THE METABOLISM OF SELECTED N-ALKYLANILINES IN THE RAT

by

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ABSTRACT

The polar compound 2-(4-hydroxyphenylamino) propanoic acid was confirmed qualitatively as a urinary metabolite of N-isopropylaniline in the rat, illustrating the metabolic pathways of aromatic hydroxylation and side chain oxidation. It was established that at least two metabolites of N-isopropylaniline are excreted in the bile, and three metabolites in the urine, all of which are likely conjugated. Preliminary studies were also carried out on the biliary metabolites of N-isobutylaniline, and on the nature of the conjugates of N-isopropylaniline metabolites in the urine and bile.

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I. INTRODUCTION

Research in the general field of drug metabolism has increased greatly in the last thirty years as man endeavours to keep stride with the metabolic fate of an ever-increasing number of drugs and chemicals in mammalian systems. The importance of such studies cannot be overemphasised. As such compounds are foreign to the normal metabolic pathways of an organism, they must be metabolised and eliminated in a form which renders the compound non-toxic to the organism. If the compound were not rendered non-toxic, poisoning or even death could result.

Detoxication may be defined as "accelerated elimination of foreign compounds and, frequently, the neutralisation of any pharmacological activity they may possess" (Parke, 1968a). Most drugs, detoxicated in the liver, are either excreted in the bile and voided in the faeces, or are taken to the kidneys and excreted in the urine. Other less important sites of detoxication are the lungs, gastrointestinal tract, kidneys and skin.

Over the past few years, research has been in progress in our laboratories on the metabolism of N-alkylaromatic amines. Aromatic amines have been found to be important intermediates in drug and chemical manufacturing, and as such could produce health hazards. The chemical 4-aminodiphenyl, formerly used as an antioxidant (Case, 1954) was found to produce a variety of tumours in rats (Walpole et al., 1952) and bladder tumours in dogs (Walpole et al., 1954). These workers expressed the view that aromatic amines are carcinogenic because of their conversion in the body to ortho-hydroxyamines, which are direct carcinogens.

Thus, 2-naphthylamine was inactive in mice as a bladder carcinogen when implanted as a paraffin pellet but bladder carcinomas were regularly produced by implantation of the o-hydroxylated metabolites of the amine (Boyland, 1958). To date, studies on N-alkylaromatic amines have been carried out on the urinary excretion of metabolites in the rat, and in some cases the major metabolites have not yet been identified. Alexander et al. (1968) were able to account for only 26 percent of the radioactivity excreted in the 24-hour urine of rats injected with N-sec-butylaniline-^{14}C hydrochloride, and Alexander and Sitar (1969), conducting similar experiments on N-isopropylaniline, identified 70 percent of the metabolites excreted in 24-hour urine. In order to obtain a complete understanding of the metabolism of these two N-alkylaromatic amines in particular, the remaining metabolites must yet be identified. In the case of both N-sec-butylaniline and N-isopropylaniline, the authors concluded that polar, water-soluble metabolites constitute the unidentified fractions. Attempts to characterise a possible polar metabolite of N-isopropylaniline comprise the first part of this thesis.

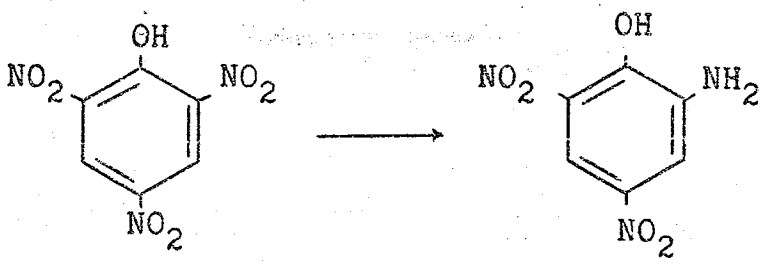
While renal excretion studies have been widely documented and reviewed (Sperber, 1959 and Peters, 1962), the study of extra-renal pathways of excretion has been ignored to a considerable extent. Biliary excretion studies, and the role of enterohepatic circulation in the detoxication of foreign compounds, are now gaining wider prominence among researchers. For practical and theoretical reasons studies on hepatic excretion have lagged behind those made on the kidney (Smith, 1966). However, a large

number of drugs are now known to be excreted in the bile and therefore the possibility that drug toxicity may be related to enterohepatic circulation must be considered (Williams et al., 1965).

There have been few reports in the literature on the biliary excretion of aromatic amines. While diphenylamine has been reported to be metabolised and excreted in the bile of rats to an appreciable extent (Alexander, 1965), studies on the metabolism of aniline have revealed very little excretion of this compound or its metabolites in the bile (Williams et al., 1965). Therefore, preliminary biliary excretion experiments were undertaken with N-isobutylaniline and N-isopropylaniline to determine if bile provided an important mode of excretion for the N-alkylaromatic amines.

II. METABOLIC FATE OF FOREIGN COMPOUNDS

The term "detoxication" formerly was used in its narrowest sense, implying that foreign compounds undergo a reduction in toxicity when transformed in the body. Two contradictions, however, became apparent: (1) some compounds, such as barium sulphate and aromatic sulphonic acids, are excreted almost totally unchanged, and (2) certain foreign substances are converted into more toxic metabolites. For example, picric acid (I.) is partially reduced to the more toxic picramic acid (II.).



I. II.

Detoxication now refers to all metabolic changes undergone by foreign compounds in the body, and it is essentially a two-phase process of metabolic transformation and conjugation, giving rise to metabolites (including conjugates) which are then excreted in the urine, bile and expired air (Parke, 1968a).

Metabolic transformations are reactions in which the foreign compound undergoes a wide variety of oxidations, reductions and hydrolyses, usually introducing functional groups which increase the polarity of the molecule and act as centres for the second phase of the process. These metabolic transformation reactions are generally referred to as phase I type reactions

(Williams, 1959a).

All transformations of foreign molecules are catalysed enzymatically, and most of these enzymes are present in the liver microsomal fraction (Williams, 1962). Fouts (1961), employing density gradient centrifugation, electron micrographs and enzyme activity studies, determined that the smooth endoplasmic reticulum had four times the ability to metabolise hexobarbital as the rough endoplasmic reticulum. Hydrolysis of amides and esters may be microsomal or non-microsomal. Reduction of nitro and azo compounds involve microsomal enzymes, while aldehyde and ketone reductions are non-microsomal in nature. Oxidation, the most general reaction undergone by foreign compounds, is microsomal as regards hydroxylation of acyclic, aromatic and alicyclic compounds, epoxidation, N-hydroxylation of amines, N-oxidation of tertiary amines, S-oxidation, dealkylation, deamination and desulphuration. Non-microsomal oxidations include deamination, oxidation of alcohols and aldehydes, and aromatisation of alicyclic compounds (Parke, 1968b).

Conjugations are syntheses by which the foreign compound or any of its metabolites is combined with endogenous molecules or groups such as glucuronic acid, sulphuric acid and amino acids, or methyl and other alkyl groups. With the exception of the latter, these synthesis reactions generally make the molecule more polar and less lipid-soluble, and thus more readily excreted. The conjugation reactions are generally referred to as phase II reactions (Williams 1959a). A foreign compound or molecule which already contains a site for phase II reactions

will not necessarily go through the phase I reaction stage. Conjugation of foreign compounds and their metabolites to one of the aforementioned endogenous substrates takes place most often by a transfer of the endogenous substrate from the co-enzymes that participate in intermediary metabolism. However, the enzymes which catalyse the transfer are usually specific for the formation of the conjugates of foreign compounds.

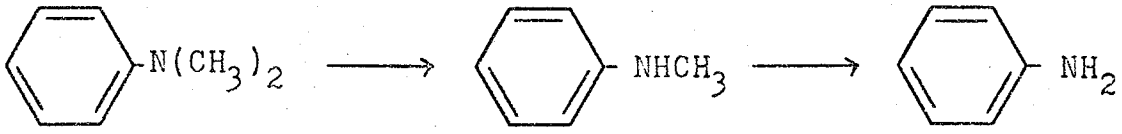
The pathways of reduction and hydrolysis are of minor importance in the metabolism of N-alkylaromatic amines. Oxidation and conjugation, however, are quite important and will be dealt with in respect to the compounds under investigation.

III. METABOLIC REACTIONS IMPORTANT TO AROMATIC AMINES

There have been few reports in the literature on the metabolic fate of the N-alkylaromatic amines. Oxidative degradations such as oxidative N-dealkylation, hydroxylation of the aromatic ring, and side chain oxidation have been reported to occur with this class of compounds (Alexander and Sitar, 1969), and at present appear to be the major types of metabolic attack involved. These types of oxidations have been found to be microsomal in nature and require oxygen and reduced nicotinamide adenine dinucleotide phosphate ($NADPH_2$) to function (Gillette, 1962).

A. OXIDATIVE N-DEALKYLATION

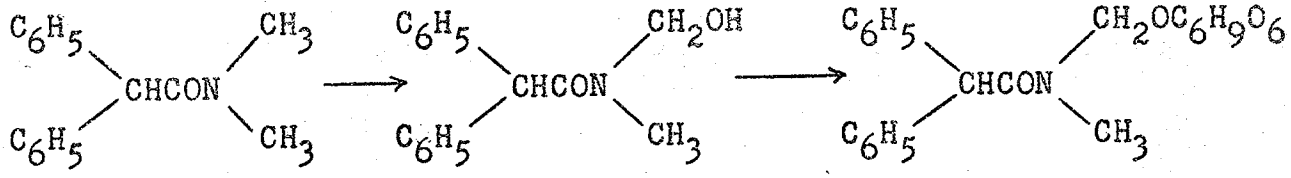
This type of degradation is typified by the following reaction, in which N,N-dimethylaniline (III.) is dealkylated to aniline (V.), with N-methylaniline (IV.) as an intermediate (Parke, 1968c):



III. IV. V.

Many different foreign alkylamines are dealkylated by the microsomal enzyme system, including N-methyl and dimethyl-barbiturates, hydantoins, adrenaline, ephedrine, aminopyrine and pethidine (Parke, 1968c). The requirements of the enzyme systems

responsible for N-dealkylation are essentially similar to those of the other oxidation systems. Natural compounds such as sarcosine and dimethylaminoethanol are dealkylated by mitochondrial enzymes, not microsomal enzymes (La Du, 1955). The N-dealkylation mechanism is thought to involve the enzymic formation of an N-oxide (Ziegler and Pettit, 1966) or hydroxymethyl intermediate (McMahon and Sullivan, 1964), which then rearranges intramolecularly to yield the amine. N-hydroxymethyl intermediates are sometimes sufficiently stable to form glucuronide conjugates, which are excreted in the urine. Diphenylamid (VI.) forms a hydroxymethyl intermediate (VII.), which can combine with glucuronic acid to form a conjugate (VIII.) (McMahon and Sullivan, 1965).



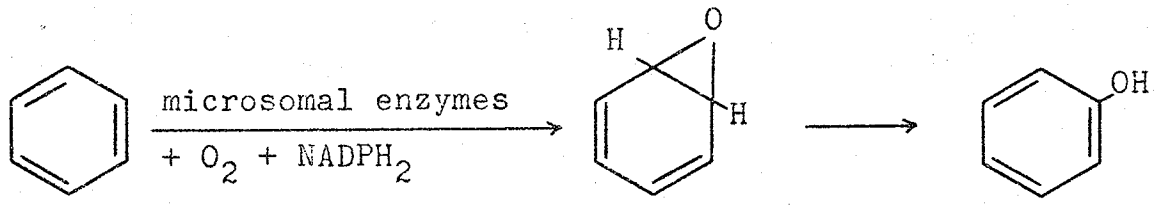
VI. VII. VIII.

In the study of the metabolism of N-alkylaromatic amines in the rat, N-dealkylation was found to occur, with concurrent para-hydroxylation of the benzene ring to yield p-aminophenol. This metabolite accounted for the following percent of radioactivity excreted in the 24-hour urine after intraperitoneal administration of each respective ^{14}C-labelled compound: N-sec-butylaniline, 13% (Alexander et al., 1968); N-isopropylaniline, 8% (Alexander and Sitar, 1969); N-tert-

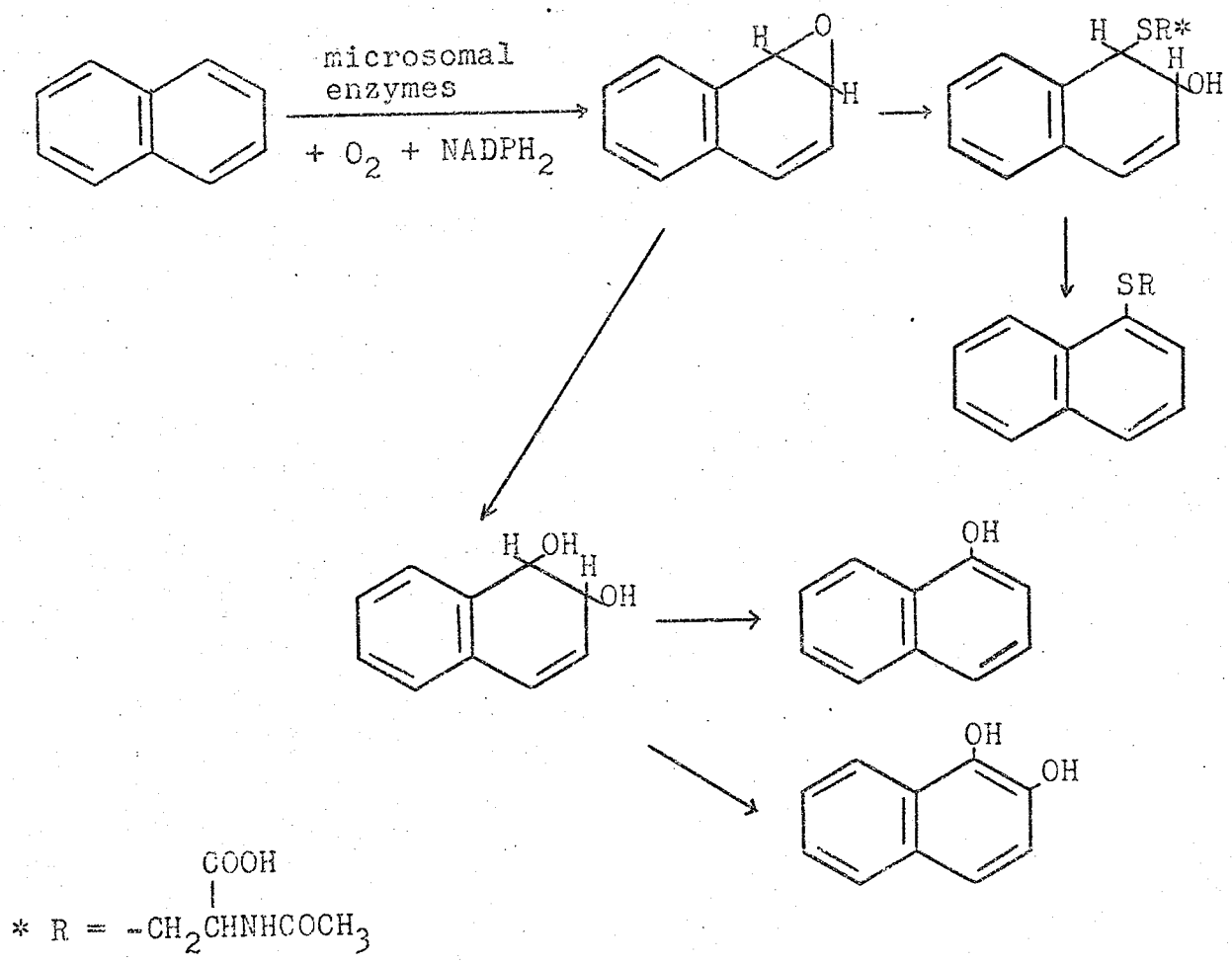
butylaniline, 1.9% (Brown, 1970) and N-isobutylaniline, 55.7% (Thiessen, 1969). This reaction has also been extensively studied with a number of unlabelled N-alkylaromatic amines such as N-n-butylaniline and N-n-propylaniline, and the production of p-aminophenol has been observed in every instance (Alexander, unpublished results).

B. HYDROXYLATION OF THE AROMATIC RING

Microsomal ring hydroxylations are currently considered to involve either: hydroxylation by a free radical (Smith, 1950); the formation of an epoxide followed by intramolecular rearrangement to form a phenol (Boyland, 1950;Mechanism 1); or a more complex mechanism in which an arene-1,2-epoxide is a common intermediate for the end production of phenols, catechols, hydrocarbons or mercapturic acids (Parke, 1968d; Mechanism 2).

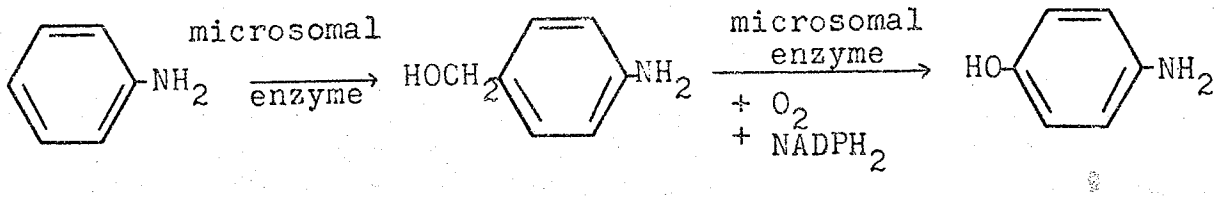


Mechanism 1.



Mechanism 2.

Another possible mechanism is one in which hydroxymethylation of the aromatic ring occurs. Guinea pig liver microsomes have been shown to convert aniline (IX.) into p-aminophenol (XI.) via the formation of p-aminobenzyl alcohol (X.) (Sloane, 1964).



IX. X. XI.

The orientation of hydroxylation of monosubstituted benzenes such as the N-alkylanilines can be determined either by the directing nature of the substituent or by species variability (Parke, 1968d). If the substituent is ortho/para-directing, these positions will be hydroxylated. If the substituent is meta-directing, for example nitrobenzene, all three positions have been found to be hydroxylated, with the hydroxyl groups entering predominantly at the para- and meta- positions. As for species variation, Parke (1960) reported that aniline is hydroxylated in various species as follows: rabbit, p-aminophenol 51%, o-aminophenol 9%; dog, p-aminophenol 11%, o-aminophenol 25%; rat, p-aminophenol 48%, o-aminophenol 19%.

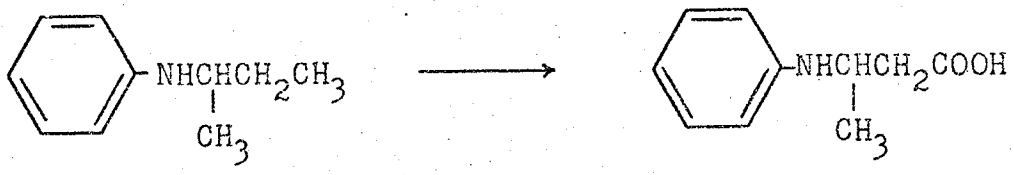
In addition to N-dealkylation, the N-alkylanilines have been shown to undergo ring hydroxylation without removal of the N-alkyl group. Thus, it has been shown that N,N-dimethylaniline is metabolised to p-hydroxy-N,N-dimethylaniline and p-hydroxy-N-methylaniline in the rabbit (Horn, 1936). Furthermore it appears that the type of reaction which occurs is species-dependent. For example, N,N-dimethylaniline yields o-aminophenol in the dog, as compared to the metabolites formed in the rabbit as described above. With respect to N,N-diethylaniline, the p-hydroxylated product is formed in both the dog and the rabbit (Horn, 1937). When the side chain is longer than ethyl, p-hydroxylation again has been demonstrated to be a major metabolic route for the parent N-alkylanilines, as demonstrated in the following series:

p-hydroxy-N-sec-butylaniline	10% (Alexander et al., 1968)
p-hydroxy-N-isopropylaniline	61% (Alexander and Sitar, 1969)
p-hydroxy-N-tert-butylaniline	89% (Brown, 1970)
p-hydroxy-N-isobutylaniline	37% (Thiessen, 1969)

Branching of the N-alkyl side chain has been shown to affect the metabolic pathway utilised. When the chain is branched on the α-carbon (the carbon adjacent to the nitrogen), p-hydroxylation predominates (e.g. N-isopropylaniline and N-tert-butylaniline). If branching is on the β-carbon (the carbon one removed from the nitrogen, N-dealkylation and p-hydroxylation combined predominate, as demonstrated by the metabolism of N-isobutylaniline (Alexander and Thiessen, 1970). It thus appears that the further the branching occurs from the nitrogen, the greater the degree of N-dealkylation.

C. SIDE CHAIN OXIDATION

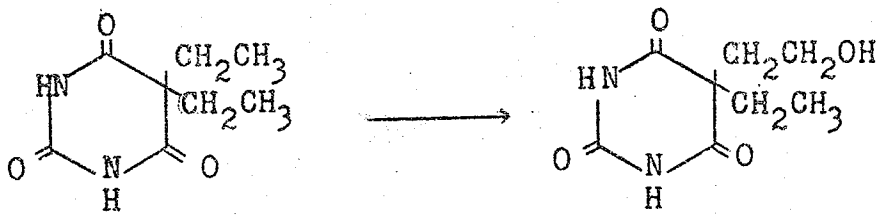
The quest for polar metabolites which have been demonstrated to occur with N-sec-butylaniline and N-isopropylaniline has led to the investigation of oxidation of the alkyl side chain. Such metabolic reactions could yield organic acids, which potentially could possess the polar properties which have been attributed to the unidentified metabolites. While literature is scarce on oxidation of these compounds, Alexander et al. (1968) found that three percent of N-sec-butylaniline (XII.) was excreted as 3-phenylaminobutyric acid (XIII.) when administered to rats.



XII. XIII.

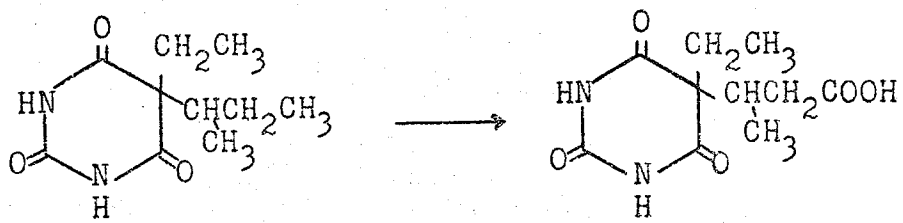
However, Alexander and Sitar (1969) could demonstrate only a small proportion of N-isopropylaniline being oxidised in the side chain to yield 2-anilinopropionic acid.

Numerous other groups of drugs containing alkyl side chains provide excellent evidence that oxidation of this type is a major degradative pathway. Terminal (ω) oxidation is exemplified by the formation of 5-ethyl-5-(2'-hydroxyethyl) barbituric acid (XV.) when diethylbarbituric acid (XIV.) is administered to rats (Goldschmidt and Wehr, 1957).



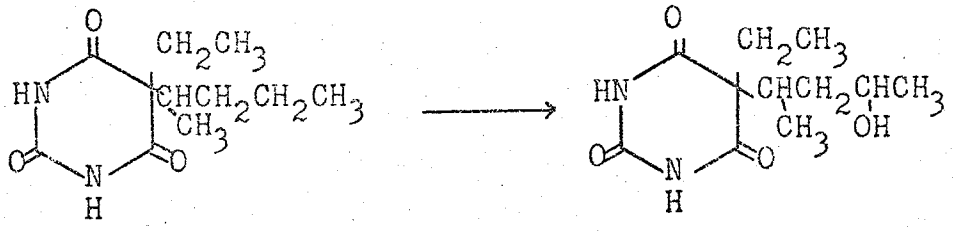
XIV. XV.

The more common barbiturate, butabarbital (XVI.), has been shown by Maynert and Losin (1955) to be metabolised in quantities of 28 to 35 percent to 5-ethyl-5-(2'-carboxy-1'-methylethyl) barbituric acid (XVII.).

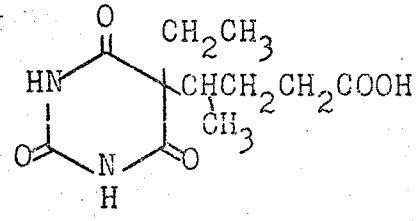


XVI. XVII.

Penultimate (ω-1) oxidation is exhibited by pentobarbital (XVIII.) in the formation of a secondary alcohol, 5-ethyl-5-(3'-hydroxy-1'-methylbutyl) barbituric acid (XIX.), as well as the terminal acid, 5-ethyl-5-(3'-carboxy-1'-methyl-propyl) barbituric acid (XX.) (Brodie et al., 1953 and Frey et al., 1959).

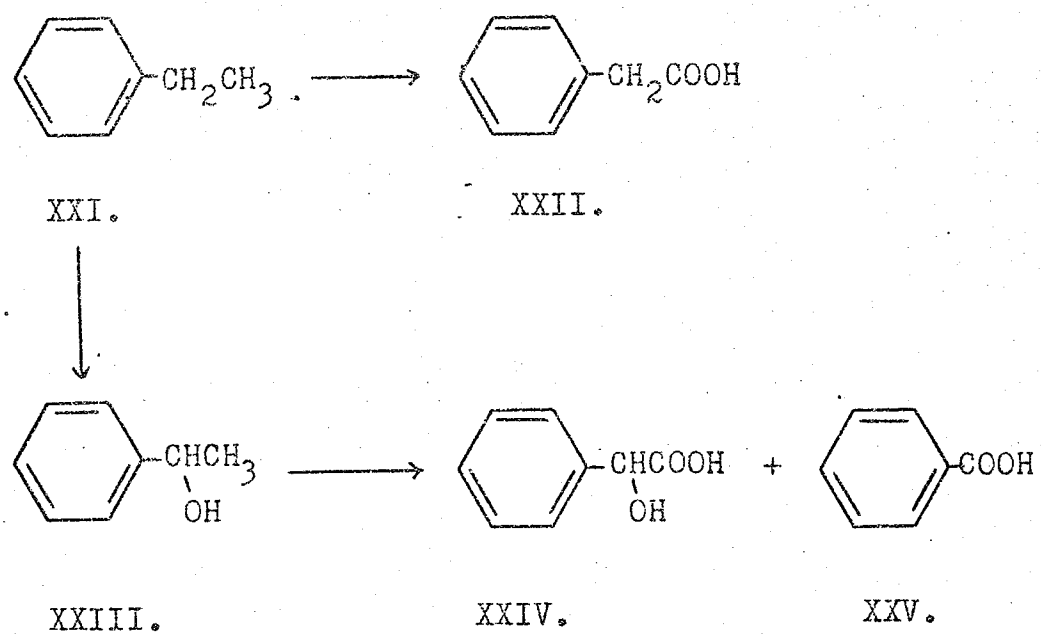


XVIII. XIX.



XX.

Ethylbenzene (XXI.), in rabbits, is metabolised to phenylacetic acid (XXII.), and to methylphenylcarbinol (XXIII.), which converts to mandelic acid (XXIV.) and benzoic acid (XXV.). The metabolites are excreted as conjugates of phenylacetic, mandelic and benzoic acids respectively (El Masri et al., 1956).



XXI. XXII.

XXIII. XXIV. XXV.

Side chain oxidations of this type are catalysed by enzymes present in liver microsomes (Mark, 1963), and Brodie et al. (1955) found that both reduced nicotinamide adenine dinucleotide phosphate (NADP) and oxygen are required for the reaction to occur. The requirements of the system, therefore, are essentially similar to those of the aromatic hydroxylation system.

D. CONJUGATION

The following general types of conjugation reactions are known to occur (Smith, 1966):

Glucuronic acid conjugation	Glucoside formation
Ethereal sulphate synthesis	Glutamine conjugation
Hippuric acid synthesis	Ornithuric acid synthesis
Glutathione conjugation	Cyanide-thiocyanate detoxication
Mercapturic acid formation	Methylation
Acetylation	

Of the above mechanisms, glucuronide, ethereal sulphate, acetate and glutathione formation have been demonstrated in the metabolism of aniline (Parke, 1960 and Boyland et al., 1963). It is possible that glycine conjugates may also form with N-alkyl side chains whose terminal (ω) methyl group has been oxidised to a carboxyl group. Ethylbenzene, which is partially metabolised in the rabbit to phenylacetic acid, is excreted as phenaceturic acid, a glycine conjugate (El Masri et al., 1956). Therefore, it seems possible that some of these reactions may be involved in the conjugation of the N-alkylanilines, which are known to conjugate since they must be hydrolysed prior to identification of the free metabolites.

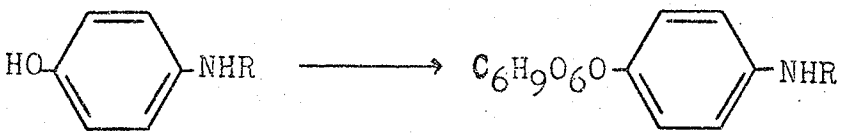
1. Glucuronic acid conjugation

Conjugation with glucuronic acid is likely the most common conjugation mechanism, occurring in all mammals and most vertebrates except fishes (Parke, 1968e). The cat is one exception, using this pathway only to a very minor extent. This species can, however, excrete the glucuronide of hydroxylated metabolites of 2-acetamidofluorene in the urine (Weisburger et al., 1964) and of progesterone in the bile (Taylor and Scratcherd, 1961).

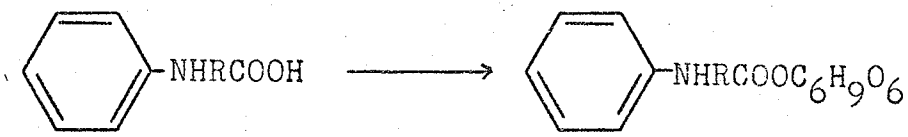
The synthesis of the glucuronide proceeds via transfer of the glucuronic acid from uridine diphosphate α-D-glucosid-uronic acid (UDPGA) to an acceptor, catalysed by the enzyme glucuronyl transferase (UDP transglucuronylase), which is present in the liver microsomes (Dutton, 1956). Glucuronide synthesis is not limited to the liver. It has been found to occur as well in

the kidney, lung, spleen, bladder and tissues of the gastro-intestinal tract (Schacter et al., 1959, and Stevenson and Dutton, 1960).

The O-glucuronide is the most probable conjugate to be formed by N-alkylaniline metabolites, either on the phenolic group formed by hydroxylation of the aromatic ring (ether type):

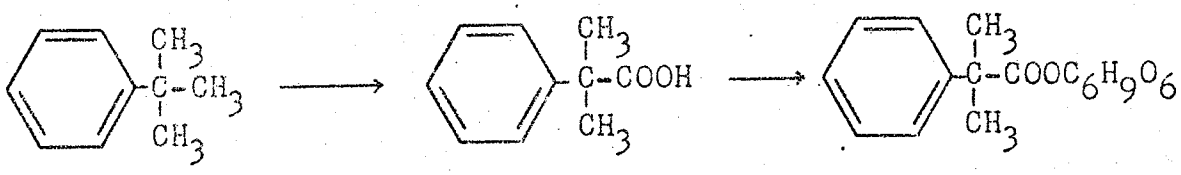


or on a carboxyl radical resulting from side chain oxidation (ester type):



R = alkyl chain

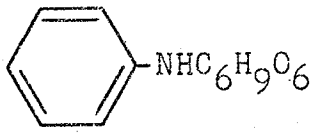
The ether type glucuronide has been shown to occur with the p-hydroxylated metabolites of N,N-dimethylaniline and N,N-diethylaniline (Horn, 1936 and 1937) and of aniline itself as p-aminophenylglucuronide (Williams et al., 1965). Tert-butylbenzene (XXVI.), in part, is first oxidised to α,α-dimethylphenylacetic acid (XXVII.), then excreted as an ester type glucuronide (XXVIII.) (Robinson and Williams, 1955).



XXVI. XXVII. XXVIII.

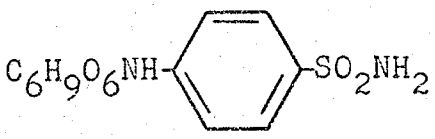
A double conjugate is formed with p-hydroxybenzoic acid, on both the phenolic and carboxylic acid groups (Parke, 1968e.).

N-glucuronides have been shown to occur on the nitrogen atom of an aromatic amino group, a sulphonamide, a carbamyl group, or in a heterocyclic ring compound. Aniline-N-glucuronide (XXIX.) comprised three percent of the dose of aniline excreted by rabbits (Parke, 1960).

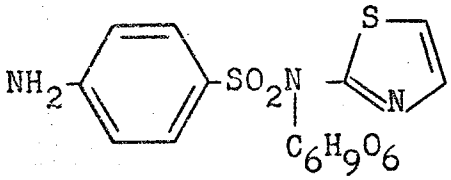


XXIX.

Conjugations of the sulphonamides may be on either nitrogen, as illustrated by sulphanilamide-N^4-glucuronide (XXX.) and sulphathiazole-N^1-glucuronide (XXXI.) (Parke, 1968e).

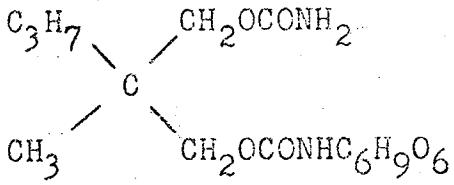


XXX.

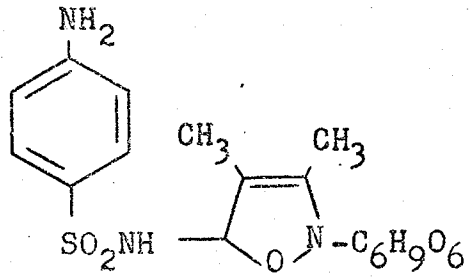


XXXI.

Meprobamate glucuronide (XXXII.) illustrates conjugation of a carbamyl group and sulphisoxazole-N^2-glucuronide (XXXIII.) conjugates on the heterocyclic ring.



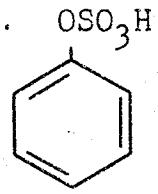
XXXII.



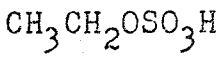
XXXIII.

2. Ethereal sulphate conjugation

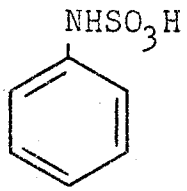
Sulphate conjugation is commonly associated with phenols (phenyl sulphate, XXXIV.) but it has also been shown to occur with aliphatic alcohols (Boström and Vestermark, 1960) such as ethanol (ethyl sulphate, XXXV.) and with aromatic amines (Boyland et al., 1957 and Roy, 1958) such as aniline (phenyl sulphamate, XXXVI.).



XXXIV.



XXXV.



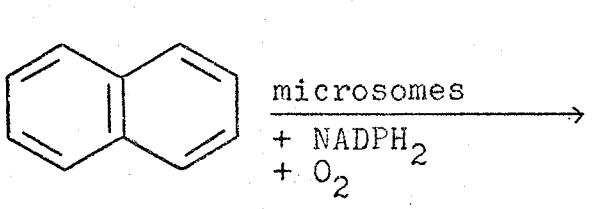
XXXVI.

This type of conjugation is thought to be the most primitive of detoxication mechanisms, in which an activated form of sulphate is transferred to an acceptor molecule, such as a phenol, under the influence of a [illegible]ferase (de Meio and Tkacz, 1950). Robbins and Lipmann (1956) showed the activated form of sulphate

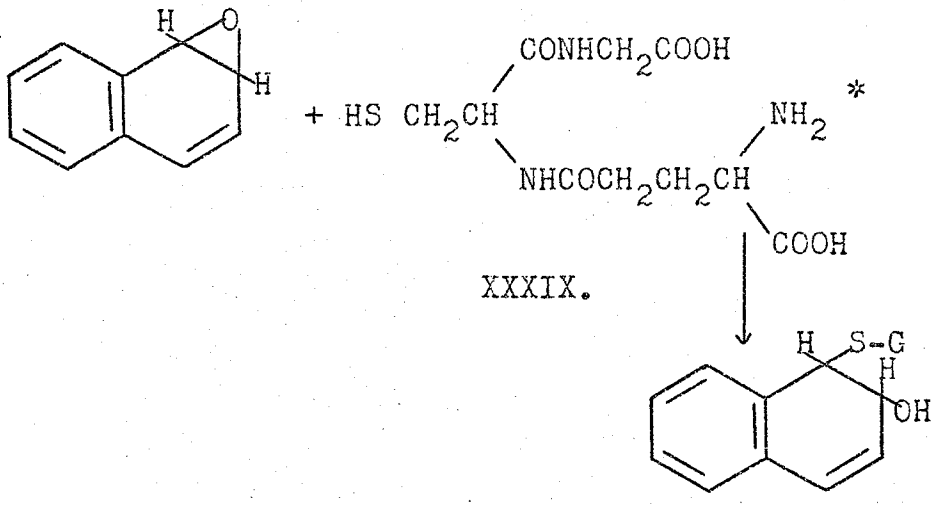
to be 3'-phosphoadenosine-5'-phosphosulphate. A number of different sulphotransferases or sulphokinase appear to exist.

3. Glutathione conjugation

The glutathione conjugation reaction is a relatively recent discovery of considerable importance, especially with respect to biliary excretion. Naphthalene (XXXVII.) is considered to form a 1,2-epoxide intermediate (XXXVIII.), which is catalysed by glutathiokinase to react with glutathione (XXXIX.), producing the conjugated compound (XL.). This conjugate is an intermediate in the formation of a premercapturic acid (XLI.). On treatment with mineral acid, either 1-naphthylmercapturic acid (XLII.) is formed by the loss of the elements of water, or 2-naphthol (XLIII.) by the loss of N-acetylcysteine (Boyland et al., 1961).



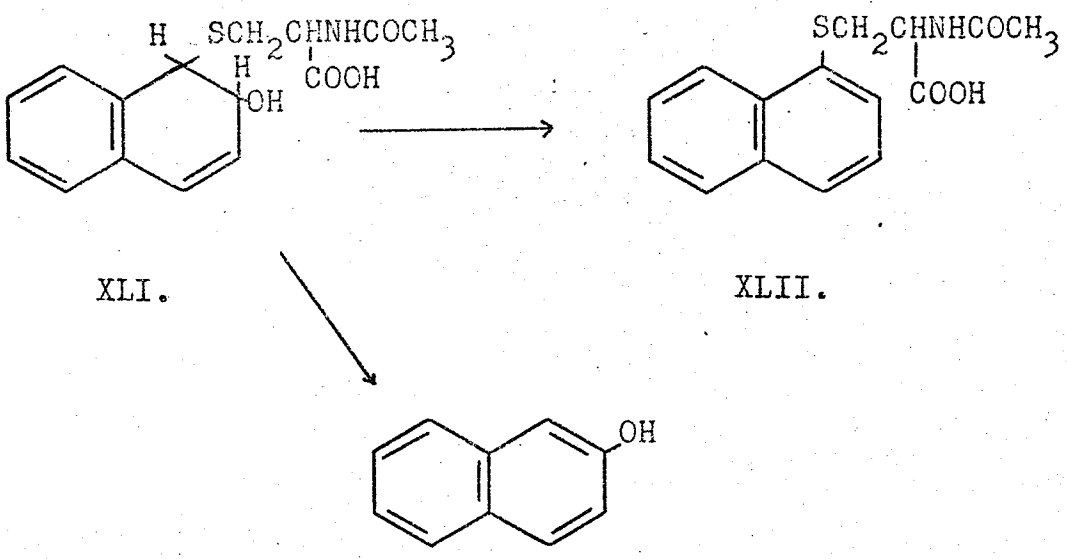
XXXVII. XXXVIII.



XXXIX.

XL.

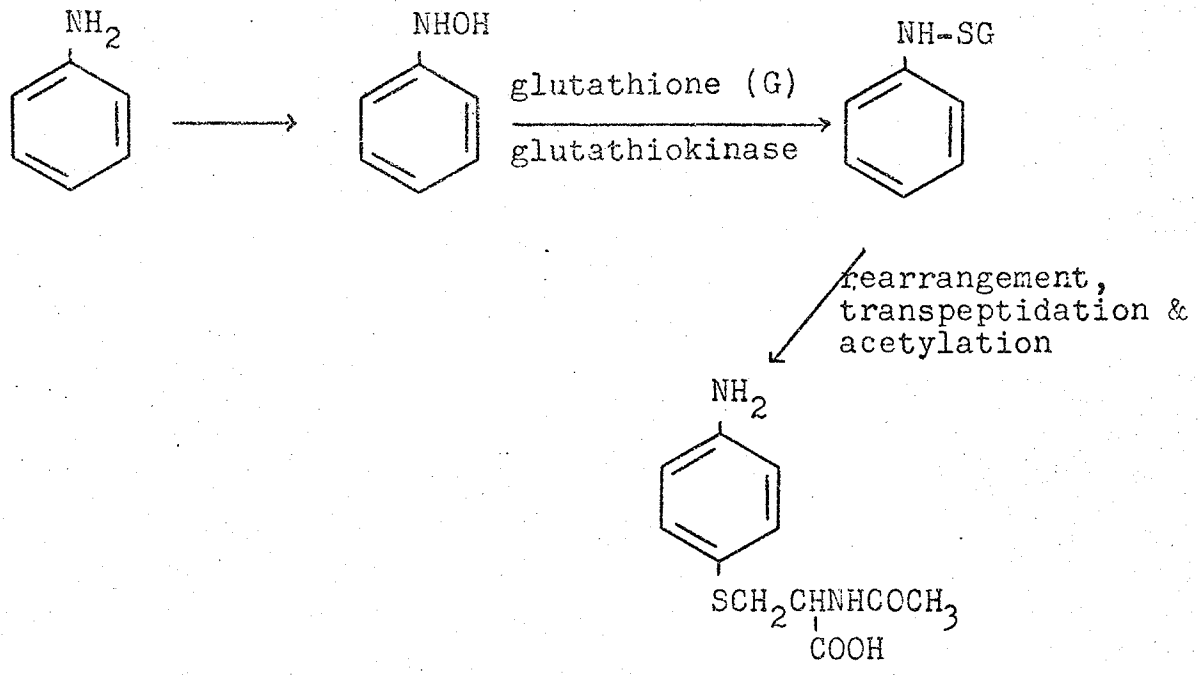
* Subsequently abbreviated as G.



XLI. XLII.

XLIII.

Compounds such as iodobenzene and pyrene (Boyland and Sims, 1964) and sulphobromophthalein (Combes and Stakelum, 1960) have been detected as conjugates of glutathione, cysteine and cysteinylglycine, before being excreted as mercapturic acids. Boyland et al. (1963) observed that trace amounts of aminophenyl- and aminonaphthylmercapturic acids appeared as metabolites of aniline and 2-naphthylamine respectively in the urine of rats. These acids must be formed similarly to aromatic hydrocarbon conjugates by combination of the hydroxylamino compounds with glutathione, followed by an intramolecular rearrangement, transpeptidation and acetylation, as illustrated by the following sequence (Parke, 1968f):

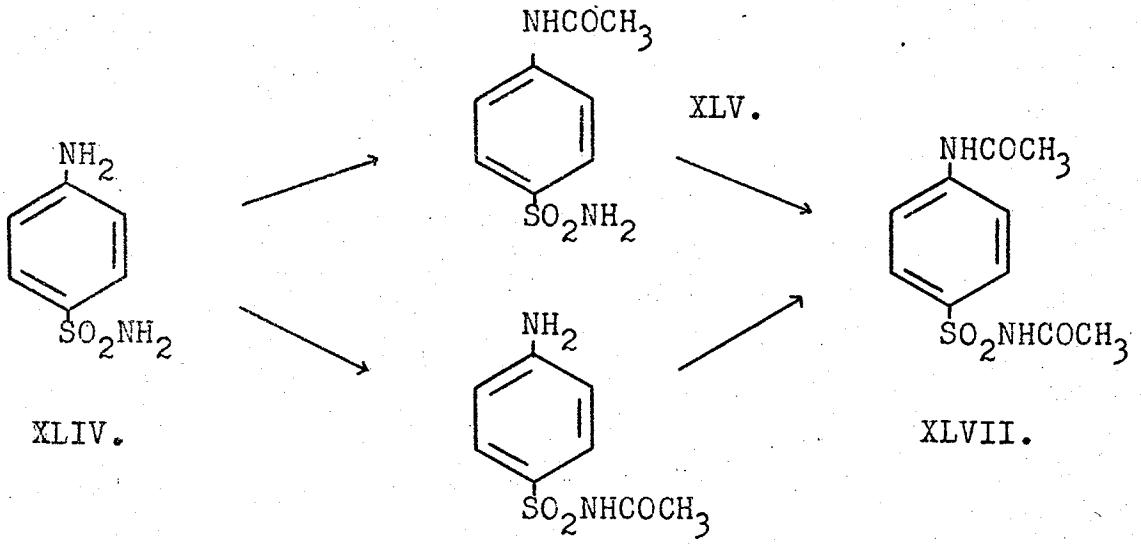


Jagenburg and Toczko (1964) found that when two gram quantities of acetophenetidine were administered to humans, two percent of the dose was excreted in 24 hours as S-(1-acetamido-4-hydroxy-phenyl) cysteine, a mercapturic acid derivative. In view of these reactions, it would not be unreasonable to assume that secondary alkylaromatic amines might undergo similar conjugation reactions.

4. Acetylation

This is a general metabolic pathway for aromatic amines, sulphonamides and some foreign amino acids such as phenyl-cysteines. In the case of aniline in rabbits, only a very small amount of acetanilide (0.2 percent) was found (Parke, 1960). This was consistent with the report of Smith and Williams (1948) that acetanilide is p-hydroxylated extensively, then metabolised to the corresponding O-glucuronide or ethereal sulphate,

constituting a double conjugation mechanism. Sulphonamides, such as sulphanilamide (XLIV.), may acetylate on either or both nitrogen atoms (Parke, 1968g; XLV., XLVI., and XLVII.).



XLIV. XLVII.

XLVI.

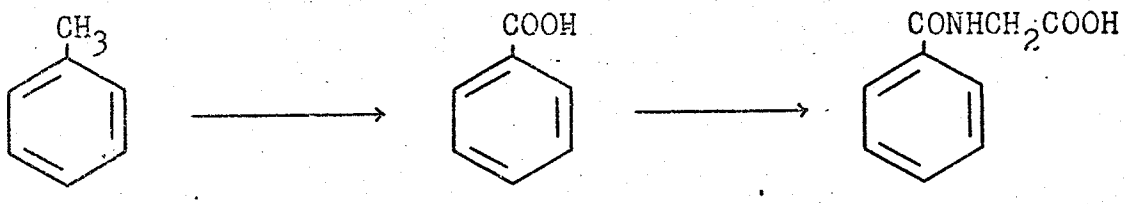
The acetylation reaction usually occurs in the liver but sulphanilamide and p-aminobenzoic acid have been shown to be acetylated by rabbit reticuloendothelial cells (Govier, 1965). As for most other biological acetylations, acetylcoenzyme A is the required intermediate in the acetylation of aromatic amines (Williams, 1958b).

5. Glycine conjugation

Conjugation with glycine and other amino acids is characteristic only of carboxylic acids and is thus a possible reaction of N-alkylaromatic amines whose side chain has undergone ω oxidation. Glycine conjugates are known as "hippuric acids", hippuric acid itself being the conjugation product of benzoic acid and glycine. El Masri et al. (1956)* have shown that

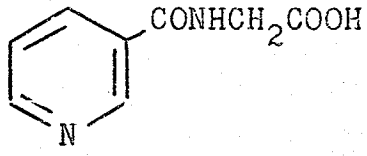
*Based on studies by Bray (1948), Biochem. J. 42, 434.

75 percent of toluene (XLVIII.) is excreted in man as hippuric acid (L), with benzoic acid (XLIX.) as the intermediate.

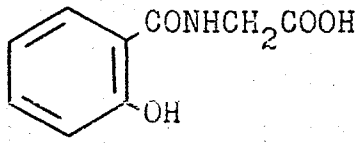


XLVIII. XLIX. L.

Nicotinic and salicylic acids form nicotinuric (LI.) and salicyluric acids (LII.) respectively (Parke, 1968h).



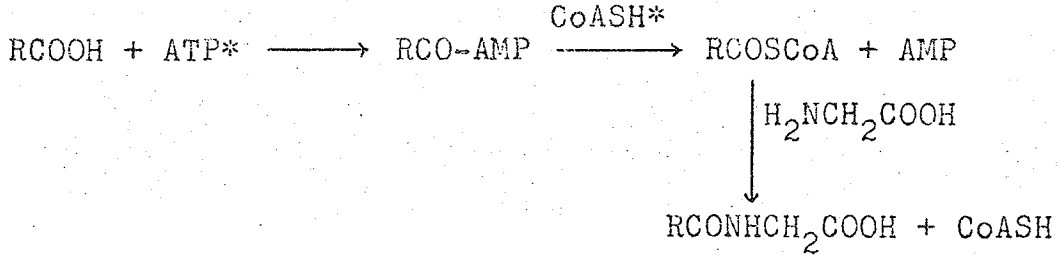
LI.



LII.

Glycine conjugates are also formed by compounds such as phenylacetic acid (a substituted acetic acid), cinnamic acid (a β-substituted acrylic acid), and cholic acid (a steroid acid). Aliphatic carboxylic acids do not conjugate with glycine (Parke, 1968h).

This type of conjugation is a three-step mitochondrial reaction: firstly, activation of the carboxyl group with ATP to form R-CO-AMP (AMP is adenosine monophosphate), which is utilised for the formation of an acyl CoA intermediate. This product is then condensed with glycine to give the hippuric acid, as shown here (Kellerman, 1958).



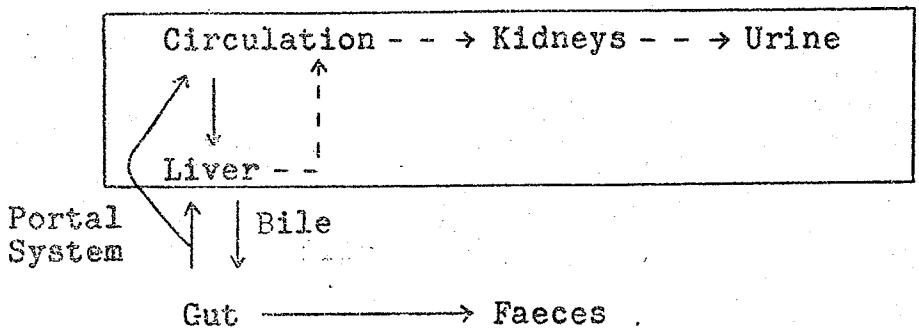
*ATP - Adenosine triphosphate

*CoASH - Coenzyme A

Although little research has been done on conjugated metabolites of N-alkylaromatic amines, the pathways described here (glucuronide, ethereal sulphate, glutathione, acetyl and glycine conjugation) are the most feasible detoxication routes insofar as the compounds in question are concerned.

IV. ENTEROHEPATIC CIRCULATION AND BILIARY EXCRETION OF FOREIGN COMPOUNDS

The secretion of a drug in the bile may be an important factor in influencing the pharmacological properties of that drug (Smith, 1966). An important consequence of biliary excretion is that a compound may undergo the process known as enterohepatic circulation. Following administration of a drug by any route, the drug enters the bloodstream. Metabolism, if it does occur, takes place mainly in the liver. If the drug enters the bile duct it will be passed into the gut, from which it may be either reabsorbed and recirculated, or excreted in the faeces. If the compound is recirculated, the efficiency of the liver in re-excreting the compound is an important factor. This efficiency must be considered in relation to the efficacy of competing excretory mechanisms such as the kidney. Extra-hepatic elimination will decrease the total amount of drug available for recycling. Thus, a compound detected in the bile could still be excreted in the kidneys in view of this competing mechanism involved in enterohepatic circulation. Usually, however, a drug involved in true enterohepatic circulation will be excreted in the faeces, and it is therefore hazardous to assume that if a drug is administered orally and is detected in the faeces, then it is not being absorbed. Parenteral administration will substantiate enterohepatic circulation as faecal excretion of the drug will still take place. The following diagram explains the process.



An obvious result of this process is that a foreign compound might persist in the body for unduly long periods. This has been found for morphine (Woods, 1954) and glutethimide (Keberle et al., 1962) in dogs and for digitoxin in man (Okita et al., 1954).

The mechanism by which compounds pass from the liver into the bile is considered to proceed in the following manner (Schanker, 1962). The compound is first absorbed from the blood of the hepatic sinusoids into the hepatic parenchymal cells, and then is either transferred in a metabolised or conjugated form into the bile, or alternately is returned to the sinusoids, ultimately to be excreted by the kidneys, as shown by the enclosed part of the diagram. The hepatic parenchymal cells have highly permeable membranes, providing a porous boundary between blood and bile. The passage of most molecules and ions smaller than proteins is thus facilitated. Many substances have been shown to appear in the bile in concentrations similar to the plasma concentration via a simple diffusion process (Smith, 1966). Such compounds include glucose, the ions Na^+, K^+ and Cl^- and the glucuronide of p-nitrophenol. But highly polar compounds such as bile salts, bilirubin glucuronide and conjugates of some

foreign compounds were found to be excreted in much higher concentrations by a process of active transport. Brauer (1959) has determined that, in order to engage in this active transport, compounds must meet three conditions: (1) be present in the blood as anions; (2) have a molecular weight greater than 300; and (3) be bound to plasma proteins. An increase in molecular weight is accompanied by an increase in biliary excretion. For example, the glucuronide of 4-hydroxybiphenyl (M.W. 346) is excreted as 58 per cent of the dose in the 24-hour bile of rats, while that of p-aminophenol (M.W. 285) is not excreted at all (Williams et al., 1965). A free hydroxyl group also appears to increase biliary excretion of a compound, as 4,4'-dihydroxybiphenyl monoglucuronide (M.W. 362) is excreted as 92 percent of the dose of the parent compound. The only conjugation mechanisms known definitely to be prominent in mammalian biliary excretion are those involving glucuronic acid, ethereal sulphate, hippuric acid and glutathione, the most important of these being glucuronic acid (Smith, 1965). Polar compounds which have become conjugated may be hydrolysed by intestinal flora or enzymes (glucuronidase, sulphatase) prior to being reabsorbed from the gut.

The phthalein dyes, fluorescein and sulphobromophthalein (BSP), have been used widely in biliary excretion studies, as well as diagnostic agents in gall bladder examination. Krebs and Brauer (1958) found at least five metabolites of BSP in the bile of the rat, cat, dog, sheep and chicken, and except for the last species, little unchanged BSP was excreted. These metabolites are likely amino acid conjugates of BSP with glycine and glutamic

acid, which have been identified on hydrolysis of the conjugates (Combes, 1959). Halogenation of fluorescein markedly increases biliary excretion with a concomitant decrease in urinary excretion (Webb et al., 1962).

Only those sulphonamides which are themselves sufficiently polar or are metabolised to polar conjugates appear in the bile to a significant extent (Williams et al., 1965). Sulphadimethoxine is one of the few sulpha drugs to be excreted more than five percent of the dose in the bile. The N^1-glucuronide of this compound is excreted extensively (66 percent) in the bile of rats, and is also the main metabolite found in human urine. In rat urine, however, the N^4-acetyl derivative is of greater importance (Bridges et al., 1964). It is possible that the long action of sulphadimethoxine is associated with biliary excretion of the glucuronide, which on hydrolysis in the gut would yield free sulphadimethoxine again. This could be absorbed and thus set up an enterohepatic circulation of the drug (Williams et al., 1965).

Little information is available in the literature on the manner in which the metabolites of the N-alkylaromatic amines are conjugated, nor is there much information concerning the biliary excretion of aromatic amines in general. Less than 0.5 percent of the dose of aniline administered to rats is excreted as biliary metabolites (Williams et al., 1965). However, 26 percent of the dose of diphenylamine administered to rats has been found to be excreted in the bile (Alexander, 1965). It was therefore considered that some of the metabolites of the N-

alkylaromatic amines which on conjugation could yield compounds with molecular weights of 300 or more might be excreted in the bile and as a result, the structure of the N-alkylaromatic amine with respect to the side chain, might have some effect on biliary excretion.

V. EXPERIMENTAL

A. SYNTHESIS

1. 2-(4-hydroxyphenylamino) propanoic Acid

2-(4-hydroxyphenylamino) propanoic acid was synthesised according to a modification of the method of Nastvogel (1890). p-Aminophenol (2.18 g., 0.02 moles) in water (30 ml.) was heated to boiling on a magnetic stirring hot plate. 2-bromopropionic acid (1.53 g., 0.01 moles) was added dropwise and the mixture warmed under nitrogen for 10 minutes. The crude product was filtered after cooling and dried under nitrogen. Recrystallisation was effected from water. m.p. 225-226°C. (decomp.)

Calculated for $C_9H_{11}O_3N$: C, 59.66%; H, 6.12%

Found for $C_9H_{11}O_3N$: C, 59.01%; H, 6.00%

The O-benzoate was prepared according to the Schotten-Baumann method (Vogel, 1966a) in which 2-(4-hydroxyphenylamino) propanoic acid (400 mg.) was dissolved in 10 percent sodium hydroxide solution (20 ml.). A large excess of benzoyl chloride (2.0 ml.) was added and the mixture shaken in a stoppered flask for one minute. The crude benzoate was filtered and recrystallised from dilute ethanol or ethanol/ethyl acetate. m.p. 203-205°C. (decomp.)

Calculated for $C_{16}H_{15}O_4N$: C, 67.36%; H, 5.30%

Found for $C_{16}H_{15}O_4N$: C, 67.72%; H, 5.53%

The equivalent weight of the O-benzoate was determined by dissolving 0.2853 g. in aqueous ethanol (25 ml.). Employing the Metrohm Potentiograph E336, the sample was titrated with standardised sodium hydroxide solution. The equivalent weight

was found to be 295.7 (theoretical, 285.3). An infrared spectrum of the sample on a Beckman IR 8 Infrared Spectrophotometer indicated significant frequencies at 3050 cm^{-1} and 1670 cm^{-1}, corresponding to secondary aromatic amine and the stretching frequency of the carbonyl group, respectively.

Several techniques were employed in attempts to prepare an acetate derivative of 2-(4-hydroxyphenylamino) propanoic acid. Refluxing the parent compound with redistilled acetic anhydride for ten minutes, then pouring the mixture into ice water (Vogel, 1966a) produced only starting material, indicating that acetylation had not been successful. Heating on a water bath in place of refluxing yielded a gummy mass which could not be recrystallised. The method of Shriner, Fuson and Curtin (1965) for the preparation of acetamides from amines, employing acetic anhydride and sodium acetate, did not give a product, even after chilling overnight. No product was obtained according to the method of Vogel (1966b) for acetylation of phenols. Under all of these conditions the isolation of a crystalline acetate was not accomplished.

Attempts to prepare a p-toluenesulphonate derivative were equally unsuccessful. Refluxing 2-(4-hydroxyphenylamino)-propanoic acid, p-toluenesulphonyl chloride and pyridine (Vogel, 1966a) for 15 minutes gave a tarry mass when the mixture was poured into ice water. Extraction of the ice water with ether, then washing the ether layer with dilute hydrochloric acid and dilute sodium hydroxide, drying over molecular sieves and evaporating the ether on a Rinco rotary evaporator failed to

clarify the tarry mass. An oil resulted under the conditions of the Schotten-Baumann reaction, replacing benzoyl chloride with p-toluenesulphonyl chloride.

2. 2-(4-hydroxyphenylamino) propanoic Acid Methyl Ester Hydrochloride

2-(4-hydroxyphenylamino) propanoic acid (2.0 g.) was mixed with methanol (200 ml.), and dry hydrogen chloride gas bubbled through for 10 minutes. The resulting solution was refluxed for 8 hours, then allowed to sit overnight. After concentrating the solution to 5 ml. on a water bath and cooling, dry ether (25 ml.) was added and the mixture stirred constantly for 10 minutes. The crude methyl ester hydrochloride, precipitating as a white powder, was filtered and recrystallised from super dry ethanol/dry ether and from ethanol/ethyl acetate. m.p. 185°C. (decomp.)

Calculated for $C_{10}H_{14}O_3NCl$: C, 51.83%; H, 6.09%

Found for $C_{10}H_{14}O_3NCl$: C, 51.98%; H, 6.05%

Attempts at the synthesis of the methyl ester hydrochloride employing diazomethane as prepared from Diazald according to the method of Aldrich Chemicals (Aldrich Chemical Co. Catalogue, 1967-68) were unsuccessful.

B. DEVELOPMENT OF REVERSE ISOTOPE DILUTION TECHNIQUES

1. Simulated Urine Experiment for Preparation of the O-Benzoate of 2-(4-hydroxyphenylamino) propanoic Acid

2-(4-hydroxyphenylamino) propanoic acid (0.5 g.), concentrated hydrochloric acid (2.0 ml.) and water (10 ml.) were subjected to hydrolysis in a Presto Pressure Cooker at 15 p.s.i.

for one hour. After cooling, the sample was benzoylated under the conditions of the Schotten-Baumann reaction, previously described. The resulting white solid was identical in all respects to the reference benzoate. Hydrolysing on a water bath rather than in a pressure cooker yielded the same results. This indicated that the parent acid was stable under the conditions which were to be utilised in urinary experiments.

2. Blank Urine Experiments

a. O-Benzoate of 2-(4-hydroxyphenylamino) propanoic acid

Many variations of the urine hydrolysis procedure previously employed by Sitar (1968) and Thiessen (1969) in these laboratories were attempted to determine whether the O-benzoate could be isolated as a suitable derivative of the suspected metabolite, 2-(4-hydroxyphenylamino) propanoic acid. A sample of this acid (97.1 mg.), blank 24-hour rat urine (10.0 ml.) and concentrated hydrochloric acid (2.0 ml.) were hydrolysed for one hour (Presto Pressure Cooker, 15 p.s.i.) and the pH adjusted to 5.0 with sodium hydroxide. Following continuous ether extraction for 3 hours, the aqueous and ether fractions were separated. The ether was evaporated to dryness and the residue taken up in water. Both this fraction and the aqueous fraction were subjected to benzoylation *via* the Schotten-Baumann reaction. Both fractions yielded oils which could not be recrystallised. By adding only enough concentrated hydrochloric acid to produce a pH of 5.0, and omitting the hydrolysis procedure, a gummy solid was isolated from benzoylation of the aqueous fraction. When run on thin

layer chromatography with water as the solvent, a faint spot was detected at R_f 0.66, the value for the reference benzoate, but most of the material remained at the origin. When the hydrolysis was carried out in a similar manner by heating on a water bath, followed by direct benzoylation, a gummy solid was again produced, which could not be recrystallised from ethyl acetate/ethanol. Bubbling nitrogen through the mixture during hydrolysis was ineffective. This gum was dissolved in ethanol 20%/ether and extracted with dilute hydrochloric acid. The acid layer was adjusted to pH 5.0 and re-extracted with ether, then both ether fractions were run on thin layer chromatography, with water as the solvent. A spot at R_f 0.70, corresponding to the reference benzoate, was detected in the second ether fraction. Both the spot and the reference gave a blue colour with Gibb's reagent. The product could not be isolated from the second ether layer and replacing ether with chloroform presented similar difficulties, even though the benzoate was again detectable on thin layer chromatography.

Purification of the crude benzoate was then attempted using thick layer chromatography. The crude benzoate produced after hydrolysis of blank urine samples on a water bath was dissolved in ethanol and the concentrated solution streaked onto thick layer plates,* which were then developed in water. Bands were noted at the origin (Band I) and at R_f 0.65 to 0.70 (Band II). Both bands were removed from the plate and extracted. The extracts were then concentrated and examined by thin layer chromatography. Band I showed a single spot at the origin, Band

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*Silica gel GF-254 acc. to Stahl was the absorbent employed.

II a single spot at R_f 0.68, indicating separation of unwanted constituents by thick layer chromatography. Recovery of the benzoate was not possible. Ultraviolet scanning of the ethanol solution from the origin (Ultrascan) failed to give a defined peak. The other ethanol fraction showed a wavelength of maximum absorption (λ_{max}) at 228 mμ. The reference benzoate has max at 241 mμ; benzoic acid at 230 mμ. As a result of this study, it was concluded that benzoic acid had been recovered. The experiment was repeated as above, except that the plates were run initially in chloroform. The origin was again removed and examined by thin layer chromatography. The reference benzoate could not be further detected.

Direct benzoylation in the urine was attempted, omitting the hydrolysis procedure. 2-(4-hydroxyphenylamino) propanoic acid (0.5 g.) was dissolved in water (10 ml.) and concentrated hydrochloric acid (2 ml.), then an equal volume of blank urine (12 ml.) added. Benzoylation was carried out as described previously. A yellow solid resulted, but on thin layer in water diffused from the origin to R_f 0.70, although a distinct spot at R_f 0.70 was detectable. Blank urine was found to have R_f values in the same solvent system of 0.30, 0.53, 0.68 and 0.90. Reference benzoate in blank urine gave values of 0.33, 0.52, 0.70 and 0.90. Benzoylation of blank urine produces a solid product, diffusing on thin layer in water with no distinct spots. These results indicated formation of a mixture of compounds which could not readily be separated, and therefore the method was rejected.

In a final experiment, 2-(4-hydroxyphenylamino) propanoic acid was added directly to blank urine and benzoylated with benzoyl chloride in the presence of sodium hydroxide. The solid was filtered and boiled in water to remove water-soluble impurities. On cooling, a white crystalline precipitate appeared. This precipitate was found to melt at 122°C., to have an equivalent weight of 122.0 (determined potentiometrically) and to give an anilide derivative melting at 159°C., thus identifying the product as benzoic acid. The yellow compound which did not dissolve in boiling water was recrystallised from dilute ethanol, and found to be the crude benzoate desired (m.p. 200-202°C., decomp.). However, the product could not be further purified.

Thus, no suitable method was found to isolate the benzoate derivative of the p-hydroxy acid from blank urine in a pure form, and therefore alternative reverse isotope dilution procedures were sought.

b. Methyl ester hydrochloride of 2-(4-hydroxyphenylamino) propanoic acid

All experiments were initiated as follows: 2-(4-hydroxyphenylamino) propanoic acid (200 mg.), blank urine (10 ml.), water (10 ml.) and concentrated hydrochloric acid were subjected to hydrolysis on a water bath for one hour. The pH was adjusted to 5.0 with ammonia, and the sample lyophilised overnight on a Virtis lyophiliser. The freeze-dried residue was refluxed for 8 hours in methanol (200 ml.) through which dry hydrogen chloride gas had been bubbled for 10 minutes, then reduced in volume (to 5 ml.) by evaporation on a water bath. Various methods were

employed to isolate the methyl ester hydrochloride at this point:

(i) The concentrate was taken up in water (20 ml.), neutralised with ammonia, extracted twice with ether and the ether fraction washed with a small amount of sodium bicarbonate solution and dried with sodium sulphate. Dry hydrogen chloride gas was bubbled through the dried ether solution to precipitate the methyl ester hydrochloride, but an oily residue resulted which could not be recrystallised from petroleum ether/ethanol or ethanol. Attempts to prepare an O-benzoate or O-tosylate of the crude methyl ester hydrochloride were also unsuccessful. An aqueous sample (treated exactly as above, but without urine) behaved identically. Examination of the ether extracts from both samples on thin layer in isopropanol 15%/benzene gave R_f values of 0.55 to 0.70, turning purple, then brown, with Gibb's reagent.

(ii) Dry ether (25 ml.) was added in an attempt to precipitate the crude methyl ester hydrochloride, as described in the preparation of the compound in question. A white solid precipitated. On spotting both this solid and the liquid on thin layer and developing in butanol, the methyl ester hydrochloride was found to be in the methanol/ether fraction. Evaporation of this fraction to dryness, however, yielded a dark brown liquid.

(iii) The concentrate was streaked onto thick layer plates and developed in butanol. The band corresponding to the methyl ester hydrochloride (R_f 0.85) was removed, boiled in ethanol, filtered, and ether added dropwise to precipitate the product. Again, no product could be isolated.

(iv) A silica gel column was prepared from a slurry in ethyl acetate. The concentrate was pipetted onto the column and eluted with ethyl acetate. Six fractions were collected. When tested with Gibb's reagent, the first three fractions gave a positive colour reaction for the methyl ester hydrochloride. On examination of these fractions on thin layer chromatograms in butanol, the product appeared only in the first two fractions. Evaporating these fractions to dryness produced a badly decomposed solid.

(v) A second column was prepared from a slurry of silica gel in benzene. The concentrate, absorbed on silica gel, was added to the top of the column. The following solvents were run through the column (50 ml. each): benzene; benzene/ethyl acetate (3:1); ethyl acetate; ethyl acetate/ethanol (3:1). Only the final eluting fraction gave a positive test for the methyl ester hydrochloride on thin layer in butanol, using Gibb's reagent for detection. This fraction, plus the contents of the column were mixed with methanol (100 ml.) through which dry hydrogen chloride gas had been bubbled for 5 minutes. After filtering, the solution was evaporated to dryness. Attempts to recrystallise the residue from ethyl acetate/ethanol were unsuccessful. As some solid did not dissolve, both this solid and the filtrate were examined on thin layer chromatograms, using butanol as the developing solvent. The methyl ester hydrochloride could not be detected in the solid portion. However, a spot corresponding to the ester was located in the filtrate. The filtrate was evaporated to 3 ml. and placed onto

a silica gel column slurried with benzene. This sample was eluted with butanol (40 ml.) and with ethyl acetate (2 x 40 ml.) to remove impurities, then the column contents were extracted with ethanol, filtered and ethyl acetate added in an attempt to precipitate the methyl ester hydrochloride. Examination of the concentrated extract indicated that the compound was still in the solution. Evaporation of this solution to dryness produced a dark brown oil, which could not be further purified. The solid which precipitated and was insoluble in ethyl acetate/ethanol was thought to be ammonium chloride, formed in the original neutralisation process following hydrolysis.

C. ANIMAL EXPERIMENTS

1. Urinary Excretion Experiments

a. Collection of urine

Male Sprague-Dawley rats (350-400 g.) were each injected intraperitoneally with an aqueous solution (1 ml.) of N-isopropylaniline-^{14}C hydrochloride (15 mg. free base/Kg.). The rats were each isolated in metabolism cages (Acme), and urine samples collected after 24 hours. The urine was then centrifuged and either made up to 25.0 ml. with water, or freeze-dried and made up to 10.0 ml. with water. The samples were frozen until used for chromatography, hydrolysis studies, or reverse isotope dilution experiments.

b. Paper chromatographic studies of conjugated metabolites

Samples of urine (0.1 ml.) were streaked onto chromatograph paper and developed by ascending chromatography in

chloroform (stationary phase); n-butanol/ammonia 20:1 (mobile phase) (solvent system I) and in n-butanol/glacial acetic acid/ water 8:2:2 (solvent system II). After development, the paper strips were scanned for radioactivity. The latter system indicated three metabolites, I (R_f 0.45, major peak), II (R_f 0.25) and III (R_f 0.69).

Larger quantities of the peak I and II metabolites were obtained by streaking chromatograms (6 in. wide) with urine (0.5 ml.) and developing in solvent system II. Portions of these chromatograms were scanned for radioactivity and the areas corresponding to the metabolites eluted from the papers with water (100 ml.). The solutions were then lyophilised.

(i) Acid hydrolysis of the urine

Hydrolysis of the urine sample (1 ml.) was carried out under acid conditions (five drops of concentrated hydrochloric acid) on a water bath for one hour. The hydrolysate (0.1 ml.) was streaked onto chromatograph paper, run in solvent system II and scanned for radioactivity.

(ii) β-glucuronidase hydrolysis of the urine

Urine samples were hydrolysed with β-glucuronidase by incubating urine (0.5 ml.) in pH 6.8 phosphate buffer (0.5 ml.) and β-glucuronidase solution (1 ml.) on a metabolic shaker for four hours at 37°C. Aliquots of the hydrolysed solutions were examined on paper chromatograms in solvent system II.

The freeze-dried residue, corresponding to peak I, the major metabolite, was taken up in water (5 ml.), then pH 6.8 phosphate buffer (5 ml.) and β-glucuronidase solution (2 ml.)

added. Following incubation, the mixture was freeze-dried. The residue was taken up in ethanol, filtered, and the filtrate reduced to 1 ml. under nitrogen. The entire solution was streaked onto paper chromatograms and developed in solvent system II.

(iii) Sulphatase hydrolysis of the urine

The single peak resulting from the β-glucuronidase experiment just described was eluted off the paper with water and freeze-dried. The residue was taken up in water (3 ml.), pH 5.0 acetate buffer (2 ml.) and sulphatase solution (2 ml.) and incubated as for β-glucuronidase hydrolysis. The residue, after freeze-drying, was dissolved in ethanol, chromatographed in solvent system II and scanned for radioactivity.

c. Reverse isotope dilution studies

An aliquot of urine (10.0 ml.), concentrated hydrochloric acid (2 ml.), water (5 ml.) and 2-(4-hydroxyphenylamino) propanoic acid (0.2005 g.) were hydrolysed, neutralised, and extracted with ether as described for blank urine experiments. The aqueous portion was freeze-dried and the residue refluxed in methanol/hydrochloric acid for eight hours. The solvent was evaporated off, the residue taken up in water, neutralised, ether extracted, and the ether layer washed with sodium bicarbonate solution, dried with sodium sulphate, and concentrated to 5 ml. Portions of this concentrate were streaked onto thin layer plates and developed in isopropanol 15%/benzene; ethyl acetate 20%/petroleum ether (66-75°C.); and water. The bands visible under short-wavelength ultraviolet light were

removed from the plates and prepared for liquid scintillation counting. Blank bands of similar width were counted concurrently. Attempts to isolate the methyl ester hydrochloride as a crystalline solid for liquid scintillation counting were unsuccessful.

In an attempt to identify the minor metabolite (II) detected on scanning of chromatographic strips, the residue from this peak (previously eluted from the paper and freeze-dried) was taken up in water (20 ml.), then 2-(4-hydroxyphenylamino) propanoic acid (0.2 g.) and concentrated hydrochloric acid (2 ml.) added. Following hydrolysis on a water bath for one hour, the sample was neutralised to pH 7.0 with sodium hydroxide and freeze-dried. This residue was refluxed in methanol/hydrochloric acid for eight hours, after filtering off the insoluble sodium chloride. The methanol solution was concentrated to 5 ml., then dry ether added in an attempt to form the crude methyl ester hydrochloride. An oil formed, which on dissolving in water and lyophilising, could not be induced to solidify. Portions of this residue were streaked onto thin layer plates and developed in butanol, in isopropanol 15%/benzene and in water. The spots which corresponded to the R_f values of the reference methyl ester hydrochloride were removed from the plates and counted for radioactivity. Attempts to recrystallise the remainder of the oily residue by addition of reference methyl ester hydrochloride were unsuccessful.

2. Biliary Excretion Experiments

a. Collection of bile

Male Sprague-Dawley rats (400-450 g.) were

anaesthetised with urethane, administered intraperitoneally (780 mg./Kg.). The bile duct was isolated and cannulated, and either N-isobutylaniline-^{14}C hydrochloride (4.0 mg.) or N-isopropylaniline-^{14}C hydrochloride (3.0 mg.) in water injected into the femoral vein. Bile was collected for periods up to five hours, then made up to 10.0 ml. in a volumetric flask and stored at 0°C.

b. Paper chromatographic studies of conjugated metabolites

A sample of bile (0.1 ml.) was streaked onto chromatograph paper and developed by ascending chromatography in either solvent system I or II, then scanned for radioactivity. Larger amounts of the metabolites were separated by streaking bile (0.5 ml.) from rats injected with N-isopropylaniline-^{14}C hydrochloride and developed in solvent system II. Sections of the chromatograms were scanned and the radioactive peaks eluted with water (100 ml.), freeze-dried, and reconstituted in 1.0 ml. of water. Separation was confirmed by rechromatographing the separated peaks and scanning. At least two metabolites were indicated in approximately equal amounts: peak I (origin) and peak II (R_f 0.26).

(i) Hydrolysis of conjugates

Experiments were carried out only on the bile of rats injected with N-isopropylaniline-^{14}C hydrochloride. A sample of bile (1.0 ml.) was hydrolysed on a water bath after acidifying with concentrated hydrochloric acid (five drops). The hydrolysate (0.1 ml.) was streaked onto paper, developed in solvent system II,

and scanned for radioactivity. Bile samples were also hydrolysed with β-glucuronidase by incubating bile (0.5 ml.) with pH 6.8 phosphate buffer (0.5 ml.) and β-glucuronidase solution (1.0 ml.) on a shaker for four hours at 37°C. The hydrolysates were examined by paper chromatography.

(ii) Identification of peak II by modified reverse isotope dilution

In a subsequent experiment with N-isopropylaniline-^{14}C hydrochloride, the residue from peak II in bile (with the same R_f value as peak II in urine) was subjected to a modified form of reverse isotope dilution similar to that described under urinary experiments (page 43). The freeze-dried residue, 2-(4-hydroxyphenylamino) propanoic acid (0.3 g.), water (20 ml.) and concentrated hydrochloric acid (2 ml.) were hydrolysed for one hour on a water bath. Following neutralisation with ammonia, the sample was freeze-dried. The residue was refluxed in methanol-hydrochloric acid for eight hours, and the volume reduced to 5 ml. The precipitating solid was recrystallised from ethyl acetate/ethanol and compared to a known sample of the compound in question. The melting point of the solid was over 300°C., and the R_f value did not correspond to reference methyl ester hydrochloride on thin layer chromatography in butanol. Evaporating the remaining solution to dryness yielded a tarry residue.

D. CHROMATOGRAPHY

1. Thin Layer Chromatography

Thin layer chromatography was employed extensively,

both in attempts to separate suspected metabolites from extraneous material and to compare suspected metabolites to reference standards whose R_f values were known. Thick layer plates (1.00 mm.) and thin layer plates (0.25 mm.) were prepared using fluorescent silica gel (silica gel GF-254 acc. to Stahl) on a Quickfit apparatus, their thickness governed and kept constant by a fixed aperture spreader. Material spotted on these plates was detected by its quenching of fluorescence as seen when the plates were subjected to short-wave ultraviolet light, or by colour reactions when sprayed with Gibb's reagent. When further purification was desired, bands or spots were scraped from the plates and extracted with ethanol. The silica gel was filtered off and the solution concentrated to a volume suitable for further chromatographic or radioactivity studies.

2. Paper Chromatography

Paper chromatography was employed in animal experiments to determine the properties of the heretofore unidentified polar metabolite(s) of N-isopropylaniline. All urine and bile preparations were spotted or streaked onto Whatman No. 3 chromatographic paper utilising an Agla micrometer syringe. The paper systems were then developed in either of the following solvent systems:

I: Chloroform (stationary phase); n-butanol/ammonia 20:1 (mobile phase).

II: n-Butanol/glacial acetic acid/water 8:2:2.

E. DETECTION OF RADIOACTIVITY

1. Liquid Scintillation Counting

a. Scintillation fluid

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

POP (2,5-diphenyloxazole)	2.0 g.
bis-MSB [bis-(o-methyl-styryl)-benzene]	0.04 g.
Isopropanol (Baker Reagent)	400.0 ml.
Toluene (Fisher Reagent) q.s.	1000.0 ml.

b. Internal standard

In order to convert counts per minute (cpm.) to disintegrations per minute (dpm.) in liquid scintillation counting, 0.1 ml. of benzoic acid-^{14}C* (approximately 3000 dpm./0.1 ml., accurately known) in toluene was added.

c. Measurement of radioactive samples

Liquid samples (0.10 to 0.40 ml.) were measured directly into counting vials using an Agla micrometer syringe and scintillation fluid (10.0 ml.) added prior to counting in a Nuclear Chicago Unilux Liquid Scintillation Counter. If the scintillation fluid was not completely miscible with the liquid samples, thixotropic gel (250 mg./vial) was added. All counts were converted to dpm. by the addition of internal standard. Samples removed from thin layer plates were extracted from the silica gel with ethanol, the silica gel removed by filtration,

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

scintillation solvent (10.0 ml.) added, and the samples counted as previously described.

d. Minimum radioactivity

In this project, the minimum valid radioactivity per sample in the glass counting vial was taken to be a count rate of double the background count. Since the background count averaged 35 cpm., and the efficiency of the instrument was about 70 percent, the minimum valid radioactivity required would be 50 dpm. per sample in the glass counting vial. Radioactivity below 50 dpm. which, including background, would yield a count rate of 70 cpm., was not considered to be conclusive or significant.

2. Scanning of Chromatograms

Paper chromatograms were scanned for radioactivity on a Nuclear Chicago Actigraph III. Results thus obtained were qualitative, unless the resulting scans were integrated with a Hughes-Owens Aristo Planimeter.

The following instrumental settings were kept constant for all scans:

High voltage - 1050 V

Time constant - 10 sec.

Collimator slit width - 12 mm.

Helium-butane pressure - 7 p.s.i.

Count rate - 150 cpm., unless otherwise specified.

Scan speed - 60 cm./hr., unless otherwise specified.

F. MATERIALS

Dry hydrogen chloride gas was generated by the action of sulphuric acid on ammonium chloride, with a sulphuric acid

drying trap placed between the generating flask and the outlet tube.

2-bromopropionic acid and p-aminophenol, employed in the synthesis of 2-(4-hydroxyphenylamino) propanoic acid, were both technical grades supplied by Aldrich Chemical Co. Inc., Milwaukee, Wisconsin.

Silica gel columns were prepared from a slurry of reagent grade silica gel (BDH laboratory chemicals) in a suitable organic solvent. The column of Quickfit glass measured 20 cm. in length, with a 2 cm. diameter.

Gibb's reagent, 2,6-dichloroquinone-4-chloroimide (Aldrich Chemical Co. Inc.) was employed for chromatographic spraying as a one percent solution in ethanol.

N-isopropylaniline-^{14}C hydrochloride (uniformly labelled) was prepared previously in these laboratories and had a specific activity of 5.19586×10^5 dpm./mg. (Thiessen, 1969).

N-isobutylaniline-^{14}C hydrochloride (uniformly labelled) was prepared previously in these laboratories and had a specific activity of 3.8048×10^5 dpm./mg. (Thiessen, 1969).

The pH 6.8 phosphate buffer* employed in β-glucuronidase hydrolysis studies 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 water to one litre.

The pH 5.0 acetate buffer* employed in sulphatase hydrolysis experiments was prepared by mixing 73.8 ml. of molar acetic acid with 50.0 ml. of molar sodium hydroxide and diluting with water to one litre.

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*Long, E. (1961) Biochemist's Handbook, E. and F.N. Spon., London, p.32.

β-glucuronidase, with an activity of 55,000 units/g., was supplied by Sigma Chemical Co., St. Louis, Missouri. This enzyme was dissolved in water and stabilised with chloroform to give an activity in solution of 55 units/ml. To verify activity, two solutions were prepared, each containing phenolphthalein glucuronide, 2 mg. (Sigma Chemical Co.) and pH 6.8 phosphate buffer (1.0 ml.). The β-glucuronidase solution (1.0 ml.) was added to one of the flasks. Both solutions were incubated on a shaker for three hours at 37°C., then three drops of ammonia added to test for colour reaction. The sample containing the enzyme turned pink, indicating that the conjugate had been cleaved to yield free phenolphthalein. The blank showed no colour reaction.

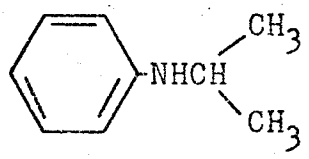
Sulphatase, with an activity of 10 units/mg., was also supplied by Sigma Chemical Co. This enzyme was dissolved in water to give a solution with 50 units/ml. of sulphatase activity. To verify activity, two solutions were prepared, each containing p-aminophenyl sulphate, 2 mg. (synthesised in these laboratories according to the method of Burkhardt and Wood, 1929) in pH 5.0 acetate buffer (3.0 ml.). Sulphatase solution (1.0 ml.) was added to one flask. Both solutions were incubated on a shaker for three hours at 37°C., then tested for free p-aminophenol by coupling with phenol to yield an indophenol dye according to the procedures of Brodie and Axelrod (1948), and Welch and Conney (1965), using the following phenol reagent: 0.2N. sodium hydroxide (8 parts); phenol solution (1 g. phenol in 88 ml. water, 2 parts); bromine solution (100 ml. N. sodium carbonate

plus 15 ml. of a 10% $^{w}/_{v}$ solution of bromine in water, 2 parts). On the introduction of this reagent (3.0 ml.) to the sample containing the enzyme, a distinct, characteristic blue colour was produced, indicating that the conjugate had been cleaved. The blank showed no colour reaction.

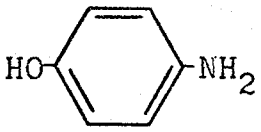
VI. RESULTS AND DISCUSSION

A. IDENTIFICATION OF 2-(4-HYDROXYPHENYLAMINO) PROPANOIC ACID AS A URINARY METABOLITE OF N-ISOPROPYLANILINE

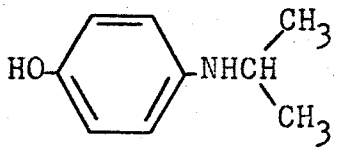
Alexander and Sitar (1969) were able to identify 70 percent of the metabolites of N-isopropylaniline (LIII.) as p-aminophenol (LIV.) and p-hydroxy-N-isopropylaniline (LV.) in the urine of rats.



LIII.

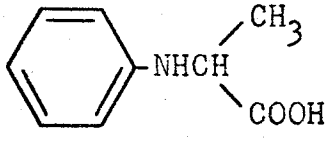


LIV.



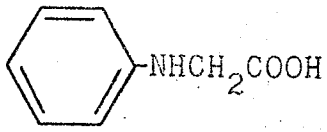
LV.

These two metabolites corresponded favourably to the amount of ether-soluble metabolites which could be extracted after acid hydrolysis. These authors concluded that the unidentified metabolite(s) were polar in nature. However, examination of the urine for 2-phenylaminopropanoic acid (LVI.) (Sitar, 1968 and Thiessen, 1969), which was suggested from related studies on N-sec-butylaniline (Alexander et al., 1968), revealed that this compound was not a metabolite.



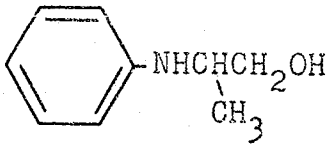
LVI.

Similarly, Thiessen (1969), using paper chromatography, demonstrated that the polar metabolites were acidic in nature. The latter author also eliminated N-phenylglycine (LVII.) as a potential polar metabolite.



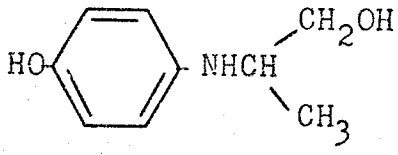
LVII.

All the evidence suggested that the unidentified metabolites involved either formation of an alcohol by side chain oxidation (2-phenylamino-1-propanol, LVIII.):

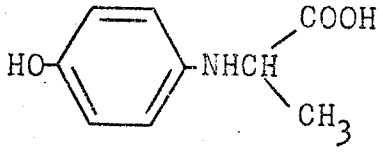


LVIII.

or simultaneous ring and side chain oxidation to form the corresponding phenolic alcohol (LVIX.) or phenolic acid (LX.):



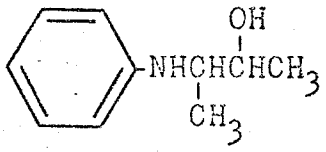
LVIX.



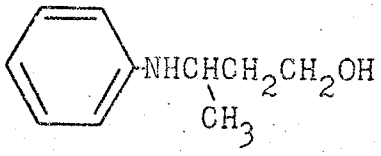
LX.

Although the oxidation of side chains to alcohols is well known in the alkylbenzenes (El Masri et al., 1956) and barbiturates

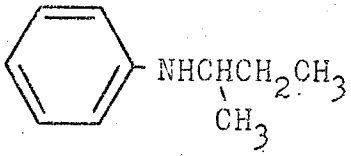
(Goldschmidt and Wehr, 1957), this reaction seemed unlikely in view of the fact that 3-phenylamino-2-butanol (LXI.) or 3-phenylamino-1-butanol (LXII.) could not be demonstrated as metabolites in the metabolism of N-sec-butylaniline (LXIII.):



LXI.



LXII.

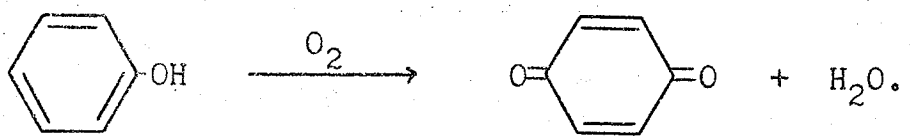


LXIII.

Therefore, 2-(4-hydroxyphenylamino) propanoic acid (referred to subsequently as the p-hydroxy acid) appeared to be the most likely prospect.

This compound could be readily prepared from α-bromopropionic acid and p-aminophenol, but proved to be quite unstable and readily darkened on exposure to air due to oxidation. A quantitative estimation of the p-hydroxy acid in the urine by reverse isotope dilution was desirable to determine if this compound represented the unidentified urinary metabolites of N-isopropylaniline. Considerable difficulty was encountered in derivative preparation for reverse isotope dilution studies,

presumably due again to the instability of the compound. Oxidative reactions are well known among phenols (Noller, 1965), especially under alkaline conditions, where the hydroxyl group can supply electrons to the nucleus, forming complex mixtures of quinonoid compounds. Phenol itself forms quinone:



Quinone forms a bright red addition product with phenol, known as phenoquinone, accounting for the discolouration of phenol, especially in solution.

Although it was possible to prepare the O-benzoate in aqueous solution, when this procedure was attempted using urine, no derivative could be isolated. While the product could be demonstrated to be present in small amounts chromatographically, there was insufficient present to isolate. Another problem encountered was that blank urine itself could be benzoylated since components with free amino or hydroxyl groups (urea, uric acid, creatinine and amino acids) are prone to react. When the impure benzoate (containing benzoyl chloride in excess, and urinary constituents) was heated in water to remove water-soluble impurities, benzoic acid was isolated. The presence of this compound was confirmed through melting point, potentiographic studies, thin layer chromatography and derivatisation studies. Attempts to isolate the O-benzoate by thick layer chromatography

also proved unsuccessful. Although small amounts of the derivative could be demonstrated chromatographically, examination of the ultraviolet spectra of the bands resulting from separation of the reaction mixture on thick layer chromatograms (Table I) showed that the various wavelengths of maximum absorption (λ_{max}) did not correspond to the reference benzoate. Therefore, it was considered that the O-benzoate was not the derivative of choice for urinary reverse isotope dilution studies.

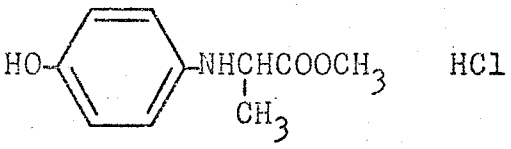
Table I. R_f values and wavelengths of maximum absorption of bands of the O-benzoate reaction mixture isolated on thin layer chromatograms developed in water.

Band	R_f value	λ_{max} (mμ)
1	0	233
2	0.05-0.10	235
3	0.10-0.33	232
4	0.90-1.00	-
Reference benzoate	0.66	241

Similarly, attempts to prepare the O-acetate and O-tosylate derivatives of the p-hydroxy acid were also unsuccessful. Under the reaction conditions considerable darkening of the reaction mixture occurred, with tarry and gummy products being obtained. It seemed likely that oxidation of the p-hydroxy acid was occurring, which could account for the inability to isolate these particular derivatives.

The methyl ester hydrochloride of the p-hydroxy acid

(LXIV.) was the most convenient derivative prepared, as it could be synthesised entirely under acid conditions.



LXIV.

Ease of synthesis and recrystallisation would have made this derivative ideally suited for reverse isotope dilution experiments, but isolating the compound from urine proved difficult. All methods described under "Blank Urine Experiments" (page 37-38) in which the methanol/hydrochloric acid solutions were neutralised above pH 5 and ether-extracted in an attempt to purify the methyl ester caused oxidation of the product. Theoretically, the methyl ester will be less water-soluble and more ether-soluble than the free acid, enabling it to be separated from aqueous urinary constituents. However, this derivative still has an unprotected hydroxyl group capable of oxidising to quinonoid products, which could explain the tarry residues constantly obtained in the blank urine experiments. The separation of the methyl ester hydrochloride on a silica gel column, although unsuccessful, should be further pursued but conditions throughout any such experiment must be kept acid to prevent decomposition.

Since the methyl ester hydrochloride of the p-hydroxy acid (LXIV.) could not be isolated in crystalline form, the residue from the reverse isotope dilution experiment was examined by thin layer chromatography. Three distinct radioactive

bands could be detected (Table II).

Table II. R_f values and radioactivity (cpm.) of ether extract of urine of rats injected with N-isopropylaniline-^{14}C, following preparation of the methyl ester of 2-(4-hydroxyphenylamino) propanoic acid, on thin layer chromatograms.

Band	R_f values*		Radioactivity (cpm.)
	Ether Extract	Reference methyl ester hydrochloride**	
I	0.60-0.72	0.55-0.70	433
II	0.47-0.51		162
III	0.36-0.40		331

*Solvent system: Benzene/Isopropanol 85:15

**R_f values of reference compounds are recorded in Appendices A, B and C.

Band I was found to possess the chromatographic properties identical to those of the authentic methyl ester hydrochloride. The presence of this ester was further confirmed by thin layer chromatography in two additional solvent systems (Table III).

Table III. R_f values and radioactivity (cpm.) of ether extract of urine of rats injected with N-isopropylaniline-^{14}C following preparation of the methyl ester of 2-(4-hydroxyphenylamino) propanoic acid on thin layer chromatograms in two additional solvent systems.

Solvent system	Band corresponding to methyl ester hydrochloride		R_f of reference methyl ester hydrochloride	Blank band (cpm.)
	R_f	cpm.		
Ethyl acetate 20%/ petroleum ether	0.20	486	0.20	43
Water	0.76	145	0.76	42

These results establish 2-(4-hydroxyphenylamino) propanoic acid as a metabolite of N-isopropylaniline in the rat. The nature of bands II and III (Table II) was not further investigated. These bands may represent the known metabolites of N-isopropylaniline (p-hydroxy-N-isopropylaniline and p-aminophenol), additional unidentified metabolites, or they may have been caused by oxidation of the methyl ester hydrochloride. The latter alternative is favoured, since the R_f values of reference p-hydroxy-N-isopropylaniline and p-aminophenol (Appendix B) do not correspond to the values obtained for bands II and III (Table II).

B. CONJUGATION STUDIES ON THE URINARY METABOLITES OF N-ISOPROPYLANILINE

Examination of 24-hour urine samples of rats injected with N-isopropylaniline-^{14}C by ascending paper chromatography indicated the presence of at least two, and possibly three,

metabolites.* As illustrated in Table IV and Figure I, a better degree of separation was achieved in the butanol/acetic acid/water system than in the chloroform/butanol/ammonia system. The movement of compounds in these systems suggests the presence of acidic substances (Betts, 1964).

Table IV. R_f values of metabolites present in the 24-hour urine samples of rats injected with N-isopropylaniline-^{14}C on ascending paper chromatography.

	Solvent system	R_f values
I.	Chloroform (stationary phase); n-butanol/ammonia 20:1 (mobile phase)	0-0.27 (diffusion, poor separation)
*II.	n-Butanol/glacial acetic acid/water 8:2:2	**0.45 and 0.25

*See Figure I.

**A very small peak (peak III) was also noted, with R_f 0.69.

Integration of peak areas in Figure I gave percentages very similar to those reported for metabolites of N-isopropylaniline by Alexander and Sitar (1969), employing reverse isotope dilution techniques. The comparison is given in Table V.

*Subsequent experiments in these laboratories have indicated three distinct metabolites by ascending paper chromatography.

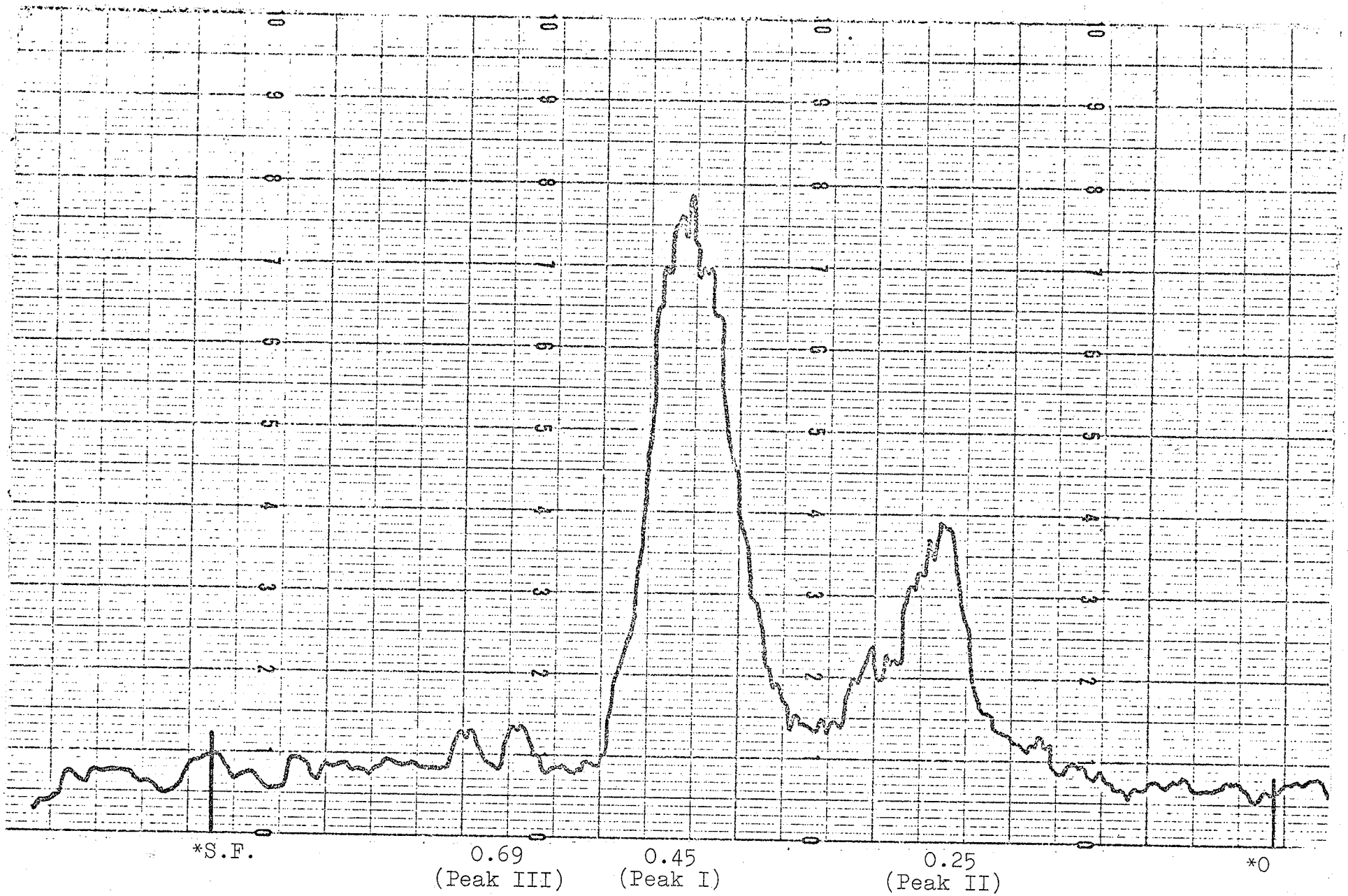


Figure I. Scanning of paper chromatogram spotted with 24-hour urine of rat injected with N-isopropylaniline - ^{14}C (300 cpm.)

*S.F. - solvent front
0 - origin

Table V. Comparison of percentages of metabolites of N-isopropylaniline in 24-hour rat urine by reverse isotope dilution and by paper chromatographic separation.*

Metabolite	Reverse Isotope Dilution (%)**	Peak	Radioactive scanning (%)	R_f value
p-hydroxy-N-isopropylaniline	61	I	65	0.45
Unidentified polar metabolites	30	II	29.8	0.25
p-aminophenol	8	III	5.2	0.69

*This represents conjugated metabolites since no hydrolysis of the urine was carried out.

**% metabolites present in 24-hour urine samples.

It seems reasonable to suspect that the major peak (peak I, R_f 0.45, 65 percent, Figure I) should correspond to p-hydroxy-N-isopropylaniline, the second peak (peak II, R_f 0.25, 30 percent, Figure I) could represent various polar metabolites, and the very small peak (peak III, R_f 0.69, 5.2 percent, Figure I) could potentially correspond to p-aminophenol.

Chromatography of acid-hydrolysed 24-hour urine samples showed a shift in the major peak from 0.45 to 0.77, as illustrated in Table VI and Figure II.

Table VI. R_f values of 24-hour urine samples of rats injected with N-isopropylaniline-^{14}C by ascending paper chromatography, following hydrolysis.

Solvent system	*Method of hydrolysis	R_f values	
		Urine sample	p-hydroxy-N-isopropylaniline
n-Butanol/ glacial acetic acid/water 8:2:2	Concentrated hydrochloric acid	0.77 and 0.25	0.77
	β-glucuronidase	0.45 and 0.23	
	Unhydrolysed urine	0.45 and 0.23	

*See Figures II and III.

It would appear that the larger peak (R_f 0.45) does, in fact, yield p-hydroxy-N-isopropylaniline on acid hydrolysis. Attempts to hydrolyse the urine with β-glucuronidase did not seem to have any effect in that the peak did not shift, but remained at 0.45 (Figure III). Larger quantities of the peak I metabolite (R_f 0.45) were isolated by preparative paper chromatography and the freeze-dried residue was hydrolysed with β-glucuronidase and sulphatase. Results are given in Table VII and Figures IV and V.

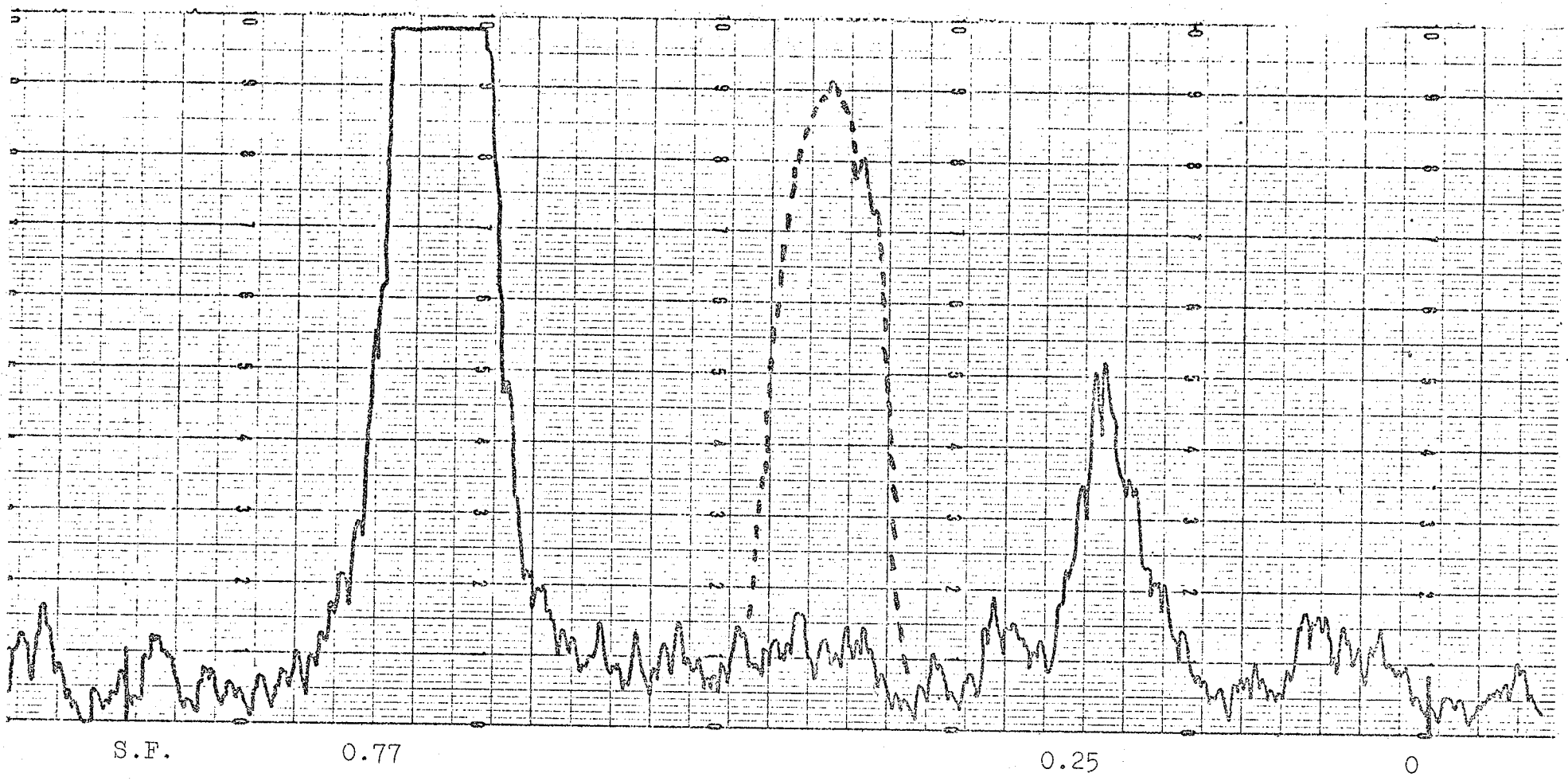


Figure II. Scanning of paper chromatogram spotted with N-isopropylaniline - ^{14}C urine sample, following hydrolysis with concentrated hydrochloric acid.

------ Indicates position of peak prior to acid hydrolysis

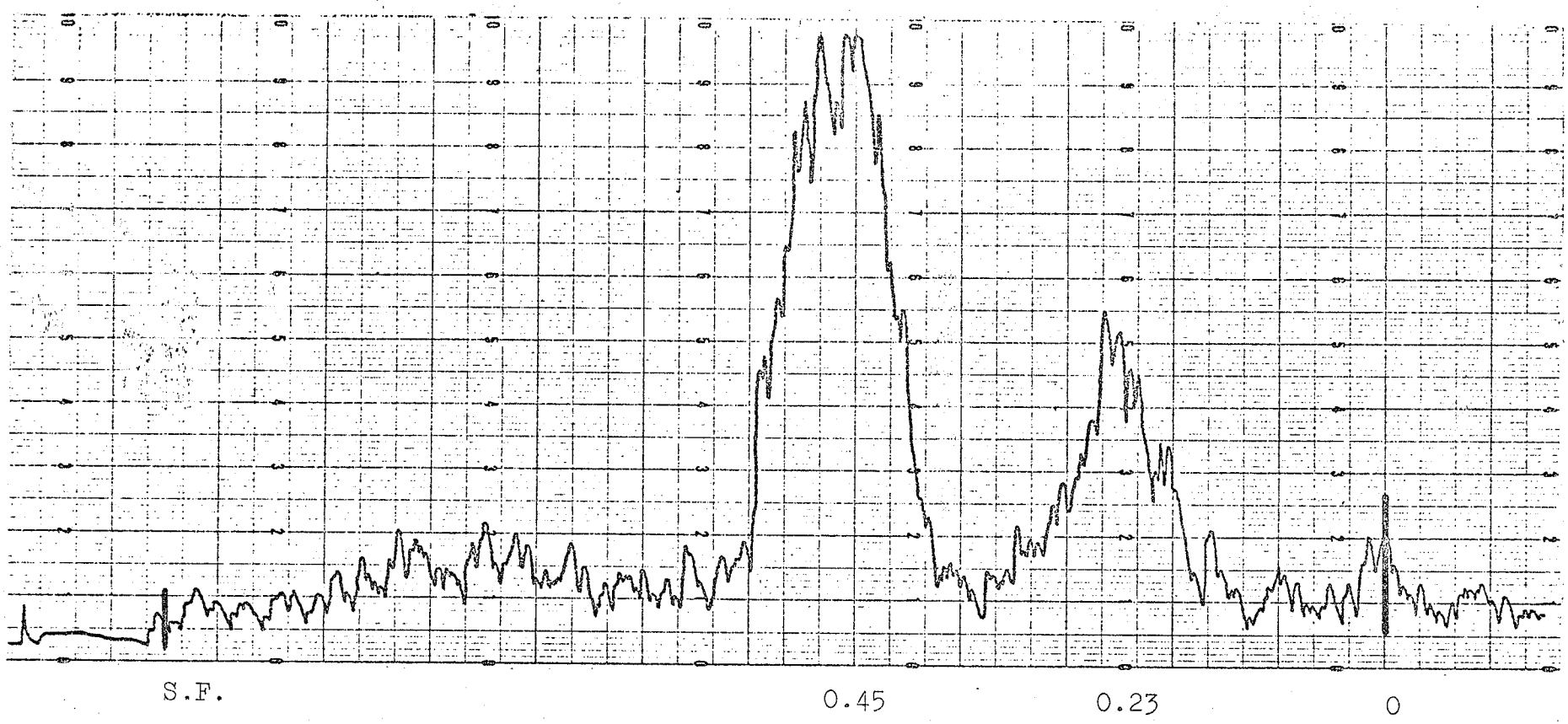


Figure III. Scanning of paper chromatograph spotted with N-isopropylaniline - ^{14}C urine sample, following hydrolysis with β-glucuronidase.

Table VII. R_f values of freeze-dried residue corresponding to peak I (R_f 0.45) by ascending paper chromatography, following hydrolysis.

Solvent system	*Method of hydrolysis	R_f values	
		Hydrolysed sample	Unhydrolysed sample
n-Butanol/ glacial acetic acid/water 8:2:2	β-glucuronidase	0.47	0.45
	Sulphatase	0.46	0.45

*See Figures IV and V.

As indicated in Table VII, incubation of the freeze-dried residue corresponding to peak I with both β-glucuronidase (Figure IV) and sulphatase (Figure V) failed to cleave this conjugate. Unless the conjugate is resistant to the action of these two enzymes, it would appear that a different form of conjugation must be involved, perhaps with glutathione or glycine (Parke, 1968f.). Since the two enzymes were proven active by cleavage of known conjugates (phenolphthalein glucuronide and p-aminophenyl sulphate), the latter alternative is favoured, although further work is necessary before any definite conclusion can be drawn.

C. IDENTIFICATION OF PEAK II IN URINE AS 2-(4-HYDROXYPHENYLAMINO) PROPANOIC ACID

The identification of the p-hydroxy acid as a metabolite of N-isopropylaniline was further confirmed by the examination of peak II (Figure I) obtained by separation of the urinary metabolites using paper chromatography. Although the crystalline methyl ester hydrochloride again could not be isolated in reverse

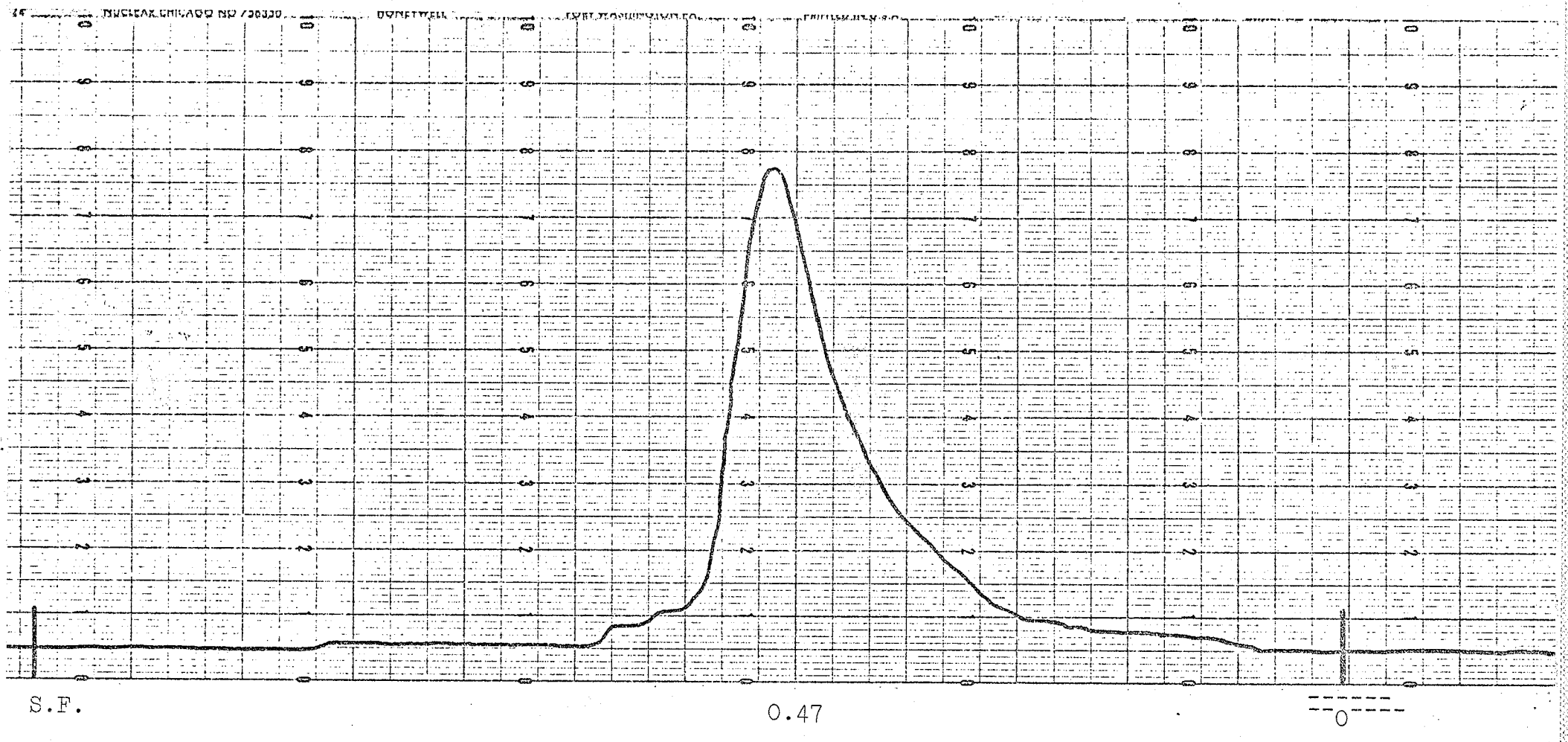


Figure IV. Scanning of paper chromatogram spotted with eluent of peak I (R_f 0.45, Figure I), following hydrolysis with β-glucuronidase. (1000 cpm., 325 cm./hr.).

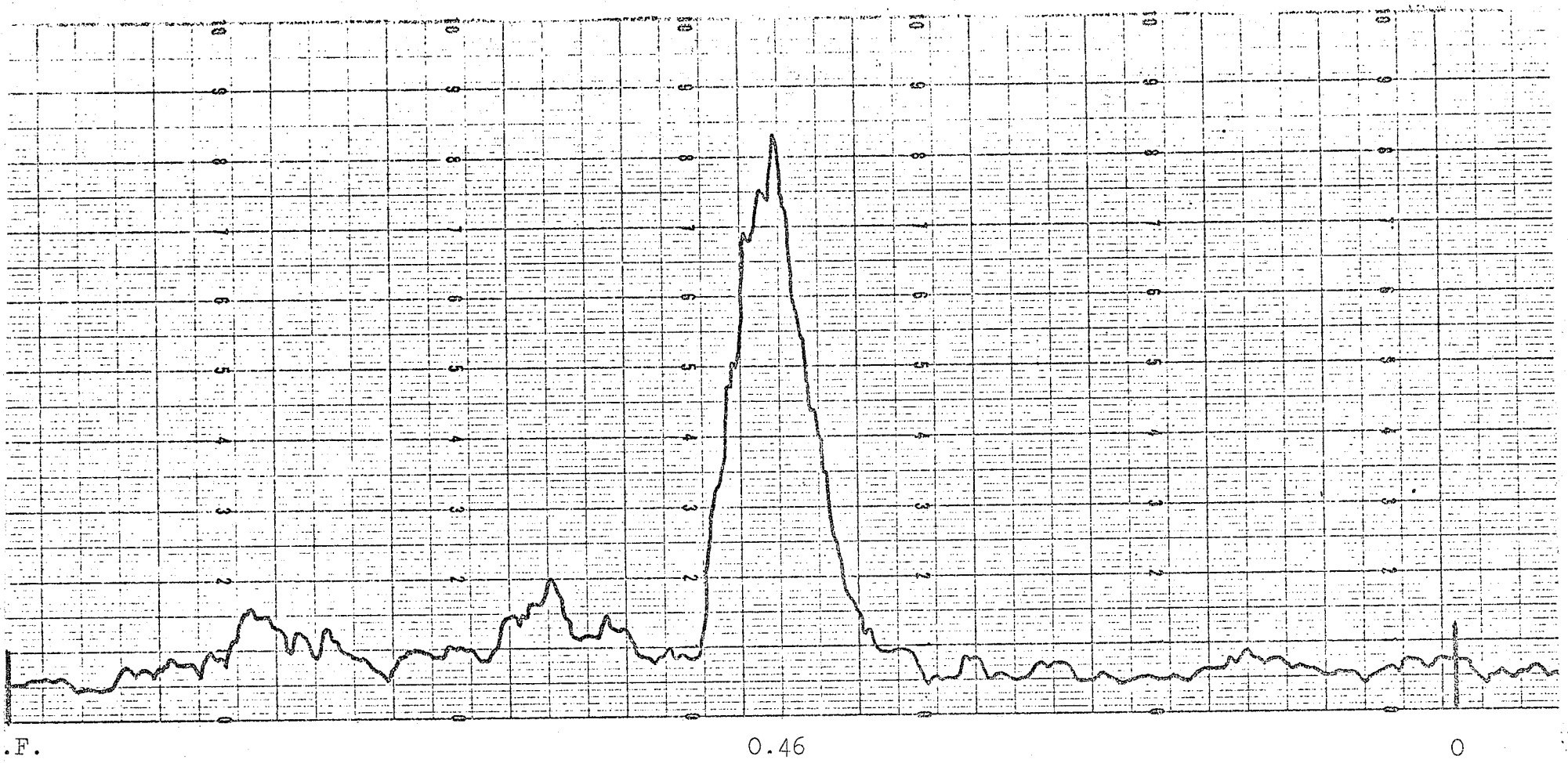


Figure V. Scanning of paper chromatogram spotted with eluent of peak I (R_f 0.45, Figure I), following hydrolysis with sulphatase. (300 cpm.).

isotope dilution studies, it was shown to be present in the reaction mixture obtained by treating the peak II extract as previously described, employing thin layer chromatography (Table VIII).

Table VIII. R_f values and radioactivity (cpm.) of suspected methyl ester hydrochloride of 2-(4-hydroxyphenylamino) propanoic acid prepared from urine of rats injected with N-isopropylaniline-^{14}C, by thin layer chromatography.

Solvent system	R_f values of sample residue	Cpm. of sample bands	R_f value of reference methyl ester hydrochloride
n-Butanol	0.85	143	0.85
	0-0.40 (diffuse)	50	
Isopropanol 15%/ benzene	0.63	100	0.60
	0	78	
Water	0.72	83	0.72
	0.90	34	

While count rates were low, activity was detected in spots whose R_f values corresponded to reference methyl ester hydrochloride in three chromatographic systems. Although quantitative results would have been preferable, it would appear that the nature of the p-hydroxy acid precludes tests of this type unless methods can be developed to prevent its ready decomposition during analysis. However, this information, together with the

quantitative area estimations of peaks in Figure I (Table V), appears to indicate that the p-hydroxy acid represents the 30 percent unidentified polar metabolites of N-isopropylaniline.

D. BILIARY EXCRETION OF N-ISOPROPYLANILINE AND N-ISOBUTYLANILINE

Studies on the biliary excretion of N-isopropylaniline and N-isobutylaniline in the rat have revealed that both compounds are excreted in the bile but to significantly different extents. N-isopropylaniline is excreted over a period of three to five hours to an extent of 12 to 15 percent, whereas up to 47 percent of the administered dose of N-isobutylaniline is excreted within the same time period (Table IX).

Table IX. Biliary excretion of radioactivity in rats (as a percentage of administered dose) following femoral vein injection of N-isobutylaniline-^{14}C or N-isopropylaniline-^{14}C.

Rat	Compound injected	Dose as administered dpm. x 10^6	Time of bile collection (hours)	Radioactivity excreted (% of administered dose)
I	N-isobutylaniline	1.12	5	47.61
II	N-isopropylaniline	1.56	3	12.40
III	N-isopropylaniline	1.56	5	15.09

Such a difference in excretion rates could be due to an increase in molecular weight from N-isopropylaniline (M.W. 135) to N-isobutylaniline (M.W. 149). Although the difference is only 14, Williams et al. (1965) determined that while only 58 percent of

4-hydroxybiphenyl glucuronide (M.W. 346) is excreted in the bile of rats, the biliary excretion of the monoglucuronide of 4,4'-dihydroxybiphenyl (M.W. 362), a closely related compound, occurs to a significantly greater extent (92 percent). The difference may also be related to the polarity of metabolites or their degree of binding to plasma proteins. Higher anionic strength and greater protein binding accompany increased biliary excretion with a subsequent decrease in urinary excretion (Webb et al., 1962).

All metabolites of both N-isopropylaniline and N-isobutylaniline, with the possible exception of p-aminophenol, should be excreted in the bile when conjugated, according to the criteria for biliary excretion reported by Brauer (1959). Formation of conjugates is facilitated by ring hydroxylation and side chain oxidation, yielding metabolites which have suitable reaction sites. Examination of the bile on paper chromatography revealed the presence of two metabolites in the case of N-isopropylaniline, when developed in n-butanol/glacial acetic acid/water 8:2:2, as illustrated in Table X and Figure VI.

Table X. R_f values of biliary metabolites from rats injected with N-isobutylaniline-^{14}C or N-isopropylaniline-^{14}C by ascending paper chromatography in chloroform; butanol ammonia 20:1 (system I) or butanol/glacial acetic acid/water 8:2:2 (system II).

Rat	Compound injected	Solvent system	R_f values
* I	N-isobutylaniline	II	0.15, 0.46, 0.54, 0.69, 1.00
II	N-isopropylaniline	I	0
		II	0 and 0.26
**III	N-isopropylaniline	II	0 and 0.27

* See Figure VII

** See Figure VI

The metabolite represented by peak I was extremely polar and did not move from the origin, whereas that corresponding to peak II had an R_f value of 0.27, which corresponds very closely to the metabolite present in urine (peak II, Figure I) possessing the same R_f value. The latter metabolite has already been identified as 2-(4-hydroxyphenylamino) propanoic acid (Section VI C). Reverse isotope dilution experiments to further verify the presence of this compound in bile were unsuccessful. Although 2-(4-hydroxyphenylamino) propanoic acid appeared to be excreted in both urine and bile, p-hydroxy-N-isopropylaniline, identified chromatographically on radioactive scans of urine (Figure I, peak I, R_f 0.45), could not be detected in the bile. Peak I in bile, similarly, could not be demonstrated chromatographically in

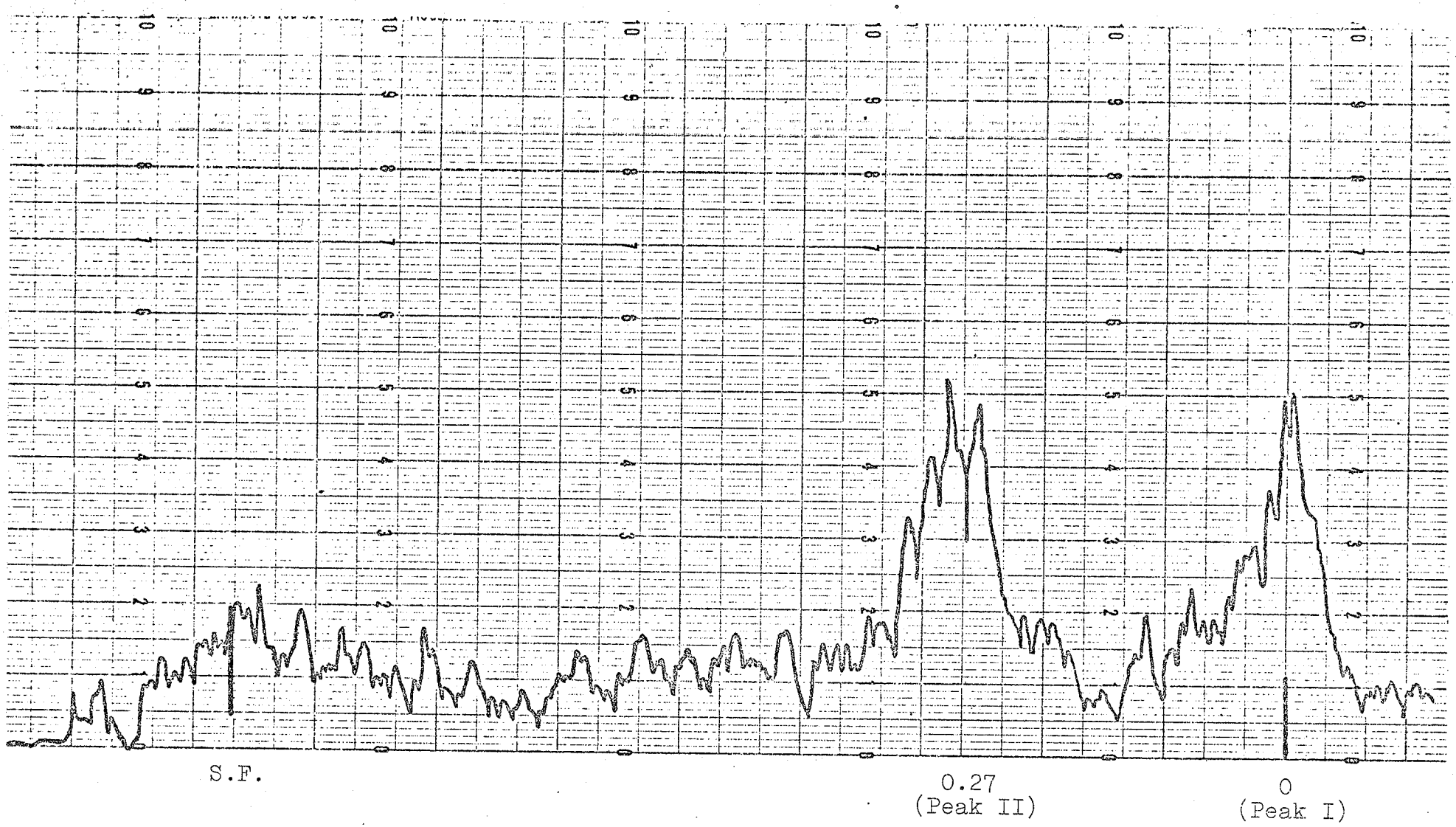


Figure VI. Scanning of paper chromatogram spotted with bile of rat injected with N-isopropylaniline - ^{14}C.

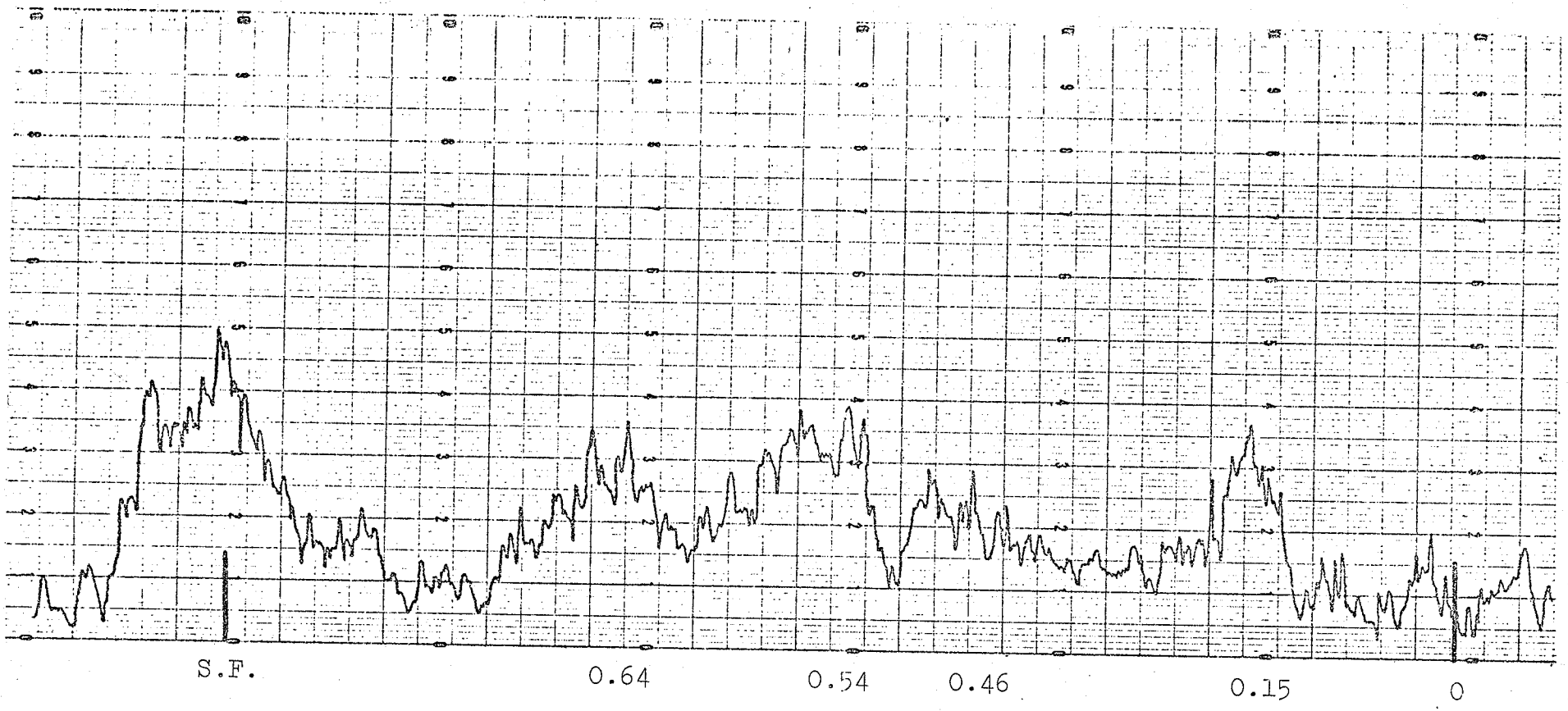


Figure VII. Scanning of paper chromatogram spotted with bile of rat injected with N-isobutylaniline - ^{14}C.

the urine. As p-hydroxy-N-isopropylaniline would be expected to appear in the bile and could form conjugates on the hydroxyl group, the possibility does exist that peak I is a conjugate of this metabolite, different from that occurring in urine. Hence, an altogether different R_f value would be expected. This aspect, however, was not pursued and peak I in the bile still remains to be identified.

On isolation of the two biliary metabolites of N-isopropylaniline by preparative paper chromatography, neither could be hydrolysed by acid or β-glucuronidase (Table XI and Figures VIII and IX), indicating that neither metabolite is conjugated in a manner detectable by these methods.

Table XI. R_f values of biliary metabolites from rats injected with N-isopropylaniline by ascending paper chromatography, following hydrolysis.

Solvent system	*Method of hydrolysis	R_f values
n-butanol/glacial acetic acid/water 8:2:2	β-glucuronidase	0, slight 0.23-0.30.
	Concentrated hydrochloric acid	0 and 0.22

* See Figures VIII and IX.

It seems unlikely that the metabolites would be excreted in unconjugated forms since the molecular weights of all known metabolites of N-isopropylaniline are under 300, the minimum value generally accepted for biliary excretion (Brauer, 1959).

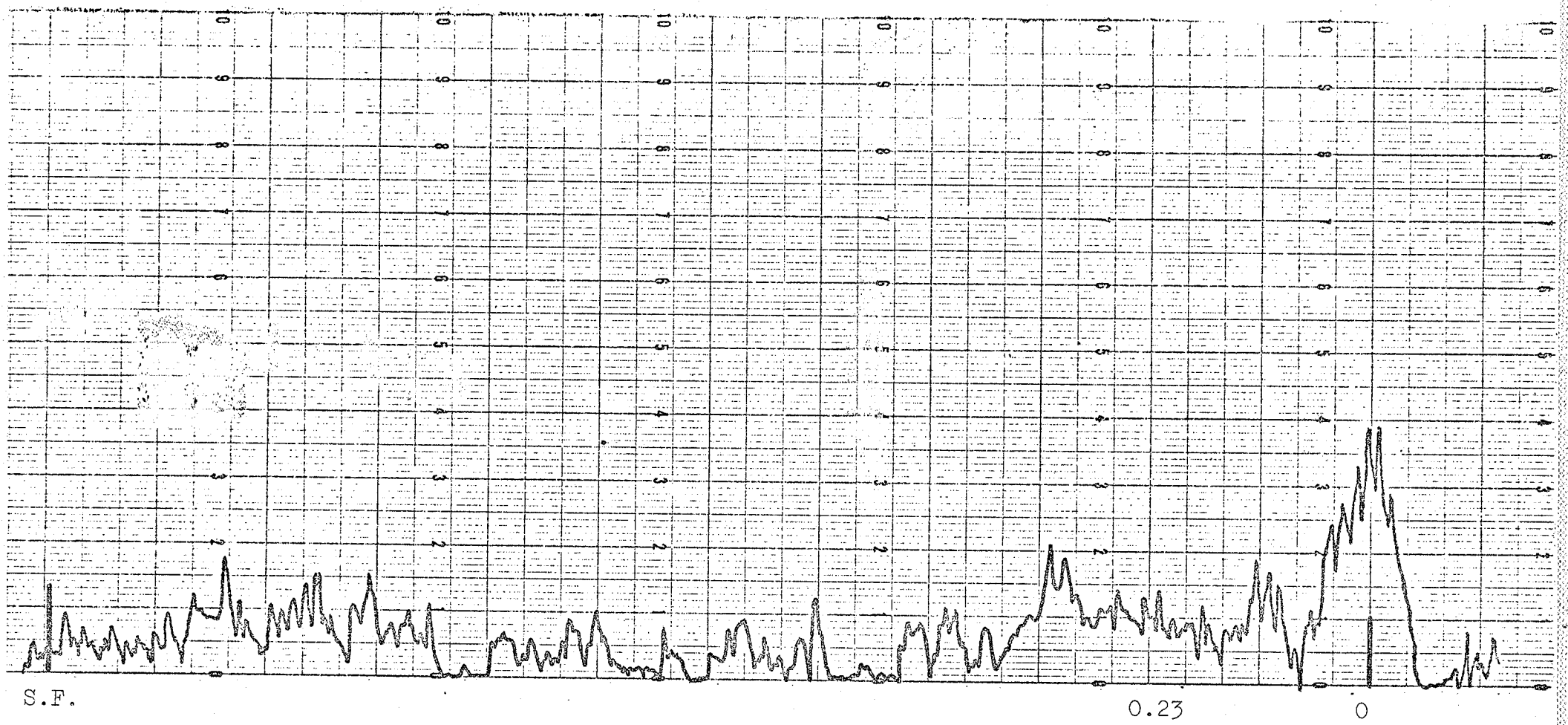


Figure VIII. Scanning of paper chromatogram spotted with N-isopropylaniline - ^{14}C bile sample following hydrolysis with β-glucuronidase.

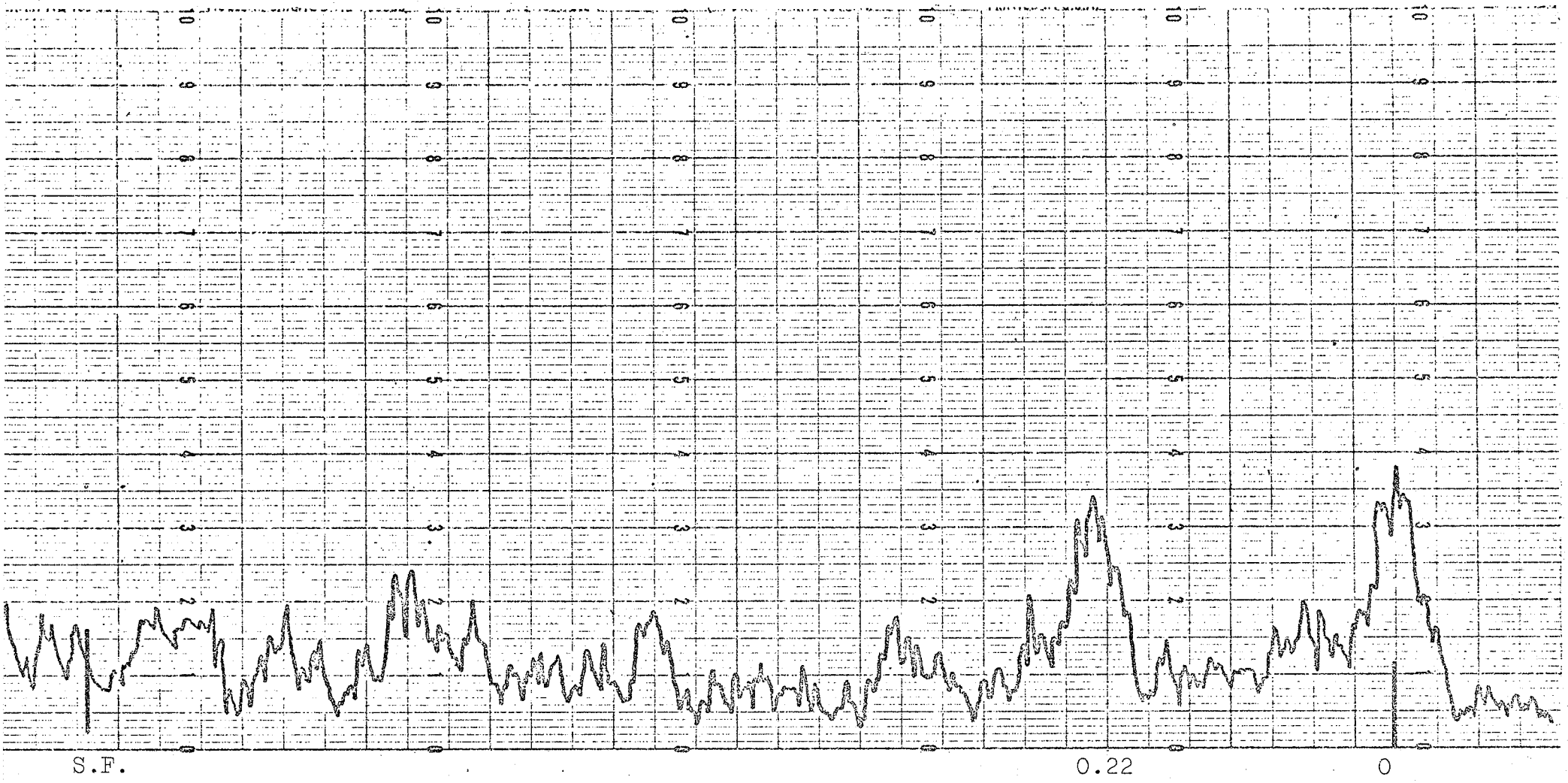


Figure IX. Scanning of paper chromatogram spotted with N-isopropylaniline - ^{14}C bile sample, following hydrolysis with concentrated hydrochloric acid.

The nature of these conjugates remains to be determined.

In the case of N-isobutylaniline, where Thiessen (1969) identified 93 percent of the 24-hour metabolites in urine as p-aminophenol and p-hydroxy-N-isobutylaniline, radioactive scanning of paper chromatograms from a five-hour bile sample (Table X and Figure VII) indicated the presence of a possible five metabolites. Since p-aminophenol has not been detected in the bile of rats following administration of aniline (Williams et al., 1965), and since 56 percent of N-isobutylaniline is excreted in rat urine as p-aminophenol, all five peaks must be attributable to other metabolites, or various conjugates of p-hydroxy-N-isobutylaniline. Whether these peaks represent five different metabolites, or more than one conjugate of a fewer number of metabolites remains to be investigated.

Although additional studies are necessary, these results suggest that the biliary excretion of N-alkylanilines is in part dependent on the structure of the N-alkyl side chain. In the case of the two compounds investigated, the higher molecular weight N-isobutylaniline which branches on the β-carbon, showed a significantly greater rate of excretion of metabolites in the bile than did N-isopropylaniline, which branches on the α-carbon. Further work must also be undertaken to explain the differences in metabolites recovered from the urine and bile of rats injected with N-isopropylaniline.

CONCLUSION

The compound 2-(4-hydroxyphenylamino) propanoic acid has been identified as at least one of the polar metabolites of N-isopropylaniline in the urine of the rat. This metabolite, formed by the metabolic pathways of p-hydroxylation and side chain oxidation, was verified qualitatively by thin layer chromatography and a modified form of reverse isotope dilution. While both the O-benzoate and methyl ester hydrochloride of this compound could be prepared, adaptation of these procedures to urinary reverse isotope dilution experiments was unsuccessful.

It has been established that there are at least two metabolites of N-isopropylaniline excreted in the bile and three metabolites in the urine. All appear to be conjugated although further studies are necessary to confirm this. While 2-(4-hycroxyphenylamino) propanoic acid appears to be a common metabolite *via* both routes of excretion, one metabolite was present in bile which was not detected in urine. Similarly, while p-hydroxy-N-isopropylaniline (conjugated) was excreted in the urine, this compound did not appear to be excreted in the bile, at least not as the same conjugate as detected in the urine. Preliminary biliary excretion studies carried out on N-isobutylaniline and N-isopropylaniline indicated the presence of five metabolites, either all different compounds, or different conjugates of a smaller number of compounds. Furthermore, the extent to which N-isobutylaniline and N-isopropylaniline are excreted in the bile differs considerably and this difference appears to be reflected by the structure of the N-alkyl side chain.

Three new compounds were synthesised and verified: 2-(4-hydroxyphenylamino) propanoic acid, and the O-benzoate and methyl ester hydrochloride derivatives of this acid.

APPENDICES

Appendix A. R_f values of the O-benzoate of 2-(4-hydroxyphenylamino) propanoic acid on thin layer chromatography.

Solvent system	R_f value
Ethanol 50%	1.0
Ethanol 95%/isopropanol 1:1	0.52-0.68, tailing.
n-Butanol/ethyl acetate 3:1	- . Extensive tailing.
Ethanol 10%	0.83
Water	0.66

Appendix B lists the R_f values of 2-(4-hydroxyphenylamino) propanoic acid methyl ester hydrochloride, p-aminophenol and p-hydroxy-N-isopropylaniline in various solvent systems for thin layer chromatography. The following systems are identified in the appendix by number:

1. Isopropanol 15%/benzene
2. n-Butanol/benzene 1:10
3. Ethyl acetate 20%/petroleum ether (66-75°C.)
4. Water
5. Benzene
6. Chloroform
7. Petroleum ether (66-75°C.)/ethyl acetate/ethanol 8:2:1
8. n-Butanol/water 4:1
9. Diethyl ether/ethanol 2:1
10. n-Butanol

Appendix B. R_f values of reference compounds on thin layer chromatography.

Reference Compound	Solvent system									
	1	2	3	4	5	6	7	8	9	10
2-(4-hydroxyphenylamino)-propionic acid methyl ester hydrochloride	0.55-0.70	0.40-0.60 (diffuse)	0.20	0.76	0	0	0.83	0.80	Diffuse throughout	0.85
p-aminophenol	0.35	-	0	0	-	-	-	-	-	-
p-hydroxy-N-iscpropyl-aniline	0.62	-	0.15-0.25	0.40	0-0.13	0	0.88	-	-	-

Appendix C. R_f values of reference compounds on ascending paper chromatography in n-butanol/acetic acid/water 8:2:2

Reference compound	R_f value
p-hydroxy-N-isopropyl-aniline hydrochloride	0.75
p-aminophenol hydrochloride	0.40
2-(4-hydroxyphenylamino) propanoic acid	0.67
2-anilinopropionic acid	0.85
p-aminophenyl sulphate	0.25

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