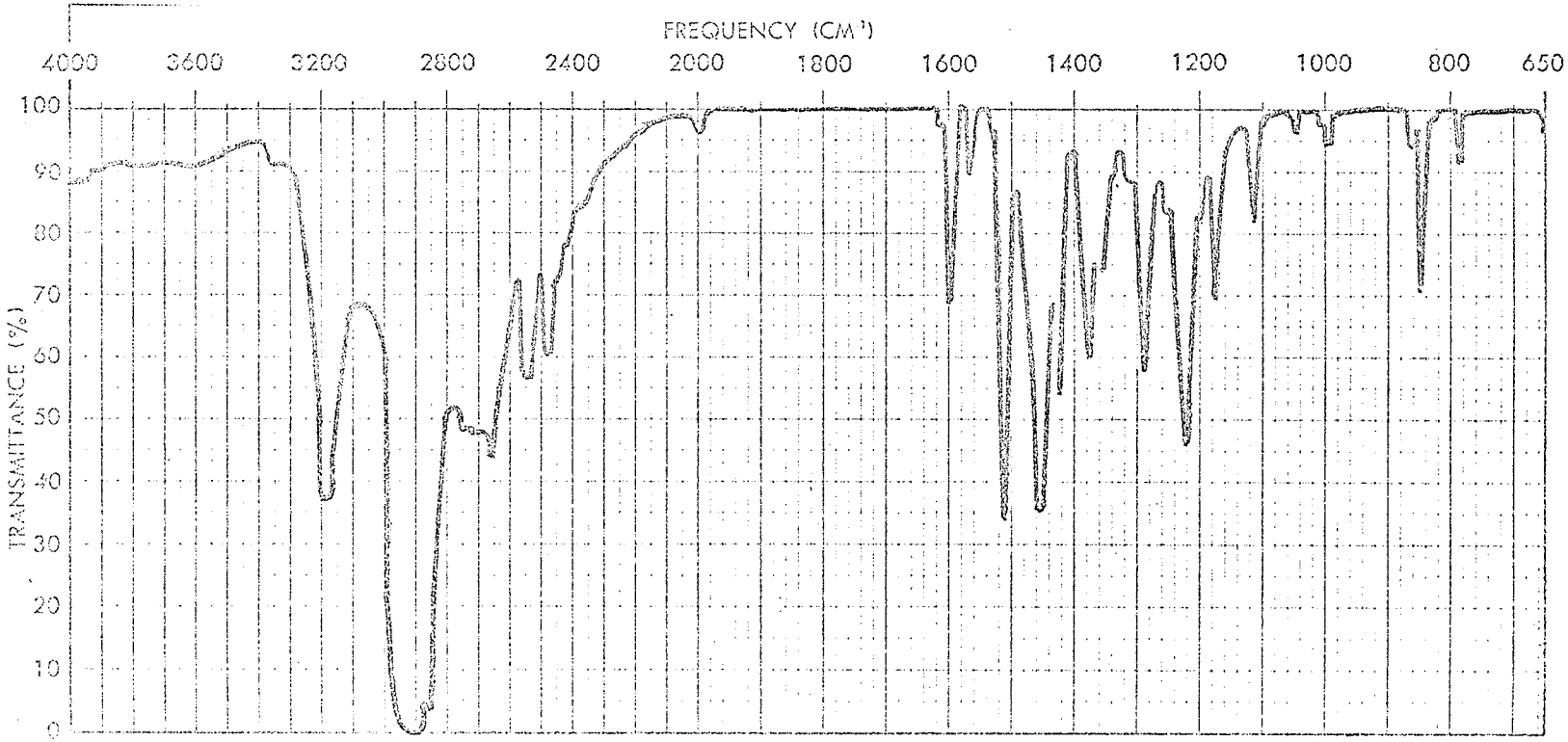


FIGURE 3: INFRA-RED SCAN OF p-AMINOPHENOL HYDROCHLORIDE IN NUJOL

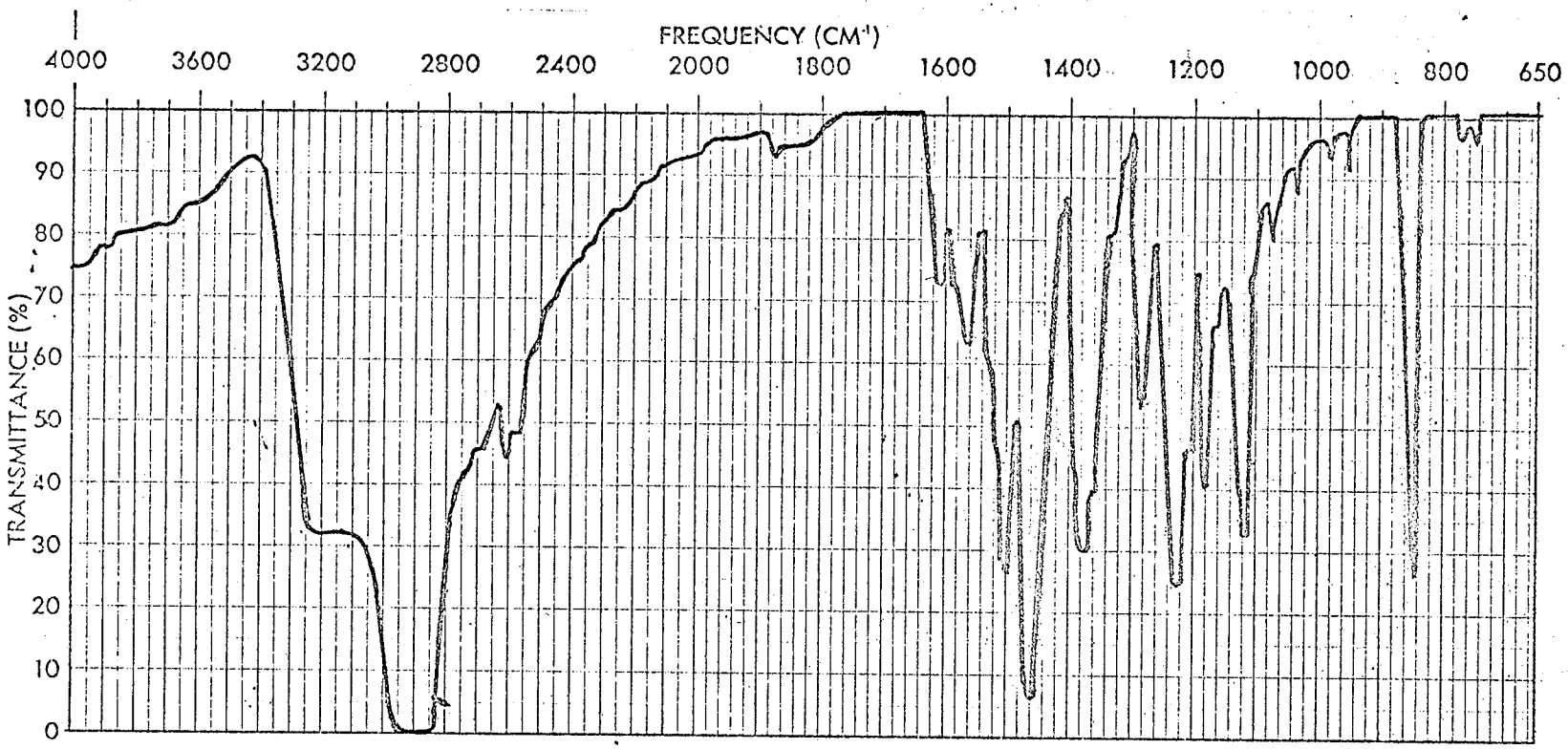


FIGURE 4: INFRA-RED SCAN OF N-CYCLOPENTYLANILINE TOSYLATE IN NUJOL

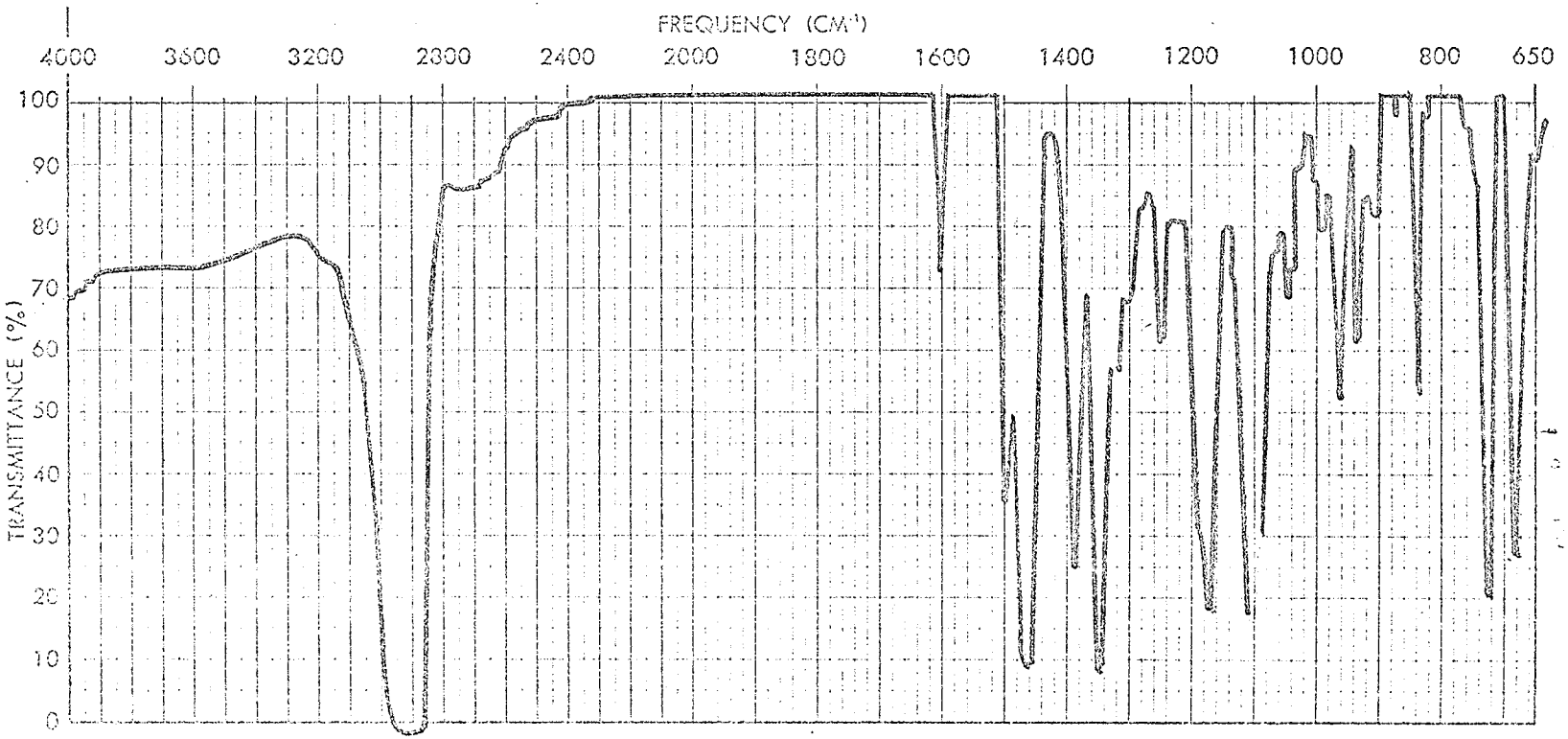


FIGURE 5: INFRA-RED SCAN OF p-HYDROXY-N-CYCLOPENTYLANILINE-N,O-DITOSYLATE IN NUJOL

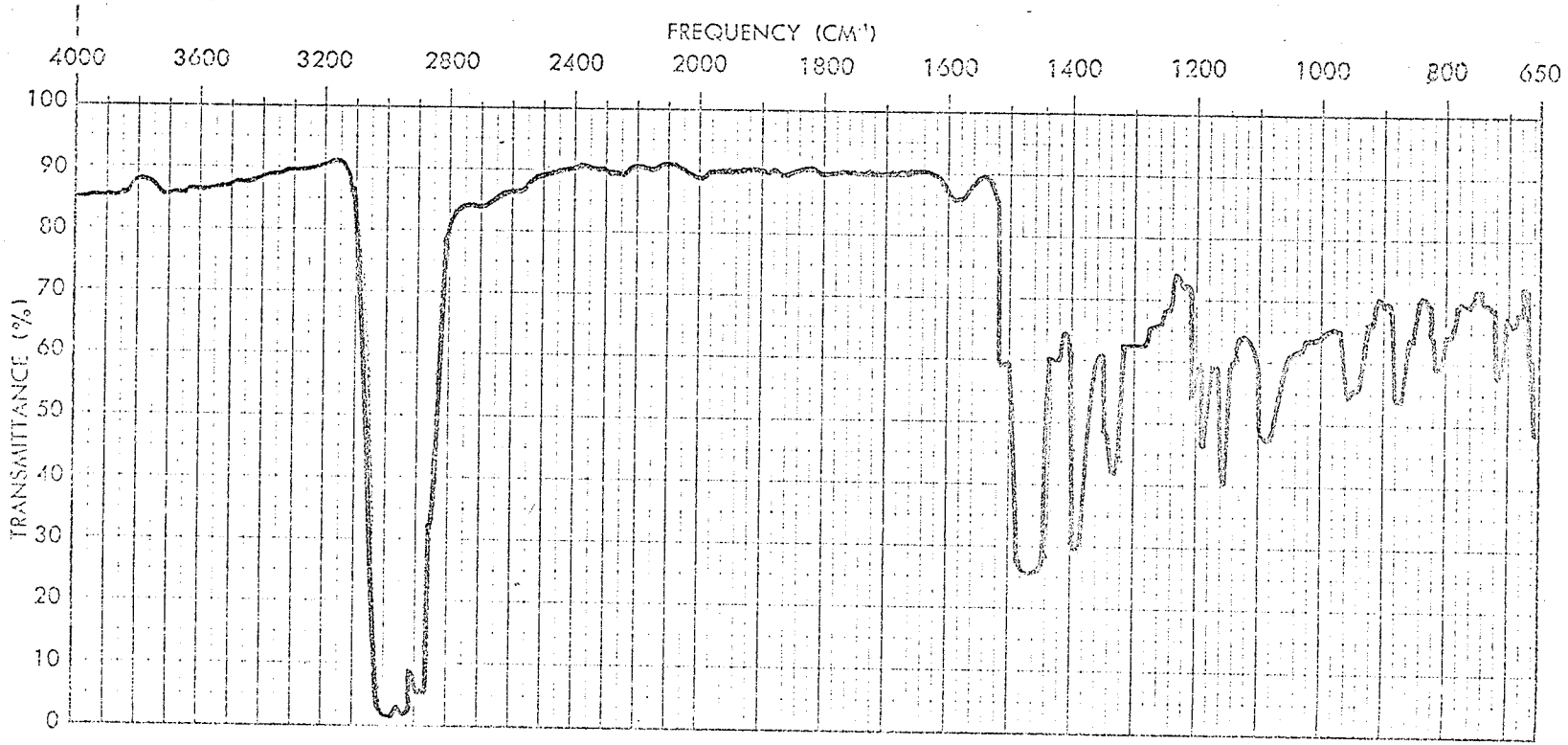
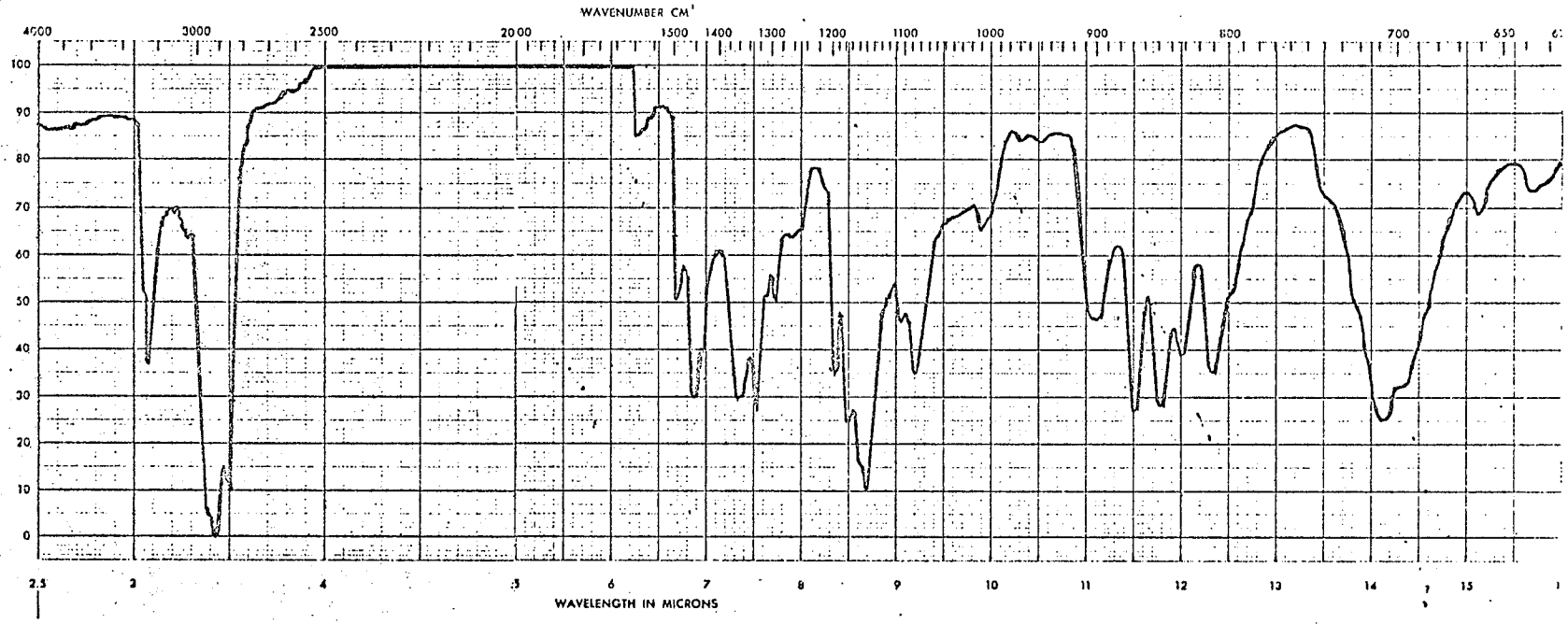


FIGURE 6: INFRA-RED SCAN OF p-AMINOPHENOL-N,O-DITOSYLATE IN NUJOL



APPENDIX III

Determination of the Efficiency of Combustion

To determine the combustion and collection efficiency of the apparatus developed to combust samples of rat feces and collect and count the carbon dioxide produced, samples of benzoic acid-^{14}C of known radioactivity (6.7×10^3 dpm/mg.) were subjected to a similar procedure. Aliquots of approximately 1 mg. were accurately weighed and combusted, and the carbon dioxide collected as described. Table I summarizes the results of benzoic acid-^{14}C combustons and indicates that the method resulted in a recovery of 99.30 ± 2.06% of the benzoic acid combusted.

TABLE I: COMBUSTIONS OF BENZOIC ACID-^{14}C TO DETERMINE EFFICIENCY OF RECOVERY OF COMBUSTION AND COLLECTION METHODS

Sample	Aliquot Number	% Recovery of Activity	Mean	Standard Deviation
1	1	97.50		
	2	93.79		
	3	99.07	96.15	1.76
	4	96.27		
	5	94.10		
2	1	98.98		
	2	95.71	98.98	2.18
	3	102.25		
3	1	101.04		
	2	103.82		
	3	104.06	102.41	1.82
	4	101.04		
	5	99.10		
4	1	102.79		
	2	103.88	101.34	2.66
	3	97.34		
5	1	100.00		
	2	97.83	97.64	1.10
	3	97.20		
	4	97.83		
	5	95.34		
Overall average			99.30	±2.06

BIBLIOGRAPHY

1. Alexander, W. E.,Ryan, A. J., and Wright, S. E. Metabolism of Diphenylamine in the Rat and Rabbit, Experientia 20,223 (1964).

2. Alexander, W. E., Ph.D. Diss., A Study on the Metabolism of Secondary Aromatic Amines and Mechanisms of Aromatic Hydroxylation, University of Sydney, Australia (1965).

3. Alexander, W. E., Ryan, A. J., and Wright, S. E. The Metabolism of N-Secondarybutylaniline in the Rat, Can. J. Pharm. Sci. 3, 20 (1968).

4. Sitar, D. S. Masters Thesis, The Metabolism of N-Isopropylaniline- ^{14}C Hydrochloride in the Rat, University of Manitoba (1968).

5. Thiessen, J. J. Masters Thesis, The Metabolism of Selected N-alkyl-aromatic Amines in the Rat, University of Manitoba (1969).

6. Murray, J. D. Masters Thesis, The Metabolism of Selected N-alkylanilines in the Rat, University of Manitoba (1970).

7. Brown, R. A. Masters Thesis, The Metabolism of N-tertiary Butylaniline- ^{14}C Hydrochloride in the Rat, University of Manitoba (1970).

8. Alexander, W. E. Unpublished data.

9. Parke, D. V. Biochemistry of Foreign Compounds, Pergamon Press, London (1968), p. 34.

10. Mellet, L. B. Comparative Drug Metabolism, Arzn. Forsch. 13, 136 (1969).

11. Mason, H. S., North, J. C., and Vanneste, M. Microsomal Mixed-function Oxidations: The Metabolism of Xenobiotics, Fed. Proc. 24, 1172 (1965).

12. Mazel, P., and Henderson, J. R. On the Relationship Between Lipid Solubility and Microsomal Metabolism of Drugs, Biochem. Pharac. 14, 92 (1965).

13. Williams, R. T. Detoxication Mechanisms, Chapman and Hall, Ltd., London, 2nd Edition (1959), p. 428.

14. Williams, R. T. Comparative Patterns of Drug Metabolism, Fed. Proc. 26, 1029 (1967).

15. Parke, op.cit. #9, p. 36.

16. Creaven, P. J., Parke, D. V., and Williams, R. T. A Spectrofluorimetric Study of the 7-Hydroxylation of Coumarin by Liver Microsomes, Biochem. J. 96, 390 (1965).

17. Creaven, P. J., Parke, D. V., and Williams, R. T. A Fluorimetric Study of the Hydroxylation of Biphenyl in vitro by Liver Preparations of Various Species, Biochem. J. 96, 879 (1965).

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

19. Parke, D. V., and Williams, R. T. Species Differences in the o- and p-hydroxylation of Aniline, Biochem. J. 63, 12P (1956).

20. Horn, J. The Breakdown of Dimethylaniline and Dimethylaniline Oxide in the Animal Body, Z. Physiol. Chem. 238, 84 (1867).

21. Alexander, W. E., Ryan, A. J., and Wright, S. E. Metabolism of Diphenylamine in the Rat, Rabbit and Man, Fd. Cosmet. Toxicol. 3, 571 (1965).

22. Parke, op. cit. #9, p. 41.

23. Sloane, N. H. Microsomal Hydroxylation of the Benzene Ring. An Intermediate Step in Enzymic Hydroxylation, Biochem. Biophys. Acta. 81, 408 (1964).

24. Guroff, G., Daly, J. W., Jerena, D. M., Ronson, J., Witkop, B. and Udenfriend, S. Hydroxylation-Induced Migration: The NIH Shift, Science 157, 1524 (1967).

25. Smith, D. J., Keasling, H. H. and Forist, A. A. The Metabolism of N-alkyl-4-bromobenzenesulfonamides in the Mouse, Correlation with Anticonvulsant Activity, J. Med. Chem. 8, 520 (1965).

26. Parke, op. cit. #9, p. 51.

27. McMahon, R. E. and Sullivan, H. R. The Metabolism of the Herbicide Diphenamid in Rats, Biochem. Pharmac. 14, 1085 (1965).

28. Clayson, D. B. The Aromatic Amines, Brit. Med. Bull. 20, 115 (1964).

29. Uehleke, H. N-hydroxylation of Arylamines by Bladder Mucosa, Life Sciences 5, 1489 (1966).

30. Maynert, E. W. and Losin, L. The Metabolism of Butabarbital (Butisol) in the Dog, J. Pharmacol. Exptl. Therap. 115, 275 (1955).

31. Robinson, D. Smith, J. N. and Williams, R. T. Metabolism of Alkylbenzenes, Isopropylbenzene (Cumene) and Derivatives of Hydrotropic Acid, Biochem. J. 59, 153 (1955).

32. Parke, op. cit. #9, p. 40.

33. Elliott, T. H., Hanam, J., Parke, D. V. and Williams, R. T. The Metabolism of 1-(^{14}C)-tetralin in Rabbits, Biochem. J. 92, 52P (1964).

34. Parke, op. cit. #9, p. 90.

35. Parke, op. cit. #9, p. 80.

36. Bär, H., Zarnack, J. and Pfeifer, S. N-Formyl-2-aminophenol- ein nuer Naturstoff, Die Pharmazie 2, 108 (1971).

37. Bray, H. G., Clowes, R. C. and Thorpe, W. V. The Metabolism of Aminophenols, o-formamidophenols, Benzoxazole, 2-methyl- and 2-phenylbenzoxazoles and Benzoxazolone in the Rabbit, Biochem. J. 51, 70 (1952).

38. Williams, R. T. The Biosynthesis of Aminophenyl- and Sulphan-aminophenylglucuronides in the Rabbit and Their Action on Hemoglobin in vitro, Biochem. J. 37, 329 (1943).

39. Mörner, K. G. H. Hoppe-Seyl.Z. 13, 12 (1889) from Detoxification Mechanisms, Williams, R. T., Chapman and Hall Ltd., London (1959), p. 443.

40. Dickens, F. Biological Aromatization of Hydroaromatic Compounds, Symp. Biochem. Soc. 5, 66 (1950).

41. Williams, op. cit. #13, p. 114.

42. Wood, R., and Reiser, R. Cyclopropane Fatty Acid Metabolism: Physical and Chemical Identification of Propane Ring Metabolic Products in the Adipose Tissue, J. Amer. Oil. Chem. Soc. 42, 315 (1965).

43. Chung, A. E. Metabolism of Cyclopropane Fatty Acids. Oxidation of Cis-9,10-methylene Hexadecanoic and Cis-9,10-methyline Octadecanoic Acids by Rat-Liver Mitochondria, Biochem. Biophys. Acta 116, 205 (1966).

44. DiCarlo, F. J., Viaw, J.-P., Epps, J. E. and Haynes, L. J. Prazepam Metabolism by Man, Clin. Pharmac. and Therap. 11, 890 (1970).

45. Kaneshiro, T. and Thomas, P. J. Methylation of Fatty Acids in a Methionine-dependent Agrobacterium Tumefaciens Controlled With Exogenous Methionine, Biochem. Biophys. Acta 187, 26 (1969).

46. Schiller, J. G. and Chung, A. E. The Metabolism of Cyclopropane-carboxylic Acid, J. Biol. Chem. 245, 5857 (1970).

47. Mahler, H. R. and Cordes, E. H. Biological Chemistry, Harper and Row, New York (1966), pp. 518, 564.

48. Gould, E. S. Mechanism and Structure in Organic Chemistry. Holt, Rinehart and Winston, New York (1959), pp. 118, 392.

49. Stumpf, P. K. Metabolism of Fatty Acids, Annu. Rev. Biochem. 38, 159 (1969).

50. Noller, C. R. Chemistry of Organic Compounds^{-0}, 3rd ed. W. B. Saunders Company, Philadelphia (1965), p. 922.

51. Williams, op. cit. #13, p. 116.

52. Parke, op. cit. #9, p.

53. Norris, D. B. and Trudgill, P. W. The Metabolism of Cyclohexanol by Nocardia Globerula CL1, Biochem. J. 121, 363 (1971).

54. Litchfield, M. H. and Swan, A. A. B. Cyclohexylamine Production and Physiological Measurement in Subjects Ingesting Sodium Cyclamate, Toxicol. Appl. Pharmacol. 18, 535 (1971).

55. Sondars, R. C., Estep, C. B. and Wiegand, R. G. Metabolism of Cyclohexylamine-^{14}C, Fed. Proc. 27, 238 (1968).

56. Weitzel, G. Hoppe-Seyl Z. 285, 58 (1950) from Detoxication Mechanisms, Williams, R. T., Chapman and Hall Ltd., London (1959), p. 115.

57. James, S. P., Jeffery, D. J., Waring, R. H. and White, D. A. Some Metabolites of Bromocyclopentane, Bromocyclohexane and Bromocycloheptane in the Rabbit, Biochem. Pharmacol. 19, 743 (1970).

58. James, S. P., Jeffery, D. J., Waring, R. H. and White, D. A. Reaction of Mono-bromo Derivatives of Cyclopentane, Cyclohexane and Cycloheptane and of Related Compounds with Glutathione in vivo and the Nature of the Sulfur-containing Metabolites Excreted, Biochem. Pharmacol. 20, 897 (1971).

59. Bernhardt, K. and Müller, S. Stoffwechselversuche mit ω-cyclopentenyl und ω-cyclopentyl-substituierten Fettsaüren, Hoppe-Seyl Z. 256, 85 (1938).

60. Burkhardt, G. N. and Wood, H. Nitroarylsulphuric Acids and Their Reduction Products, J. Chem. Soc. 1, 141 (1929).

61. Long, E. (Ed.). Biochemists Handbook. E. and F. N. Spon Ltd., London (1961), p. 32.

62. ibid. p. 31.

63. Brodie, B. B. and Axelrod, J. The Estimation of Acetanilide and Its Metabolic Products in Biological Fluids and Tissues, J. Pharm. Exptl. Therap. 94, 22 (1948).

64. Welch, R. M. and Conney, A. H. Simple Method for the Quantitative Determination of N-acetyl-p-aminophenol in Urine, Clin. Chem. 11, 1064 (1965).

65. Schellenberg, K. A. The Synthesis of Secondary and Tertiary Amines by Borohydride Reduction, J. Org. Chem. 28, 3259 (1963).

66. Emerson, W. S. and Uraneck, C. A. Secondary and Tertiary Amines from Nitro Compounds, J. Am. Chem. Soc. 63, 749 (1941).

67. Alexander, W. E. unpublished data.

68. Heilbron, T. and Bunbury, H. M. Dictionary of Organic Compounds. Vol. 1. Eyre and Spottiswoods, London (1963), p. 194.

69. Vogel, A. T. A Textbook of Practical Organic Chemistry. 3rd Ed. J. Wiley and Sons, Inc., New York (1966), p. 653.

70. ibid. p. 684.

71 Audette, R. C. S. unpublished data.