METABOLISM OF VANILLYL COMPOUNDS IN THE RAT

By

Kim Ping Wong

METABOLISM OF VANILLYL COMPOUNDS IN THE RAT

By

Kim Ping Wong

Thesis Submitted
to the Faculty of Graduate Studies in Partial
Fulfillment of the Requirements for the
Degree of Master of Science

Allan Memorial Institute of Psychiatry Department of Biochemistry
McGill University
Montreal, Quebec.

August, 1965.

M.Sc. BIOCHEMISTRY

ABSTRACT

By Kim Ping Wong

METABOLISM OF VANILLYL COMPOUNDS IN THE RAT

In the rat, intraperitoneal injection of vanillin gave rise to a number of urinary products, the most important being vanillic acid (free and conjugated). Some vanillin and vanillyl alcohol were excreted as conjugates. Catechol was a minor metabolite; it was produced not only by normal rats, but also by animals treated with neomycin and streptomycin, to reduce the intestinal flora. Catechol was also formed from protocatechuic aldehyde, protocatechuic acid, vanillic acid and guaiacol.

Treatment with antabuse prior to injection of vanillin led to an inhibition of aldehyde oxidase, manifested by the subsequent channelling of the aldehyde into the reductive pathway with the formation of vanillyl alcohol.

Administration of adrenalone and noradrenalone (the β-keto analogues of adrenaline and noradrenaline) led to the excretion of a small excess of vanilmandelic and vanillic acids.

ACKNOWLEDGEMENTS

To Dr. T.L.Sourkes, I would like to express my very sincere gratitude and thanks for his guidance in the course of this work.

I thank Mr. George Berci for his help in the animal experiments, and my fellow-workers at A.M.I. for their "miscellaneous" but valuable assistance.

To Miss Elizabeth Wagmann, my thanks for the typing of this manuscript.

I would also like to express my gratitude to the Canadian Commonwealth Scholarship and Fellowship Committee, and the External Aid Office, Ottawa, for giving me this opportunity to study in Canada.

TABLE OF CONTENTS

Page

LIST OF ABBREVIATIONS USED IN THIS THESIS

CA	catecholamine
COMT	catechol-O-methyl transferase
DHMA	3,4-dihydroxy mandelic acid
DHPAA or dopac	3,4-dihydroxyphenylacetic acid
DHPG	3,4-dihydroxyphenyl glycol
dopa	3,4-dihydroxyphenylalanine
dopamine	3-hydroxytyramine
5-HIAA	5-indoleacetic acid
HVA	homovanillic acid, (3-methoxy-4-hydroxyphenyl-acetic acid)
MAO	monoamine oxidase
m-HPAA or mopac	meta-hydroxyphenylacetic acid
MHPG	3-methoxy-4-hydroxyphenyl glycol
MN	metanephrine
MMN	N-methyl-metanephrine
NMN	normetanephrine
PA	protocatechuic acid (3,4-dihydroxy benzoic acid)
TLC	thin-layer chromatography
VA	vanillic acid (3-methoxy-4-hydroxybenzoic acid)
VLA	vanil-lactic acid
VMA	vanilmandelic acid
VPA	vanil-pyruvic acid

PREFACE

Many aromatic acids have been found to be normal urinary constituents. They are the degradation products of a class of biogenically active amines, the catecholamines, indolylethylamines and others. As a large part of the catecholamines are acted upon by the catechol-O-methyl transferase, the aromatic acids derived from these substances occur primarily as vanillyl derivatives in the urine. They have proved to be of diagnostic importance e.g. an elevation of urinary vanilmandelic and homovanillic acids suggests phaeochromocytoma, and neuroblastoma respectively.

These vanillyl acids are derived from the catechol and vanillyl aldehydes, themselves the immediate products of oxidation of the catecholamines, and their O-methyl derivatives respectively, through the action of monoamine oxidase. One purpose of this study was to investigate the metabolism of vanillin, as the prototype of such catecholamine-derived aldehydes.

Besides the endogenous sources mentioned above, many edible fruits and other foodstuffs also contain these aromatic acids, or the corresponding amines. Vanillin is a normal dietary constituent, occurring in ice-cream, chocolate, cakes, etc. Its ingestion may interefere with the measurement of VMA, as vanillin is metabolised to vanillic acid, and this latter compound may be estimated as VMA in some methods. In

another method,metanephrine and normetanephrine (two other major metabolites of the catecholamines) are oxidised to vanillin and measured as such. Thus, dietary vanillin excreted unchanged in the urine could conceivably interefere with these measurements. As information on the metabolism is still somewhat limited, as witnessed by the paucity of data since the study by Sammons and Williams in I94I, an investigation of this subject was undertaken, an an attempt to solve some of these problems, and to evaluate the contribution of an exogenous source of a widely consumed vanillyl compound to the urinary vanillyl acids.

I. INTRODUCTION

The term "vanillyl" or "vanil" refers to any 3-methoxy-4-hydroxy derivative. These compounds constitute a spectrum of acidic, basic and neutral substances, which are the intermediates and end products of a class of bio-active amines, the catecholamines (CA). The CAs most extensively studied include adrenaline, noradrenaline, and dopamine. They are widely distributed in higher animals and have been reported in invertebrates (1) as well as in many edible fruits (2). In higher animals, large amounts of these hormones are found in the adrenal medulla, (3) and in certain parts of the brain (primarily the hypothalamus and basal ganglia). Evidence by U.S. von Euler (4,5) showed that noradrenaline is the neurohormone of the sympathetic nerve.

1. BIOSYNTHESIS OF THE CATECHOLAMINES

(a) Main Pathway

The catecholamines are all derived from the essential amino acid, phenylalanine, by a sequence of reactions which involve aromatic and aliphatic hydroxylations, decarboxylation and N-methylation.

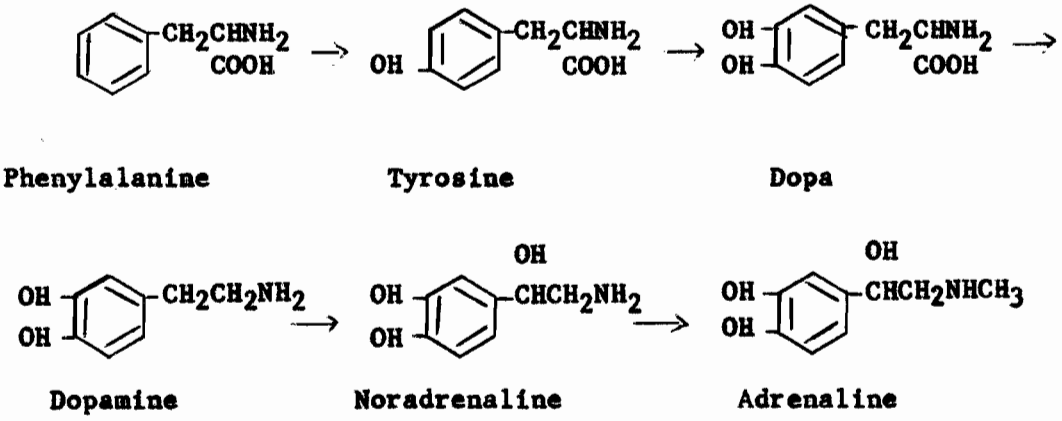


Fig. 1. BIOSYNTHESIS OF THE CATECHOLAMINES FROM PHENYLALANINE

This scheme was first proposed by Blaschko (6,7) and Holtz (8). The formation of CA *in vivo* has been demonstrated using phenylalanine (9), tyrosine (10,11), 3,4-dihydroxyphenylalanine (dopa)(10,12), and 3,4-dihydroxyphenylserine (13) as precursors. *m*-Tyrosine (3-hydroxyphenylalanine) has been considered as another possible precursor, for it is decarboxylated by mammalian organs *in vitro* (14), and *in vivo* (15), and its decarboxylation product, m-tyramine, is found in normal (16) and phenylketonuric (17) urines, and in urines of subjects who have received an inhibitor of monoamine oxidase (18). Sourkes *et al.* (19) found excess dopamine is excreted in the urine after administration of DL-m-tyrosine.

(b) Alternate Pathways

The possibility of alternate or side pathways exists for the biosynthesis of these amines (20).

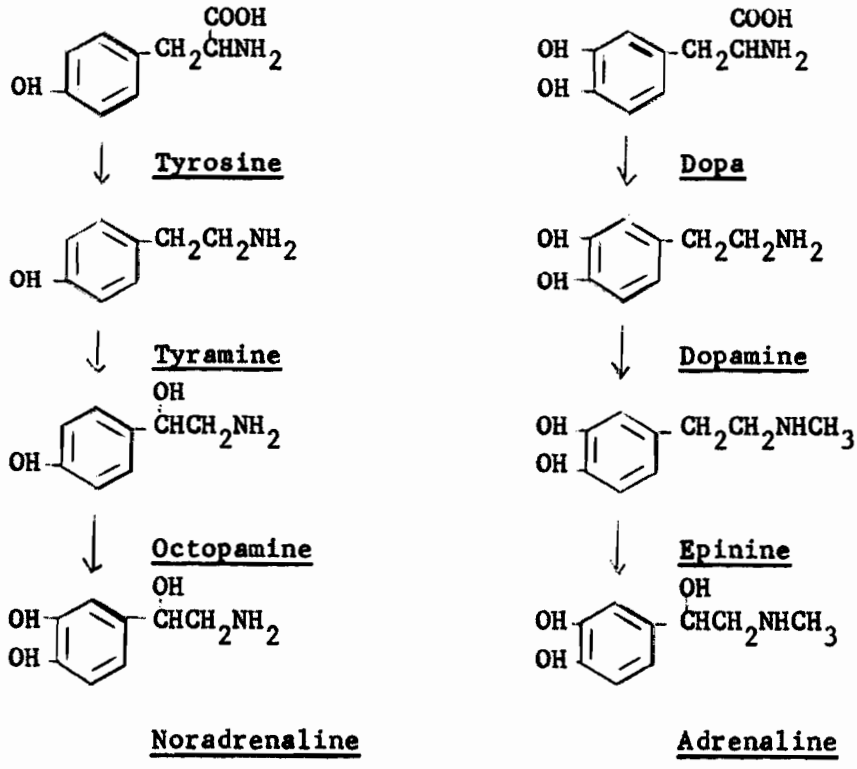


Fig. 2 ALTERNATIVE PATHWAYS FOR THE BIOSYNTHESIS OF THE CATECHOLAMINES.

It can be seen from above that if tyrosine is decarboxylated the product is tyramine, which if hydroxylated in the β position of the side chain, yields octopamine. This latter compound was originally discovered in the salivary gland of the octopus, and has been found to be present in both human and other animal urines. The conversion of tyramine to octopamine was shown to be catalysed by the dopamine hydroxylase enzyme. Likewise, epinine (N-methyldopamine) is also hydroxylated by this enzyme, the product being adrenaline directly, without the intermediate formation of noradrenaline. Axelrod has demonstrated that there is an enzyme system, capable of forming epinine from dopamine. Thus, the second alternate pathway was postulated.

2. METABOLISM OF THE CATECHOLAMINES

(a) Precursors of Vanillyl Compounds

These catecholamines are precursors of the vanillyl compounds, through the mediation of the catechol-O-methyl transferase (COMT). Monoamine oxidase (MAO) was at one time thought to be the main catabolic enzyme in the metabolism of the CA (21). However, with the discovery of the COMT by Axelrod (22) and Pellerin and D'Iorio (23), and the recognition of O-methylation as the principal pathway, the understanding of the metabolism of the CAs was revolutionised, towards the identification of a host of 3-methoxy-4-hydroxy, or vanillyl derivatives of the catechols. The action of COMT alone on the catecholamines results in the formation of basic vanillyl amines. These include metanephrine (MN), normetanephrine (NMN) and 3-Methoxy-

tyramine. When its action is coupled to that of MAO, the vanillyl aldehydes are formed, the oxidation and reduction of which produce acidic and neutral vanillyl compounds, respectively. These three classes of vanillyl compounds (with the exception of the aldehydes) are presumably the end products of metabolism of the CAs, as suggested by their presence in the urine. The likelihood of their serving as new physiologically active metabolites remains to be established. Because of the important roles played by these two enzymes, the two processes which they catalyse will be discussed presently.

(b) Oxidation

Blaschko, Richter and Schlossmann (24) were among the first to study the oxidation of adrenaline and other amines. A number of enzymes were originally thought to participate in the oxidation of these amino compounds. These include:-

(1) Adrenaline Oxidase

(2) Tyramine Oxidase

(3) Aliphatic amine Oxidase

(4) Histaminase

(5) Amino-acid Oxidase

In an extensive study of the distribution and kinetics of these enzymes, it was concluded that the first 3 systems are identical and the name monoamine oxidase was given to it. Richter, 1940 (25) suggested that oxidation of adrenaline may involve the catechol oxidase, cytochrome system, peroxidase or pseudo phenolases. Bacq (26) in his review of the metabolism of adrenaline suggested that

deamination of this CA is unlikely, but an important fraction is sulfo-conjugated and another fraction is simultaneously oxidised to indole substances. Further reviews on this subject were by Blaschko (27) and more recently by Sourkes (28). The latter has discussed the significance of oxidative pathways in the metabolism of these biogenic amines, from biochemical, physiological, pharmacological and endocrinological evidence.

According to Schayer and his colleagues (21), both adrenaline and noradrenaline share the same pattern of radioactive urinary metabolites, when isotopically labelled amine is administered parenterally. They concluded that MAO may constitute a major pathway. However, recent studies have all emphasized O-methylation as the principal pathway.

(c) Adrenalone and Noradrenalone

Both adrenalone (3,4-dihydroxy-α-methyl-amino-acetophenone) and noradrenalone (3,4-dihydroxy-amino-acetophenone), the 2-keto analogues of adrenaline and noradrenaline, also have presser activity. Two groups of workers in Japan have reported finding adrenalone, as an oxidation product formed by enzymes in blood. Imaizumi and coworkers (29,30,31,32) described an enzyme in rabbit plasma which reversibly oxidizes L-adrenaline to adrenalone in the presence of diphospho pyridine nucleotide (DPN), and which they called adrenaline dehydrogenase. According to Kashiwagi and Habu the adrenaline dehydrogenase could oxidise noradrenaline but could not oxidise neosynephrine (33). Weil-Malherbe and Bone (34) did not succeed in

confirming the claims of these Japanese workers. They have searched for this enzyme in rabbit and human plasma, and in extracts of rat liver, heart, spleen and brain. Experiments were done anaerobically to prevent any activity of amine oxidase.. Adrenaline was incubated with a large excess of DPN, but no disappearance was found by fluorimetric measurement.

Kawamoto (35) postulates that adrenalone is metabolised either to adrenaline, or along a pathway leading successively to 3,4-Dihydroxyphenylglyoxalic acid and finally to 3,4-dihydroxybenzaldehyde (Protocatechuic aldehyde). This last compound could undergo oxidation in the tissues to 3,4-Dihydroxybenzoic acid (Protocatechuic acid).

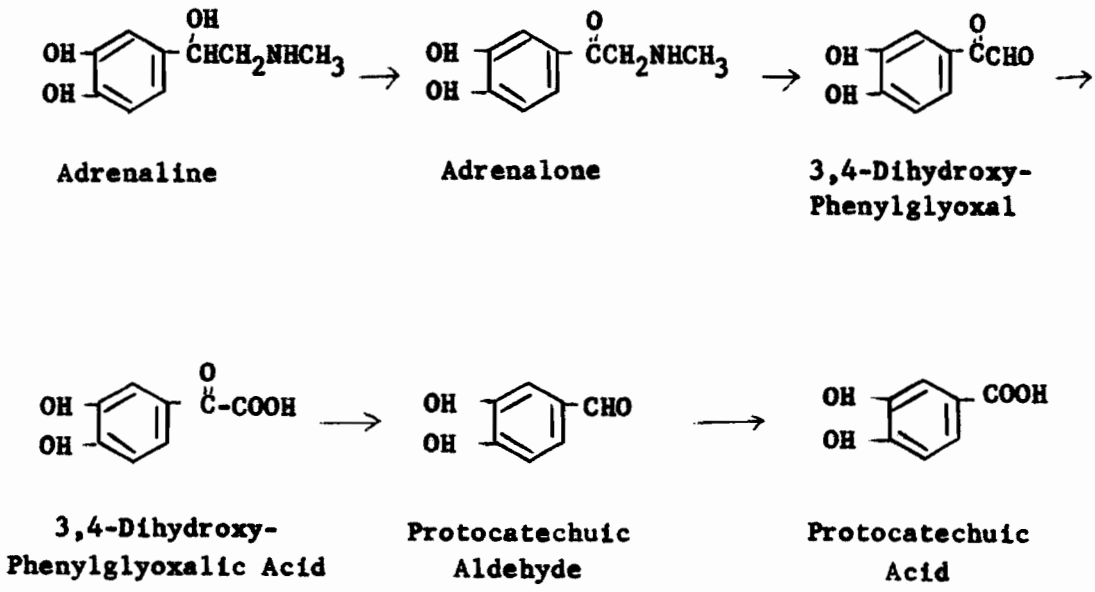


Fig. 3. METABOLIC DEGRADATION OF ADRENALINE THROUGH ADRENALINE DEHYDROGENASE AS PROPOSED BY THE JAPANESE WORKERS.

Takaoka et al. (36) on the other hand claimed that in human red blood cells, there is a DPN enzyme which catalyses the reverse reaction by which an adrenaline-like substance is formed from adrenalone.

If adrenalone is indeed reduced to adrenaline, it would share a similar metabolic pattern. Smith (37) showed in a preliminary experiment that oral administration of adrenalone to a human subject has been found to result in the excretion of grossly abnormal quantities of vanillic, isovanillic, and vanilmandelic acids. Since the last compound has been established by Armstrong (38) to be the major metabolite of both adrenaline and noradrenaline, it is likely, though not necessary, that adrenalone follows the same mode of degradation as adrenaline. In the guinea pig, however, Smith found that the injection of adrenaline or adrenalone produced protocatechuic aldehyde. It would appear that the second alternative of Kawamoto, operates in the guinea pig. This seems to be peculiar in the light of the finding of Wylie (39) who reported that both adrenalone and noradrenalone are inhibitors of COMT, presumably by virtue of their ability to act as methyl acceptors. Yet in the guinea pig, no methylation seemed to have occured. It is interesting that these ketone analogues of adrenaline and noradrenaline had a much greater affinity for the enzyme than their β-hydroxy counterparts. It appears that both adrenalone and noradrenalone undergo p-O-methylation in the intact rat (40).

Kawamoto and his co-workers (41) in their study of the action

of amine oxidase on adrenaline and adrenalone found that the destruction of adrenalone by liver amine oxidase was faster than that of adrenaline. On the contrary, Blaschko et al. (24) found that the relative rates of oxidation of adrenalone and noradrenalone by liver enzyme were less than for adrenaline and noradrenaline. Three years ago, Goldstein et al. (42) investigated the content of norepinephrine-H^3 formed from dopamine-H^3 in several organs of rabbits treated and untreated with adrenalone. Their results showed that adrenalone is an effective inhibitor in vivo of noradrenaline synthesis.

It can be seen that the situation with regard to the metabolism and physiological importance of these keto analogues of adrenaline and noradrenaline is not clear at present. It could be further postulated that adrenalone and noradrenalone might owe their greater ability to potentiate both amines to the fact that they chemically resemble the amines and may compete with them for both the enzyme and binding sites.

(d) Methylation

Methylation of a phenolic OH in the human body was first demonstrated by Maclagan, and Wilkinson (43). When n-butyl-3,5-diiodo-4-hydroxybenzoate was administered to patients with thyrotoxicosis, 3,5-diiodo-4-methoxybenzoate was excreted in the urine. Later, m-O-methylation was found to occur when 3,4-dihydroxyphenylacetic acid (DHPAA) was administered to rats (44). Oral administration of rutin and quercetin gave rise to DHPAA, the methylation

and p-dehydroxylation of which yield HVA and m-hydroxyphenylacetic acid (mopac) respectively. Booth and his co-workers (45) claimed that methylation of 3-OH occurs when the side chain has 1, 2 or 3 C. They demonstrated the formation of vanillic acid (VA) from protocatechuic acid (PA). Dopa is oxidised to DHPAA and this is methylated to form HVA. Though all urinary metabolites contain methoxy group on the meta position, several instances occur, e.g. in which iso-ferulic and dihydroferulic acids were formed when dihydroferulic, ferulic and caffeic acids were given to rats, which implicate methylation on p-hydroxy group (46). Also 3-hydroxy-4-methoxycinnamic acid has been identified after the administration of caffeic acid (47). Thus, methylation is not necessarily confined to the 3-OH group. There is a possibility that two O-methyl transferases exist with varying specific affinities toward different substrates. DeEds *et al.* (47) studied the selective para- or meta-O-methylation with COMT from rat liver and came to the conclusion that phenolic substrates having 3 adjacent hydroxyl groups direct the enzyme to methylate the middle hydroxyl group, while with ortho-dihydroxyphenolic substrates, methylation of either hydroxyl group may take place.

The significance of O-methylation of the CAs manifests itself with the discovery of Axelrod (22) of an enzyme from rat liver which catalyses the O-methylation of epinephrine and other catechols *in vivo* and *in vitro*. The corollary of this was the establishment of O-methylation as the principal pathway for the CA metabolism (48).

The enzyme is called catechol-O-methyl transferase. It is present in the soluable supernatant fraction of tissue homogenates. Liver is the chief source of COMT. Other sources include the brain, spleen, and kidney. It is stimulated by Mg^{++}, Co^{++}, and Mn^{++}, and requires S-adenosylmethionine as the methyl donor. All catechols, irrespective of the substituents on the aromatic nucleus were O-methylated. Since O-methylation occurs on the parent amine as well as on the acid end products, it remains to be seen which of the two routes predominates *in vivo*. Udenfriend *et al*. (49) suggested that O-methylation of noradrenaline converts it to a substance which is more effectively acted upon by MAO. Axelrod *et al*. (50) demonstrated that O-methylation of the CA occurs prior to oxidative deamination, and indicated that this pathway is indeed the principal route of adrenaline and noradrenaline metabolism.

The endogenous CA hormones undergo O-methylation as the CAs, adrenaline, noradrenaline, and dopamine have been found to be excreted in the urine as their methoxy metabolites (50). Both MN, and NMN were present in rat urine as glucuronides. They are also found in rat adrenal glands. NMN was found as a glucuronide in patients with phaeochromocytoma (52). In addition, methyl-metanephrine (MMN) was also notably increased (53). Kopin *et al*. (54,55) studied the metabolic fate of noradrenaline in the rat, using simultaneous injection of DL-adrenaline-7-H3 and DL-MN-methoxy-C^{14}, and concluded that 70% of adrenaline was O-methylated. Other reports (56) (57) confirmed that O-methylation was indeed a major route

of metabolism of noradrenaline.

Bacq, as early as 1936 (58,59) described augmentation of the adrenaline effect by polyphenols in pharmacological tests. This is probably due to an increased life span of adrenaline, because of its "protection" from methylation (60). Inhibition of COMT in vivo and in vitro by pyrogallol was shown by Axelrod (61). Other COMT inhibitors include catechol, flavonoids, quercetin, glycocyamine, all of which effectively block metabolism of adrenaline in vivo. Therefore, the potentiation of the action of adrenaline by flavonoids is most probably due to the inhibition of COMT. The result of such a block is a relative increase in the dihydroxy derivatives.

In man, the methoxy compounds accounts for about 90% of the total radioactivity excreted in the urine. These were free and conjugated MN (40%), VMA (41%) and 3-methoxy-4-hydroxyphenylglycol (MHPG) (7%). Only about 2% of the catechol metabolites, 3,4 dihydroxymandelic acid (DHMA) and 3,4 dihydroxyphenylglycol (DHPG) were found in the urine. Administration of MAOI elevate the excretion of the O-methylated amines, metanephrine and normetanephrine (48), while inhibitors of COMT increase the excretion of the catecholamines and catechol acids and reduce the formation of the O-methylated products (62,63).

3. INACTIVATION

Inactivation of CA is a multiple process, involving enzymatic transformation, binding or conjugation. Richter (1940),(25) concluded that conjugation is the main physiological process by which adrenaline

is inactivated in the body. Bacq (1949), (26) likewise believed that an important fraction of adrenaline is sulfoconjugated. When labelled noradrenaline-H^3 was administered to animals it was found to be selectively taken up by various tissues, e.g., heart, spleen, and adrenal gland, but only trace amount in the brain. It appears that binding is a mechanism of inactivation of circulating CAs (64).

On administration of noradrenaline-H^3, NMN-H^3 was found in all tissues. In the case of adrenaline-H^3, it was found that 2 minutes after a rapid injection, the concentration of MN-H^3 was higher in most tissues than that of adrenaline-H^3 (51). Deamination during this time was negligible. That this O-methylation is a form of inactivation appears probable because the O-methylated compounds have very weak pharmacological action. However, in the case of the brain, MAO was implicated as the enzyme mainly responsible for the physiological inactivation of serotonin and adrenaline (65).

4. SURVEY OF THE VANILLYL COMPOUNDS

As can be seen from above, the metabolism of adrenaline and noradrenaline produces basic, neutral and acidic dihydroxy as well as vanillyl intermediates and end products with a preponderance of the latter. Analogous pathways of metabolism of dopamine result in the corresponding vanillyl compounds.

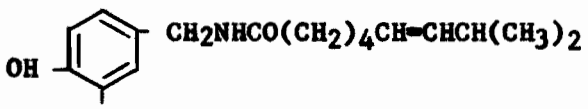
(a) Basic Vanillyl Metabolites (Amines)

These are produced by the exclusive action of COMT on the catecholamines. Adrenaline, noradrenaline and dopamine on methyl-

ation form metaphrine, normetanephrine and 3-methoxy-tyramine respectively (22,48,50,54). It is now generally believed that in man (66,67) and in animals (68,69) one of the major catabolites of noradrenaline is NMN, present in urine as the glucuronide. In addition, N-methyladrenaline has been found to be formed from adrenaline on incubation of beef adrenal glands with adrenaline and S-adenosylmethionine (70). Its O-methylated product, MMN has also been detected in human urine and phaeochromocytoma tissues (53).

According to Hertting (71), isoproterenol is metabolised exclusively by O-methylation in the rat. The isopropyl group attached to the N seems to prevent oxidative deamination. Consequently, 3-methoxy-isoproterenol and isoproterenol are both excreted in the rat urine, free or conjugated as glucuronides. Deamination plays no role in the degradation of isoproterenol.

A typical vanil amine found naturally occuring in plant is vanillylamine itself. It occurs in capsaicin in paprika (capsicum annuum, and capsicum fastigiatum). Capsaicin is a condensation product of vanillylamine (3-methoxy-4-hydroxybenzylamine) and a decylenic acid (72).



Capsaicin

Vanillylamine has been detected in human urine (73,74) and in vanilla extract (75). Kakimoto and Armstrong (73) reported a

marked increase in the excretion of vanillylamine after the ingestion of vanillin, a common flavoring agent. It appeared to be excreted only as a conjugate.

(b) Neutral Vanillyl Compounds

These include the "transient" intermediates of catecholamines, - the aldehydes, and their reduction products, the substituted glycols and ethanols. Axelrod et al. were the first to demonstrated MHPG as a new metabolite of adrenaline and noreadrenaline (76,77). The phenylglycol is excreted primarily as a sulfate conjugate by man, while VMA has been found to be excreted only unconjugated. In man, the major metabolite of noradrenaline is VMA, but in the white rat, it is MHPG. MHPG sulfate has been confirmed as a catabolite of noradrenaline by Smith (78) and Rosenblatt et al. (79).

In 1960, Goldstein and his co-workers characterised a new metabolite of dopamine, analogous to MHPG from noradrenaline (80). It is the neutral vanillyl compound 3-Methoxy-4-hydroxyphenylethanol (MHPE). It is presumably formed by reduction of 3-methoxy-4-hydroxy-phenylacetaldehyde, the oxidation of which would produce HVA. MHPE has been found in human urine. Dopamine in rats gives rise to MHPE but no DHPE (81). Apparently, dopamine is an excellent methyl acceptor. The O-methylation of dopa itself would produce "vanil-phenylalanine", and, in fact, this compound has been shown to be formed in vivo under certain circumstances (82, 83, 84).

(c) Inhibition of Aldehyde Dehydrogenase

An approach to the studies of these neutral compounds was the use of disulfiram, or antabuse, and calcium carbimide, both of which enhance the reductive pathway (a minor pathway) by inhibition of the aldehyde dehydrogenase. Both these drugs had been used in treatment of alcoholism (85). Goldstein et al. (81) reported that with calcium carbimide treatment of rats, more DHPE and MHPE were formed from dopamine and less HVA and DHPAA (the oxidation products). Smith (86) on the other hand had found disulfiram to be also effective in the inhibition of the 3-methoxy-4-hydroxymandelic aldehyde oxidase. As a result of disulfiram treatment, there was an increase in the formation of the glycol sulfate and a decrease in VMA from NMN in the guinea pig. Disulfiram-treated rats also formed more tryptophol from tryptamine, with a concomitant decrease in indole-3-acetic acid (87). Apparently, the oxidation of the indole-3-acetaldehyde was inhibited.

(d) Acidic Vanillyl Compounds

In 1956, Armstrong, Shaw and Wall described the chromatographic behaviour of 49 phenolic acids found in urine and provided preliminary identification of 23 of them, including HVA and VMA (16); the latter represents a major metabolic product of both adrenaline and noradrenaline metabolism (38). It is highly elevated in phaechromocytoma (38,88) and its measurement has been widely used in the diagnosis of this disease. HVA is derived in metabolism from L-dopa, D-dopa, meta-O-methyl, DL-dopa and 3,4-dihydroxyphenylacetic acid (dopac)

(89,90). The conversion of exogenous dopa to urinary HVA has been confirmed in the rat (23) and rabbit (45). Other metabolic sources have been identified as meta-O-methyldopamine (50) and dopamine (91). The feeding of rutin and its aglycone quercetin (44) results in an increase in the amount of certain normal urinary constituents. They are meta-hydroxyphenylacetic acid (mHPAA), HVA, and dopac. It was also shown in these experiments that administration of dopac causes excretion of urinary HVA, and mHPAA. Booth and his co-workers believed that some of the dopac is methylated in the meta-position to give HVA, and another portion is dehydroxylated in the para-position to give mHPAA.

Gjessing (82) in his systematic studies of functional tumors found a whole spectrum of vanil metabolites in 10 cases of functional tumors. In addition to all the 3-methoxy-4-hydroxyphenyl derivatives so far mentioned, he also identified vanil-lactic (VLA) (92) and vanil-pyruvic acids (VPA) (83,93) probably derived from dopa metabolism. Choremis et al. also found VLA excreted in urine in collagen diseases (94).

Vanillic Acid

This vanillyl acid was found in the free state in normal rabbit (95) and human urine, (16) mainly in the conjugated form (82). It has been considered by many workers to be an end metabolite of the catecholamines. Goodall et al. (96) in their scheme of alternative pathways for the metabolism of noradrenaline indicated that vanillic acid may

be formed from (i) 3-methoxy-4-hydroxyphenyl glycol

(ii) " " mandelic aldehyde

(iii) " " mandelic acid.

Sammons and Williams (97) showed that injection of vanillin into rabbits results in the excretion of vanillic acid, which accounts for 70% of the administered dose. Injection of vanillic acid resulted in its excretion in the urine, 56% being free and 27% conjugated as ethereal sulfate and glucuronide. A small amount was demethylated, producing probably protocatechuic acid. The administration of caffeic acid to rats also resulted in the formation of free vanillic acid and vanilloylglycine (Booth, 46). Williams (98) believed that vanillic acid, unlike other aromatic acids, e.g. veratric, benzoic, etc. does not conjugate with glycine. Booth et al. on the other hand, had identified vanilloylglycine on the basis of its chromatographic characteristics. There, thus, seems to be some discrepancy with regard to the nature of the conjugation of vanillic acid between these two groups of workers. This is probably a species difference. Vanillic acid was also detected in the urine only in the conjugated form, after the administration of shikimic and quinic acids (99).

Smith (100) claimed that subjects exposed to long periods of stress excrete abnormally large amounts of vanillic acid. Shaw and Trevarthen (101) considered this relation of excretion of vanillic acid to noradrenaline metabolism was unjustified because

no dietary restrictions had been imposed and because of the numerous likely precursors of vanillic acid that could be present in uncontrolled diets. Caffeic acid and chlorogenic acid are quantitatively the most important exogenous sources of vanillic acid (102), apart from vanillin, a flavouring substance which is readily oxidised to vanillic acid. However, on re-examination of the matter, Smith and his colleagues (103) found normal amounts of vanillic acid in "stress" urine, but still contended that this substance has an endogenous origin. "Normal" or the usual amount of vanillic acid in human urine is 2 mg/day. "Ethyl vanillin" (3-ethoxy-4-hydroxybenzaldehyde), another flavouring agent, when ingested, gives rise to "ethyl vanillic acid" (3-ethoxy-4-hydroxybenzoic acid). These workers believed that both vanillic acid and ethyl vanillic acid are of endogenous origin, though, there is no evidence that ethylation, as opposed to methylation, occurs in the animal organism.

Pellerin and D'Iorio (104) in their investigation of O-methylation of catechol acids found that when labelled DHMA was incubated with the COMT under optimal conditions with S-adenosylmethionine as the methyl donor, vanillic acid accounts for 60% of the radioactivity. It was therefore suggested that prior to methylation or following methylation, this compound could undergo oxidation and decarboxylation. Since DHMA is a metabolite of adrenaline and noradrenaline, probably vanillic acid is also derived from these catecholamines.

Perfusion experiments on rat liver showed that VMA, the "supposed" end product of CA metabolism could be further degraded to vanillic acid by dehydrogenation and oxidative decarboxylation (105). Also, it was demonstrated that homogenates of liver tissue were capable of producing vanillic acid. Dirscherl and Brisse (106) considered vanillic acid as the final end metabolite of adrenaline and noradrenaline since incubation of VMA with rat and human liver homogenates gave rise to vanillic acid in addition to free VMA. Another diago positive compound was formed also. The excretion of a significantly elevated amount of vanillic acid has been reported in a confirmed or histologically proven case of metastasing phaeochromocytoma (107).

All these reports implicate vanillic acid as an end-product of catecholamine metabolism. Its significance with regard to its endogenous origin is yet to be established, as many edible fruits contain compounds which serve as its potential precursors, including vanillin, which will be dealt with presently.

5. EXOGENOUS SOURCES OF PHENOLIC DERIVATIVES

This section is included because of the increasing evidence of exogenous amines present in fruits and other foodstuff. Some of these amines contribute to the urinary vanillyl metabolites. This is particularly important in the measurement of urinary indoles and catechols.

Following the ingestion of coffee, increased excretion of

m-hydroxy hippuric acid was found (46, 108). In 1957, Anderson and his co-workers found increased urinary excretion of 5-hydroxy-indole-acetic acid (5-HIAA) following banana feeding to monkeys (109). 5-HIAA is a catabolite of serotonin, the presence of which in bananas was confirmed by Udenfriend et al. (110). In addition, the latter detected large amounts of noradrenaline, dopamine and dopa in the banana, but no adrenaline nor histamine. These amines appear to be concentrated in the peel rather than in the pulp (111). Following this discovery, a number of physiologically active amines were sought in common fruits and vegetables (2). Vanillylamine was found in vanilla extract and paprika (75), synnephrine in oranges and p-hydroxybenzylamine in mustard (75), tryptamine and tyramine were also found in banana in large amounts. Shaw and Trevarthen (101) studied the exogenous sources of urinary phenolic and indolic acids and reported that ingestion of bananas gave rise to increased urinary VMA. However, the normal range is not exceeded. Booth observed that ingestion of caffeic acid, (which occurs as a quinic acid ester with chlorogenic acid) but not coffee, gave rise to vanillic acid (46). Coffee also gave rise to isovanillic, isoferulic and 3-ethoxy-4-hydroxybenzoic acid (37). The last named compound, ethyl vanillic acid, is undoubtedly derived from foodstuffs flavoured with ethyl vanillin (103).

Many phenylacetic derivatives, e.g. HVA, mHPAA, are metabolic end-products of quercetin metabolism (112). Petrakis and co-workers (113) claimed that orally administered quercetin gave rise to HVA and

mHPAA, as reported by Booth, but intravenous injection of quercetin produced vanillic acid.

Other sources of phenolic acids inlude tea, which is the main dietary source of 1,3-dihydroxy compounds which could give rise to resorcinol in vivo (114) and oranges which contain considerable amounts of p-sympatol and p-hydroxymandelic acid (115). Many edible plants contain quinic and shikimic acids, which are the precursors of catechol in urine (99).

Vanillin

Phenolic aldehydes occur widely in plant glycosides and these may be the source of some of the urinary amines and acids derived from plant foods. Vanillin (3-methoxy-4-hydroxybenzaldehyde) is a typical aromatic aldehyde of plant origin. Many of the vanillyl and dihydroxy aromatic aldehydes, which presumably are intermediary metabolites of adrenaline and noradrenaline, have not been detected in urine, because they are rapidly oxidised to the corresponding acids, or reduced to the corresponding alcohols or glycols. Thus, the metabolism of vanillin was undertaken in this study as an example of a vanillyl aldehye.

Vanillin has found wide application in perfumery and confectionary. It is considered to be responsible for vanillaism, a dermatosis affecting persons handling vanilla pods, (116,117).

Preusse (1880) first studied the fate of vanillin in the animal body

(118). He fed both vanillin and an alcoholic extract of vanilla pods to rabbits. Both vanillin and the extract increased the output of ethereal sulfate, and vanillic acid. This acid was excreted mainly free, but some as an ethereal sulfate. Preusse also stated that a very small amount of vanillin was excreted unchanged. Cohn (1893) confirmed Preusse's results, showing that vanillin was oxidised to vanillic acid in vivo (119). Kotake (1905) isolated the conjugated glucuronide of vanillic acid which he called glucurovanillic acid (120).

Sammons and Williams (97) made a quantitative study of the fate of vanillin, and vanillic acid in the rabbit. In the case of vanillic, 69% was oxidised to vanillic acid (44% free and 25% conjugated), 14% of the vanillin was excreted unoxidised, but conjugated mainly as glucurovanillin, 8% of the vanillin accounted for was excreted as ethereal sulfate, and 31% as glucuronide. Two conjugated glucuronides were excreted, namely, glucurovanillin and glucurovanillic acid. They suggested that conjugation of these substances may be a necessary stage in their oxidation.

6. BACTERIAL ACTION

In the study of urinary phenolic and vanillyl metabolites, it is important to establish whether it is an end-product of endogenous metabolism or is derived from food constituents, or whether it occurs as a result of the action of intestinal micro-organisms. The exogenous sources of urinary metabolites had been dealt with in the previous section. Here, the action of micro-organisms in

the gut will be considered.

Though Booth and co-workers have shown that HVA, DHPAA and m-hydroxyphenylacetic acid are metabolic end-products of quercetin and rutin, (112) Nakagawa *et al.* have been unable to identify any of the m-hydroxyphenyl acids from quercetin in neomycin-treated rats (121). Rats so treated excrete instead dihydro-ferulic, p-hydroxyphenylpropionic and p-hydroxybenzoic acids. These results strengthen the suggestion by Shaw (122) that urinary m-hydroxyphenyl acids appear to be primarily bacterial metabolites of certain dietary precursors, including flavonoid compounds. Booth and Williams (123) also attributed dehydration as a reaction of the micro-organisms in caecal extracts.

As mentioned before, tea is the dietary source of 1,3-dihydroxy compounds. Urinary resorcinol could be derived from these 1,3-dihydroxy compounds. Curzon and Pratt (114) reported a decrease in resorcinol sulfate after the administration of 500 mg by mouth of the intestinal antibiotic frammycetin, (soframycin). They therefore concluded that resorcinol, like certain other aromatic constituents of urine, including catechol and m-hydroxy aromatic acids, is formed *in vivo* by intestinal bacteria. Both the m-hydroxy acids and resorcinol are derived from dietary vegetable matter. It appears that urinary resorcinol sulfate originates from the action of particular intestinal bacteria on dietary tea polyphenols.

Smith (124) found that urinary pyrocatechol decreases after

the administration of large doses of neomycin to subjects at 8 gm. daily. Therefore, it seems that the major fraction of urinary catechol is dependent upon the activity of gut bacteria. Nevertheless, a small proportion of catechol is normally excreted in rabbit (95) and human (125) urines in the conjugated form. Garton and Williams (126) believed that catechol is formed and excreted principally as a mono-glucuronide or mono-sulfate derivative in urine of rabbits receiving benzene. Other precursors of catechol include halogeno benzenes(127), vertric acid, and veratraldehyde (128), and quinic and shikimic acids (99). From these results, it is tempting to conclude that the catechol in human and cow urines may be formed from quinic acid and related compounds in the diet, with the participation of the intestinal micro-organisms.

In conclusion, it is apparent that any investigation of urinary metabolites in an attempt to elucidate the metabolic pathway necessitates the elimination of two paramount sources of interferences incurred, firstly, by the exogenous precursors of these urinary metabolites, and secondly by the action of the gut bacteria on them.

II. METHODS

The method of thin-layer chromatography (TLC) as developed by Sankoff and Sourkes (129) for measuring urinary HVA was adopted, both for qualitative and quantitative analyses. However, a few modifications were found necessary.

p-Vanillin (3-methoxy-4-hydroxybenzaldehyde) was injected intraperitoneally in a dose of 100 mg./kg. into rats of average weight of 100 g. Control rats received an equivalent volume of 0.9% NaCl. Groups of 3 rats were kept in metabolic cages, consisting of plastic and stainless steel parts. Urine was collected in 3 ml. of 5N HCl, for 24 hours, during which period only water was given. Restriction of food was necessary to prevent interference by phenolic compounds of exogenous origin. The pooled urine samples were diluted to equal volume with water and adjusted to pH 1 with 5N HCl.

1. ETHYL ACETATE EXTRACTION

Five ml. of urine was saturated with NaCl and extracted with 6 ml. and 5 ml. of ethyl acetate successively. In all cases, to eliminate artefacts which may be formed spontaneously in the urine, authentic compounds are added to the urine, and the whole procedure of extraction carried out simultaneously. For quantitative analysis, it was found necessary to extract three times with 6 volumes of ethyl acetate. This was to ensure complete extraction, as the content of urinary phenolic derivatives formed after injection of vanillin was high, particularly after hydrolysis. The pooled volume of solvent

was evaporated to dryness in a flash evaporator, under reduced pressure, and the residue dissolved in 0.5 ml. methanol. Here again, a modification was necessary: 1 ml. of methanol was used in the case of hydrolysed urine extracts. This prevents "over-loading" of the TLC plate when 25 µl. of the methanolic extract was applied. Besides, it was essential from the quantitative aspect, for otherwise the colorimetric reading of the VA spot would be in the upper range where the sensitivity of the method is low, and inaccuracy would be incurred. Of course, an alternative would be to dilute the solution before reading or to apply a smaller volume of the extract to the plate. However, to ensure uniformity in the method, dilution of the methanolic extract was preferred.

This method extracts essentially the free phenolic acids and their conjugates, as well as the neutral components. To liberate the phenolic acids, aldehydes, alcohols or amines from their conjugates, hydrolysis of the urine before extraction was necessary. Acid hydrolysis is often carried out by heating the urine in a boiling water bath with HCl at pH 1, for 30 mins. This form of hydrolysis often causes destruction of some of the more labile urinary constituents. Besides, acid hydrolysis gave inconsistent effects. Alternatively, enzymatic hydrolysis with β-glucuronidase, or sulfatase (Mylase P) or a commercial combination of the two i.e. Glusulase may be useful. This yields more information with regard to the type of conjugate - either a glucuronide, or sulfate, or both.

Enzymatic hydrolysis will be discussed in detail in a later section.

The amines or basic components are not extracted at this pH. However, their extraction could be performed at alkaline pH. The amount of phenolic amines in normal urine is often so small that a much larger volume is necessary. Also, they are very unstable compounds, and the ampholytic nature of these phenolic amines precludes a satisfactory application of solvent extraction. Kakimoto and Armstrong (73) used Dowex resin for the adsorption, concentration and purification of these amines. Likewise, Perry et al. (74) combined ion-exchange and paper chromatography in their investigation of urinary amines and amines of exogenous sources (75).

2. NEUTRAL COMPONENTS

These may be completely extracted at pH 6. They are also extracted together with the acidic compounds at pH 1. They could be separated, however, from the acidic derivatives by the procedure of Nakajima and Sano (130). The ethyl acetate extract obtained in the usual way was washed with 2 ml. of 0.2 M sodium borate, and then with 2 ml. of water. The acidic compounds were extracted into the sodium borate solution, while the neutral compounds remained in the organic phase. This ethyl acetate extract was then evaporated to dryness, and the residue dissolved in 200 µl. methanol. 20 µl. of this was applied on TLC plates, using Benzene-propionic acid-water (10:9:1) as the solvent system.

3. THIN-LAYER CHROMATOGRAPHY

Glass plates 20 x 20 cm were laden with silica gel G. (Merck) by means of the DeSaga applicator. A thickness of 0.25 mm was found to be most satisfactory. The consistency of the silica gel was 20 g in 40 ml. of water. This is sufficient for the preparation of 5 plates. Instead of water, borate or acetate buffer may be used. However, no significant improvement in the separation was found with these "buffered" plates. These plates were then air-dried and, before use, were activated by heating in an oven for 30 mins. at 105° C.

Standard solutions of vanillic acid, p-vanillin, HVA, VMA, etc. were prepared at a concentration of 1 μg./μl. These standard solutions and urine extracts were applied by means of a micro-pipette on the plates, about 1.5 cm from the base, and the plates were dried in a stream of hot air. Five to 10 μl. (equivalent to 5 to 10 μg.) of the standard solutions, and 25 μl. of the methanolic solution of urinary extract were sufficient to produce distinct spots when the plates were sprayed with Folin-Cioclateu phenol reagent, i.e. 1 N "phenol reagent" followed by 10% sodium carbonate (131).

4. QUANTITATIVE ANALYSIS

The identified spots, which were to be analysed quantitatively, were scraped from the TLC plates, eluted overnight with 3 ml. of water, mixed thoroughly, and centrifuged. 0.25 Ml. of 1 N phenol reagent and 1.0 ml. of 20% sodium carbonate were added to the supernatant. The tubes were immersed in boiling water for 1 min. to catalyse the reaction. Precipitates so formed were centrifuged

and discarded. Readings were then taken in a Coleman Spectrophotometer at 695 mμ. The color is stable for 90 mins.

Normally, a standard curve and a recovery curve are obtained at the same time. The former is achieved by spotting 5, 10, 15, 20 and 25 μg. of the authentic compound directly on the TLC plates. For the recovery curve, which provides information with respect to the percentage of recovery through the extraction procedure, 4 tubes were set up, each containing 5 ml. of pooled normal urine. To three tubes were added 100, 200 and 300 μg. of the standard compound, and the whole procedure of extraction, evaporation, development, elution and colorimetric measurements performed. The recovery of standards carried through the whole procedure is more than 85%.

5. SOLVENT SYSTEMS

As the identities of most of the urinary metabolites were deduced from their chromatographic mobilities as compared to authentic compounds, a large number of solvent systems were used. This is not by itself a sufficient criterion. However, if 2 compounds have similar Rf values in 5 or more solvent systems, and particularly if the latter are widely varied in polarity and pH, the substances may be tentatively claimed as one and the same compound. On this basis, coupled to the similar color reactions using different spray reagents, most of the urinary metabolites were identified.

The following were the solvent systems tried in this study. Chromatographic tanks need to be equilibrated for half to one hour

before development of the plates. A longer period of equilibration does not improve separation. In fact spots tend to be diffuse if too long an equilibration period precedes development, e.g., 3 hours or more. Apparently, this must be due to the alteration in the composition of the solvent system, by evaporation of the more volatile components. Dipping, (irregular front) which usually occurs, may be lessened with well equilibrated tanks. It is presumably due to the faster rate of evaporation of solvent from the vertical margins of the plates during the advance of the solvent front. Plates were removed from the tank when a solvent front of 16 to 16.5 cm was achieved. They were then air-dried.

The solvent systems used include:-

1.	Benzene-propionic acid-water	(2:3:1)
2.	Benzene-acetic acid-water	(2:3:1)
3.	Chloroform-acetic acid-water	(2:2:1)
	" " " "	(2:1:1)
	" " " "	(8:8:3)
4.	n-Butanol-acetic acid-water	(4:1:1)
5.	Benzene-methanol-acetic acid	(45:8:4)
6.	Benzene-dioxane-acetic acid	(90:25:4)
7.	Benzene-propionic acid-water	(10:9:1)
8.	Isopropanol-ammonia-water	(8:1:1)
9.	n-Butanol-pyridine-water	(10:3:3)
10.	20% aqueous KCl	
11.	5% aqueous NaF	

Solvent 1 was found to give the best separation of the urinary extract. However, 2 and 3 were also satisfactory. Solvent 4, though widely recommended for paper chromatography, was unsuitable for TLC because of the big diffuse spots formed, most of which were in the upper half of the plate, and which overlapped in many cases. 5, 6 and 7 were satisfactory for both acidic and neutral extracts. Limited movement of these compounds, concentrated within 8 cm from the origin, rendered the basic solvent 8 unsuitable. With 9, greater movements of the spots were achieved, and vanillic acid and p-vanillin, which have very similar Rf's in the acidic solvents, were well separated in this system.

The inorganic solvents 10 and 11 tend to deposit salts on the plates, which somehow inhibit the movement of the solvent front, and consequently yield poor results.

6. SPRAY REAGENTS

The following spray reagents, many of which required to be freshly prepared, were used for the identification of spots, formed on TLC plates, after development in different solvent systems.

(a) Phenol Sprays

1. Folin's Phenol Reagent (131)

1 N "phenol reagent" followed by 10% sodium carbonate.

2. Tetrazotised Benzidine (132)

(i) 5 gm. benzidine was dissolved in 14 ml. conc. HCl. and diluted to 1 litre with water.

(ii) 10% aqueous sodium nitrite solution.

Equal volumes of (i) and (ii) are mixed before use.

3. Nitraniline Reagent (133, 134)

(i)	p-nitraniline, 0.1% in 1 N HCl	1 vol.
(ii)	sodium nitrite, 0.2% in water	1 vol.
(iii)	sodium carbonate, 10% in water	2.5 vol.

(i) and (ii) are mixed at 5°C, and kept cool for 5 mins. (iii) is then added. This is used immediately. This reagent must be prepared fresh each time.

4. Sulphanilic Reagent (134)

(i)	sulphanilic acid, 9 gm. conc. HCl, 90 ml. water, 900 ml.	1 vol.
(ii)	sodium nitrite, 5% in water	1 vol.
(iii)	sodium carbonate anhydrous, 10% in water	2 vol.

When required, (i) and (ii) are mixed in the cold for 5 mins. and then (iii) is added carefully as the mixture effervesces vigorously. It is essential to keep the chromatograms away from phenolic vapours before using this spray reagent.

5. James Reagent (135)

5% ferric chloride solution, followed by 5% potassium ferricyanide.

6. Potassium Permanganate (132)

0.1 N potassium permanganate in sodium carbonate solution.

Spray reagents 1, 2, 3, and 4 were all satisfactory. The Folin's phenol reagent, though non-specific, as all phenols produce similar

blue coloration, was most extensively used because of its sensitivety, and quantitative analysis was based on the Folin's reaction carried out subsequently. It must be mentioned here that the Folin's reaction on the plate does not in any way interfere with subsequent running of the extract in material eluted from the gel.

Reagents 2, 3 and 4 produce spots ranging in color from yellow, orange, grey, to brown and red. The James reagent 5 revealed all phenolic compounds as deep blue spots, readily visible 2 to 5 mins. after spraying. However, these spots were soon obliterated by the development of a diffused Prussian blue background, which became more intense with time. Potassium permanganate reacts with the phenols, producing yellow spots on a pink background. Here again, the color reaction is not specific and the products are unstable.

(b) Stable Diazo Salts

In addition to the above, 6 stable diazo salts were tried (136). 0.1% aqueous solutions of the following 6 stable diazo salts, obtained from Irwin Dyestuff Division, Montreal, were used. Differences in hue or tint occur with different concentrations of the same compound. (See Table I).

Reagents No. 1 and 5 gave best results. Intense colors were obtained when 0.1% aqueous solution was used, followed by saturated sodium carbonate. Reagents 4 and 6 gave ill-defined colors because of the development of a greyish-purple background on the plates

TABLE I

Stable Diazo Salts

Reagent No.	Commercial Name	Stabilized Salt of:
1.	Red Salt GG	p-nitroaniline
2.	Red Salt RL	2-amino-3-nitrotoluene
3.	Yellow Salt GC	o-chloroaniline
4.	Black Salt K	4-amino-2,5-dimethoxy-4'-nitroazobenzene
5.	Bordeaux Salt BD	4-amino-2,5-dimethoxy-benzonitrile
6.	Corinth Salt V	4-amino-2,4'-dimethyl-5-methoxy-2'-nitroazo-benzene

(c) Specific Spray Reagents for Vanillin

The "phenolic" spray reagents previously mentioned were not sufficiently sensitive for vanillin, which constitutes only a small percentage of the urinary metabolites after its intraperitoneal injection. It was therefore necessary to distinguish if from the other extracted phenolic compounds by utilising reagents which would specifically produce colored complexes with the carbonyl group of vanillin. Few, if any, carbonyl compounds are present in normal rat urine to interfere. Nevertheless, when identical coloration is reinforced with identical chromatographic characteristics, a more or less conclusive identification could be established.

The following three reagents react with the carbonyl group of

vanillin to yield intense and characteristic colors. They are:-

1. Naphthoresorcinol Reagent (92)

   Solid naphthoresorcinol was dissolved in methanol (5% solution). The TLC plate was first sprayed with this solution, followed by conc. HCl. Aromatic aldehydes with a free para-hydroxy or para-O-methoxy group give a deep red color.

2. 2,4-Dinitrophenylhydrazine

   A 0.5% solution in 2N HCl was prepared. The 2,4-dinitrophenylhydrazone formed by condensation with vanillin is a bright orange product, very distinct on TLC plates.

3. Aniline-Diphenylamine Reagent

   Aniline, 1% (1 ml.) plus 1% diphenylamine in acetone 10 vol.

   Phosphoric acid 85% (Merck) 1 vol.

   This reagent is used for the detection of sugars which give blue or green colors. Apparently, it is the CHO group which reacts to produce the color. Similarly, vanillin, p-hydroxybenzaldehyde and protocatechuic aldehyde (3,4-dihydroxybenzaldehyde) react, but they produce bright canary-yellow colors.

Utilization of these 3 spray reagents, each of which is highly sensitive and specific, made it possible to draw direct conclusions as to the presence or absence of vanillin, which would otherwise be doubtful.

(d) Ethylenediamine Spray Reagent

Catechol was suspected as a metabolite of vanillin. As catechol derivatives like noradrenaline, dopa, dopamine and 3,4-dihydroxy-

phenylacetic acid condense with ethylenediamine to form fluorescent compounds (137, 138, 139, 140), catechol itself should likewise give a fluorescent spot if sprayed with an alkaline solution of ethylenediamine. This spray solution was prepared as follows:-

(i) 2 parts of ethylenediamine

(ii) 8 parts of ammonium hydroxide (10%)

100 ml. of ammonium hydroxide is diluted to 580 ml. with water (10%). From this, take 560 ml. and dilute to 700 ml. with ethylenediamine.

The TLC plate, after development was sprayed with this solution. It was then viewed under U.V. light.

7. RE-CHROMATOGRAPHY

Spots on plates which were developed in Benzene-propionic acid-water (2:3:1) were eluted and re-extracted. They were then re-applied onto new plates and developed in different solvent systems. These spots include both the authentic (standard) spots, as well as those from urine extracts which had mobilities similar to those of the standard spots. Thus, re-chromatography could further confirm the identity of many unknown compounds.

8. TWO-DIMENSIONAL CHROMATOGRAPHY

A number of combinations of different solvent systems were tried on TLC plates. These include combinations of acidic and basic, and organic and inorganic solvent systems. As very good separation was achieved in Benzene-propionic acid-water (2:3:1), this was chosen as the first run, followed by:-

1. Isopropyl alcohol-ammonia-water (8:1:1)
2. n-Butanol-acetic acid-water (4:1:1)
3. 20% aqueous KCl
4. 5% aqueous NaF.

Except for 2, which gave fairly good results, all the other systems, which were extensively used for 2-D paper chromatography, were found to be unsatisfactory. Tailing or streaking occured. Probably the composition of the silica gel was affected by the first run, so that any subsequent run was unsuccessful. Two other factors made 2-D chromatography impractical:

1. It was not possible to "re-activate" these plates, as heating would destroy many of the compounds.
2. The gel coating no longer adheres to the glass plate on heating after the first run.

Discrete spots were obtained however if spots were eluted and re-applied on fresh plates, and subsequently developed in a different solvent system.

9. PAPER CHROMATOGRAPHY

Whatman No. 1 paper was used for 1-D chromatography using the same solvent system of Benzene-propionic acid-water (2:3:1) which gave excellent separation of the phenolic acids on TLC plates. In this case, hardly any separation was achieved when the solvent front was 16 cm. A longer strip of paper or a different solvent system may improve separation. However, both paper chromatography and 2-D thin-layer chromatography were abandoned after these

preliminary trials.

10. HYDROLYSIS

(a) Acid Hydrolysis

Five ml. of urine was adjusted to pH 1 with 5N HCl. Hydrolysis was carried out in a water bath (100°C) for 30 mins. The sulfate conjugates are supposed to be split by this procedure in 10-15 mins., while more drastic conditions are required to hydrolyse the glucuronides and glycine conjugates. Because of the uncertainty of the conditions, it is not possible to draw convincing conclusions as to the nature of the conjugates hydrolysed by these means. Enzymatic hydrolyses to be described presently are preferred in most cases.

(b) Enzymatic Hydrolysis

(i) By Glusulase - Enzymatic hydrolysis was carried out on 5 ml. of urine with Glusulase of activity equivalent to 5,000 units of β-glucuronidase and 2,500 units of sulfatase. This enzyme preparation was obtained from the Endo Laboratories Incorporated. Each c.c. of Glusulase contains 100,000 units of β-glucuronidase and 50,000 units of sulfatase. A 100-fold dilution of this gives an activity of 1,000 units of glucuronidase per ml. For each ml. of urine, 1,000 units of glucuronidase is sufficient to ensure complete hydrolysis. Therefore, for 5 ml. of urine, 5 ml. of the diluted Glusulase solution was used. Five ml. of urine was first adjusted to pH 5.5 with 2N NaOH. It was then pre-incubated at 37°C for 10 mins. The enzyme was added,

followed by a few drops of chloroform to prevent bacterial growth during the further incubation at 37°C for 24 hours. As one of the urinary conjugated products was an alcohol, extraction with 2 volume of ethyl acetate was performed on the hydrolysate prior to its re-adjustment to pH 1 with HCl. Extraction was again carried out at this strongly acidic pH. In this way, it was hoped that complete extraction of both the neutral as well as the acidic components can be accomplished.

(ii) By β-glucuronidase

β-glucuronidase (Sigma Chemical Co.) was employed at three concentrations: 1,000, 2,000 and 3,000 units/ml. urine. The above procedure for Glusulase was carried out except that the optimal pH was 5.2.

(iii) By Mylase P (or sulfatase)

Mylase P was obtained from the Nutritional Biochemicals Corp. Unfortunately, no specific activity of the enzyme is stated on the label. Consequently, three concentrations of the enzyme were again used, the lowest being equivalent to 1,000 units of β-glucuronidase on a weight basis. The two higher concentrations were 2 and 3 times this. This is, however, a poor criterion for quantitative analysis, though it may suffice for qualitative purposes. An assay of the sulfatase activity in both Mylase P and Glusulase would be necessary to determine the activity of this enzyme in these two commercial products.

(c) Zone Hydrolysis

Unhydrolysed urine extracts were first chromatographed on TLC

plates as a band, and developed in Benzene-propionic acid-water (2:3:1). Horizontal bands (about 1/2 inch) were eluted, centrifuged and the supernatant subjected to Glusulase hydrolysis as before. For comparison, these "Zone" hydrolysed extracts were re-chromatographed in the same solvent system as that used for the first run. In this way, any difference in Rf's prior to and after hydrolysis would be readily detected. Such a difference would be attributed to the presence of a conjugate.

(d) Hydrolysis of the Aqueous Fraction of Urine after Ethyl Acetate Extraction at pH 1

Since "Zone" hydrolysis failed to locate both vanillin and vanillyl alcohol conjugates, it appeared that these may be present in the aqueous fraction, not extracted by ethyl acetate at pH 1. Consequently, hydrolysis of the aqueous fraction was performed subsequent to the extraction with 6 vol. of ethyl acetate at pH 1. Glusulase hydrolysis was carried out as before, followed by re-extraction. Any significant difference between hydrolysis of the whole urine, and that of the aqueous fraction subsequent to ethyl acetate extraction would suggest that a fraction of the conjugate could be extracted. Should both hydrolysates yield the same results, then it may be concluded that the conjugate was present primarily in the aqueous phase. In the case of vanillic acid, which is present in high concentration in the free form, it is absolutely necessary to ensure complete extraction of this free vanillic acid before

hydrolysis is carried out. Otherwise conclusions may be erroneous.

11. REDUCTION OF VANILLIN BY SODIUM BOROHYDRIDE

(a) Qualitative

Vanillyl alcohol, a likely metabolite of vanillin was obtained by the reduction of vanillin by sodium borohydride. The reduction was performed as follows:-

Fifty mg. of vanillin was dissolved in 2 ml. of methanol. A solution of 0.2 g. sodium borohydride in 0.2 ml. of 2N NaOH, diluted to 1.8 ml. of water, was added slowly to the vanillin solution, the temperature being kept at 18-25° C. NaOH was used here as sodium borohydride is most stable at alkaline medium. It is also relatively stable at neutral pH. The methanol was then evaporated off, and the resulting solution was extracted with ethyl acetate. This ethyl acetate extract was then evaporated to dryness and the residue dissolved in 1 ml. methanol. Five μl. of this methanolic solution, when applied on TLC plate and developed in Benzene-propionic acid-water (2:3:1), gave rise to two spots, one of which was more distinct than the other. The fainter spot had an Rf corresponding to that of authentic vanillin, indicating incomplete reduction. The more distinct spot was presumably its reduction product, vanillyl alcohol. This had an Rf of 0.56. As the two spots were well separated in this system, the alcohol could be eluted from the plate and subsequently served as "standard" vanillyl alcohol.

(b) Quantitative

As vanillyl alcohol is not available commercially, reduction of vanillin to vanillyl alcohol, using sodium borohydride was attempted to provide a standard for quantitative analysis. In such a case, complete reduction was essential, or at least desirable. To ensure this, reduction was carried out as before, using only 10 mg. of vanillin. All the other concentrations of reactants and conditions were maintained. The reduction mixture was then extracted with 5 vol. of ethyl acetate, evaporated to dryness and the residue dissolved in a volume of methanol, such that 1 μl. of this solution contains 1 μg. (assuming complete reduction at this stage). 5, 10, 15, 20 and 25 μl. of this "standard" solution were applied on TLC plate. Simultaneously, 5, 10, 15, 20, 25 μl. (1μl = 1 μg) of vanillic acid standard solution were applied on another plate. The plates were developed in Benzene-propionic acid-water (2:3:1) and the quantitative procedure mentioned before was used.

Chromatographically, vanillin appeared to have been completely reduced, as judged by the absence of the vanillin spot when the extract of the reduction mixture was applied to the TLC plate. This was confirmed colorimetrically by the readings obtained by two independent sets of reduction mixtures, which were themselves almost identical, and when plotted against vanillic acid standard curve, ran almost coincident with it. Consequently, and subsequently, this solution was applied as

vanillyl alcohol "standard" for quantitative analysis. It was found that 10 μg. of vanillic acid and 10μl of this vanillyl alcohol "standard" solution consistently give a colorimetric reading of 0.15 on the Coleman Spectrophotometer at 695 mμ.

(c) Confirmation of "Standard" Vanillyl Alcohol

Vanillyl alcohol was finally purchased from K.and K. Laboratories Inc. This was chromatographed adjacent to the previously prepared vanillyl alcohol, obtained by sodium borohydride reduction. The two compounds were found to have similar Rf's in Benzene-propionic acid-water (2:3:1) and in Chloroform-acetic acid-water (2:2:1). Similar color reactions were also obtained with p-nitraniline, tetrazotised benzidine, and diazotised sulfanilic acid sprays.

(d) Quantitative Comparison of Authentic Vanillyl Alcohol and the "Standard" Vanillyl Alcohol.

"Standard" vanillyl alcohol solution, containing 5,10,15, and 20 μg. were pipetted into a series of 4 tubes. Corresponding quantities of authentic vanillyl alcohol were put into another set of 4 tubes. Three ml. of water was added to each, followed by 0.25 ml. of 1 N phenol reagent, and 1 ml. of 20% sodium carbonate. The tubes were immersed in boiling water for 1 min., and readings taken at 695 mμ on a Coleman Spectrophotometer.

TABLE II

Concentration in µg.	Authentic Vanillyl Alcohol	"Standard" Vanillyl Alcohol
5	0.102	0.105
10	0.210	0.193
15	0.320	0.280
20	0.370	0.364

As can be seen from the above results, there was very close correlation between the two sets of readings, indicating that almost complete reduction of vanillin by sodium borohydride was possible by the previously mentioned procedure. Besides, we can state with confidence that results so far obtained using our own "standard" vanillyl alcohol are still valid.

III. RESULTS

1. QUALITATIVE ANALYSIS

Visual comparison of the chromatographic pattern on TLC plates with regard to Rf values, color reactions with different spray reagents and U.V. fluorescence forms the basis of identification of most of the urinary metabolites. This may at first appear simple and straigth-forward. However, with different solvent systems, varying numbers of significant spots appear with different Rf values. It must be emphasized here that the enumeration of spots in each solvent system is characteristic and bears no relation to the same numbering of spots in the other solvent systems. Not until all the spots are identified could there be a unified scheme for their numbering. Even then, the relative mobilities of a few compounds in different solvent systems may differ. Nevertheless, numbering will be arranged in ascending order of Rf's.

(a) Free Phenolic Compounds

1. Benzene-acetic acid-water (2:3:1)

Using the solvent system, Benzene-acetic acid-water (2:3:1) two very intense spots were observed in the urine extract of the animals receiving vanillin. This material is henceforth to be termed "vanillin-injected-extract." As can be seen from Table III, these spots have Rf's of 0.08 and 0.79. The former Rf was similar to that of VMA, and the latter that of VA.

2. Benzene-propionic acid-water (2:3:1)

This system, though very similar to (1), enhances movements of all the phenolic compounds, resulting in slightly better separations. (See print in Fig. 4).

TABLE III

Preliminary Experiments in Different Solvent Systems

Solvent Systems	VMA	HVA	Vn	VA	Spot 1	Spot2	Spot 3
1. Benzene-acetic acid-H_2O (2:3:1)	0.08	0.66	0.71	0.80	0.08	0.79	-
2. Benzene-propionic acid-H_2O (2:3:1)	0.16	0.76	0.78	0.90	0.21	0.63	0.90
3. Chloroform-acetic acid-H_2O (2:2:1)	0.29	0.87	0.96	0.93	0.75	0.92	-
4. n-Butanol-pyridine-H_2O (10:3:3)	0.35	0.43	0.71	0.52	0.51	0.76	-

Vn = vanillin

As can be seen from fig. 5 (p.65), and Table III, 3 spots were clearly evident in the "vanillin-injected-extract" compared to the control. The most prominent had an Rf of 0.90. The other two minor spots had Rf's of 0.21 and 0.63. Here 3 spots were encountered instead of two. However, the one with Rf 0.21 probably corresponds to spot 2 in Benzene-acetic acid-water (2:3:1). In this solvent system, authentic VMA co-chromatographed always had a smaller Rf than spot 1. Because the difference in Rf was too small, a series of six runs were performed in this system. In each case, spot 3 had an Rf similar to that of VA, while spot 1 consistently moved ahead of VMA.

3. Chloroform-acetic acid-water (2:2:1)

In this system, again spot 2 appeared to be VA. However, because of the very similar Rf's of VA and vanillin, we cannot conclude that it is VA. Spot 1 is yet unidentified.

4. n-Butanol-pyridine-water (10:3:3)

Vanillic acid is well separated from vanillin in this particular system. Vanillin has a greater Rf than the acid, unlike the findings with the other solvent systems mentioned before, where VA always moved ahead of or beside vanillin. However, very poor separations of the components of the urine extract occured when this system was used. It is only useful as a means of distinguishing between VA and vanillin.

From the results obtained so far, the only tentative inference made, based on Rf's compared with those of authentic compounds, was that VA was present in the urine in the free form after intraperitoneal injection of vanillin. This was further confirmed by the addition of VA to the "vanillin-injected-urine", followed by the usual procedure of extraction and chromatography. In all cases, VA so added did not in any way interfere with the chromatographic pattern so far obtained except to increase the intensity of the spot identified as VA.

(b) Re-chromatography

Elution-cum-re-extraction of the VA spot from plates developed in Benzene-propionic acid-water (2:3:1), as well as the authentic VA spot was carried out in methanol. Results obtained by re-chromatographing these, together with freshly applied authentic VA in several other solvent systems were further evidence of the presence of free VA in the "vanillin-injected-extract." All the three spots had identical

Rf's in the solvent systems tested, as shown in Table IV.

TABLE IV

Rf Values of VA in different Solvent Systems on TLC

	Solvent Systems		Rf's of VA
(1)	Benzene-propionic acid-water	(2:3:1)	0.90
(2)	Benzene-acetic acid-water	(2:3:1)	0.80
(3)	Benzene-methanol-acetic acid	(45:8:4)	0.81
(4)	Benzene-dioxane-acetic acid	(90:25:4)	0.72
(5)	Chloroform-acetic acid-water	(2:2:1)	0.93
(6)	n-Butanol-pyridine-water	(10:3:3)	0.52
(7)	Isopropyl alcohol-NH_3-water	(8:1:1)	0.45

(c) Spraying of Plates

Six different spray reagents were used in the course of these qualitative studies in addition to the stable diazo salts mentioned before. Each color reaction by itself is not very informative, as a general shade of color is produced, e.g., a blue coloration is produced by all phenols with the Folin-Ciocalteu phenol reagent. The James reagent yields deep blue spots on a diffuse blue background, while the potassium permanganate spray gives yellow spots on a pink background. Tetrazotised benzidine and p-nitraniline showed a wider spectrum of coloration, but still limited, as only yellow, orange or brown spots are formed. The same holds for diazotised sulfanilic acid.

TABLE V

Color Reactions with several Spray Reagents and Stable Diazo Salts

Spray Reagents	VMA	HVA	p-Vanillin	VA	*Spot 1	*Spot 2	*Spot 3
Folin's Phenol Reagent	Blue	Blue	Blue	Blue	Blue	Blue with br. center	Blue
James Reagent	Blue	Blue	Blue	Blue	Blue	Blue	Yellow
Potassium Permanganate	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow	Yellow
Tetrazotised Benzidine	Yellow-brown	Grey-brown	Yellow	Rusty-brown	Yellow-brown	Grey	Rusty-red
p-Nitraniline	Pale-orange	Pink-yellow	Bright-yellow	Brown	Yellow-orange	Dark-grey	Yellow-brown
Diazotized Sulfanilic Acid	Reddish brown	Grey-yellow	Bright-yellow	Bright-orange	Yellow	Grey	Orange-yellow
**Reagent No. 1	Brown-yellow	Pale-orange	Yellow	Pale-orange	Yellow	Green-brown	Orange
Reagent No. 2	Yellow	Yellow	Yellow	Yellow	Yellow	Brown	Yellow
Reagent No. 3	Yellow	Pale-orange	Yellow	Bright-yellow	Yellow	Yellow-brown	Orange
Reagent No. 5	Pale-orange	Yellow	Yellow	Pink-yellow	Pale-orange	Grey-green	Yellow

* Spots 1, 2 and 3 are those obtained in Benzene-propionic acid-water (2:3:1).

** Reagent numbers are those referred to on Page 36.

(d) Conjugated Phenolic Compounds

Conjugation, a form of physiological inactivation of many active substances, occurs principally in the liver. The conjugated products have always been regarded as inactive or detoxication products, as manifested by their presence in urine. However, investigations of the steriods, whose conjugated urinary metabolites have been most extensively studied, have provided increasing evidence of these compounds, hitherto considered to be inactive end-products, as possible active intermediates. These conclusions have been drawn mainly from experiments using doubly-labelled compounds.

Analogously to this, phenolic conjugates may likewise prove to be important intermediates. Like the steroids, these conjugates constitute a major fraction of the total phenolic metabolites excreted. The phenolic conjugates include the glucuronides and sulfates, while the carboxyl group may be conjugated with glycine. Not all phenols appear in urine as conjugates, e.g. VMA is excreted free. The glycols and ethanols are usually sulfate-conjugated, while MN and NMN are conjugated as glucuronides.

In this study both acid and enzymatic hydrolyses were carried out.

(i) Acid Hydrolysis

Hydrolysis with HCl at pH 1 at 100° C for 30 mins., followed by extraction and chromatography, revealed that there was an increase in the intensity of the VA spot, compared to the unhydrolysed "vanillin-injected-extract." However, the existence of large amounts of free VA in the urine rendered any conclusion of the presence of a VA conjugate

unjustified. Nevertheless, an increase in both intensity of color as well as area of the VA spot was unmistakable, using Folin's phenol reagent spray. With acid hydrolysis, an undesirable background on TLC plate occured along the line of separation on development of the extract.

In addition, spot 2 (Rf 0.63) was also increased. This spot appeared dark on TLC plate prior to any spray - an indication of a dihydroxy compound. Two new spots with Rf's of 0.42 and 0.56 became apparent after acid hydrolysis.

(ii) Enzymatic Hydrolysis

Enzymatic hydrolysis yielded better results, as shown by the very distinct appearance of the spot with Rf 0.56. However, enzymatic hydrolysis by Glusulase did not reveal the spot with Rf 0.42. This is probably a glycine conjugate.

(iii) Vanillic Acid Conjugate

Free vanillic acid was found to be formed by rats which received intraperitoneal injections of vanillin. As the amount of free VA in urine was considerable, any VA liberated by hydrolysis from its conjugate would not be easily detected, as it would be masked by the existing large quantity of VA. Since extraction by ethyl acetate presumably removes both the free and conjugated products, the problem cannot be resolved except by quantitative analysis. The difference between hydrolysed and unhydrolysed urines should indicate the presence of a conjugate.

In this study, both acid and enzymatic hydrolyses seemed to increase the area as well as the intensity in color of the VA spot over that of

the unhydrolysed urine. Thus, a VA conjugate must be considered. The phenolic group is likely to be the site of conjugation, though the carboxyl group cannot be ruled out, as many aromatic acids are conjugated with glycine. Should conjugation occur via the phenolic OH, detection by the usual phenolic reacting sprays would be of no avail. Zone elution, followed by enzymatic hydrolysis was next attempted, to locate such a conjugate. This would only be possible if the free compound had a different mobility from that of the conjugate. This is to be expected, as the presence or absence of a hydroxyl or carboxyl group affects the Rf considerably.

(iv) Zone Elution and Hydrolysis

Results showed that zone elution of a horizontal band of the unhydrolysed "vanillin-injected-extract," between 1.5 to 2 cm from the origin of a plate which had previously been developed in Benzene-propionic acid-water (2:3:1), and which had a solvent front of 16 cm (i.e. the Rf of the band was 0.11) produced, after Glusulase hydrolysis, and re-chromatography in the same solvent system, a spot with an entirely different Rf. This was 0.90, similar to that of authentic VA.

In six other solvent systems, this spot had the same Rf's as authentic VA as shown on Table VI. It was clearly differentiated from vanillin when re-chromatographed in n-Butanol-pyridine-water (10:3:3). These results signify the presence of a VA conjugate. The quantitative significance of this was not evaluated. However, in spite of the losses incurred through the processes of extraction, elution, hydrolysis, re-extraction and re-chromatography, a VA spot

was clearly distinguishable when a horizontal band equivalent to approximately 0.8 ml. urine was employed.

Apart from the disclosure of a VA conjugate by this method, no other significant result was shown. The identity of the VA conjugate is yet to be resolved and confirmed by quantitative estimation.

TABLE VI

Rf Values of Vanillic Acid, Vanillin and Spot obtained from Zone Hydrolysis

Solvent Systems		Authentic VA	Authentic Vanillin	Spot from Zone Hydrolysis
Benzene-propionic acid-water	(2:3:1)	0.90	0.78	0.90
Benzene-acetic acid-water	(2:3:1)	0.8	0.71	0.81
Benzene-methanol-acetic acid	(45:8:4)	0.81	0.89	0.81
Benzene-dioxane-acetic acid	(90:25:4)	0.72	0.73	0.71
Chloroform-acetic acid-water	(2:2:1)	0.94	0.96	0.93
n-Butanol-pyridine-water	(2:1:1)	0.52	0.71	0.50
	(2:1:1)	1.0	1.0	1.0
n-Butanol-acetic acid-water	(10:3:3)	0.80	0.80	0.81

(v) Vanillyl Alcohol Conjugate

Authentic vanillyl alcohol, and vanillyl alcohol produced by sodium borohydride reduction of vanillin, had an Rf similar to one of the spots formed after hydrolysis, i.e. Rf 0.56 - 0.60 in Benzene-propionic acid-water (2:3:1). As can be seen from Table VII, this spot had a very similar Rf to authentic vanillyl alcohol in all the

seven solvent systems tested. Besides, it gave positive Folin's postassium permanganate and ferric chloride reactions and yellow colors with tetrazotised benzidine, p-nitraniline and diazotised sulfanilic acid.

The ethyl acetate extract of the Glusulase hydrolysate, obtained in the usual procedure, was washed with 2 ml. of 0.2M sodium borate, and then with 2 ml. of water. The neutral compounds remained in the organic phase. The ethyl acetate extract, on evaporation to a small volume and chromatographed, showed an increase in the spot with Rf 0.55 in Benzene-propionic acid-water (10:9:1). This further confirms the neutral nature of the vanillyl alcohol spot.

Based on these results, vanillyl alcohol was tentatively identified as an exclusively conjugated urinary product of vanillin. p-Glucuronidase yielded this vanillyl alcohol spot almost as distinct as that produced by Glusulase. Mylase P, on the other hand, showed only a very small and ill-defined vanillyl alcohol spot. This, however, may be due to the low sulfatase activity in the preparation. (See Table VII).

(vi) Catechol Conjugate

The spot with Rf 0.6 - 0.62 in Benzene-propionic acid-water (2:3:1) always appeared dark on unsprayed TLC plates, which indicates that it may be a dihydroxy compound. PA, DHMA, and DHPG, when applied as standards, gave similar dark spots on TLC plates. This is probably due to "pigmentation" of the spot as a result of quinone formation, by aerial oxidation of the two adjacent hydroxyl groups. Another charac-

istic of these o-dihydroxy compounds is that they produce a positive Folin's reaction with 1 N phenol reagent, before spraying with sodium carbonate.

TABLE VII

Rf Values and Color Reactions of Vanillyl Alcohol

Solvent Systems		Rf's A*	Rf's B**
Benzene-propionic acid-water	(2:3:1)	0.56	0.55
" " " "	(10:9:3)	0.55	0.55
Benzene-acetic acid-water	(2:3:1)	0.34	0.32
Benzene-methanol-water	(45:8:4)	0.68	0.66
Benzene-dioxane-water	(90:25:4)	0.57	0.54
Chloroform-acetic acid-water	(2:1:1)	0.78	0.78
n-Butanol-acetic acid-water	(4:1:1)	0.80	0.81
Spray Reagents			
Folin's Ciocalteu Reagent		Purple	Purple
James Reagent		Blue	Blue
Potassium Permanganate		Yellow	Yellow
Tetrazotised Benzidine		Br. Yellow	Br.Yellow
p-Nitraniline		Yellow	Yellow
Diazotised Sulfanilic Acid		Bright Yellow	Bright Yellow

* A above represents authentic vanillyl alcohol.

**B represents one of the new spots formed by Glusulase hydrolysis.

A number of o-dihydroxy compounds, which are likely to be formed from vanillin were then screened. These include PA, protocatechuic aldehyde, and catechol. As shown on Table VIII, catechol appears to be the likely dihydroxy compound formed, with respect to the Rfs and color reactions. The presence of this compound from vanillin would implicate a process of demethylation, and oxidative decarboxylation, the former being a process rarely encountered in animal organisms.

Judging from the intensity in color of the spot produced with the Folin's phenol spray, catechol appeared to be one of the major metabolites. However, as shown on Fig. 5, (Page 65), equal concentrations of VA and vanillyl alcohol produced visually the same intensity of coloration with the phenol spray, while catechol appeared almost twice as intense. This is probably due to the two hydroxy groups of catechol, each of which is capable of reacting with the phenol reagent. Conversely, vanillin produced a less intense spot. No logical explanation, however, could be given for vanillin. This was further confirmed by the dissimilar readings obtained when the Folin's reaction was performed on equal concentrations of VA, vanillin and catechol. That produced by catechol >VA> vanillin. Thus, very erroneous conclusions may result based on visual comparisons alone.

(vii) U. V. Fluorescence

A number of fluorescent spots were clearly evident on TLC plates under U. V. light, both before and after hydrolysis. There was no striking difference between the unhydrolysed urine extract and the hydrolysate, nor was there any difference between each of these extracts when compared to the controls. However, after spraying

TABLE VIII

Rf's and Color Reactions of Catechol, Protocatechuic Acid and Protocatechuic Aldehyde

Solvent Systems and Spray Reagents		Catechol	Protocatechuic Acid	Protocatechuic Aldehyde	C *
Benzene-propionic acid-water	(2:3:1)	0.62	0.53	0.47	0.63
Benzene-propionic acid-water	(10:9:1)	0.70	0.54	0.49	0.71
Benzene-acetic acid-water	(2:3:1)	0.52	0.46	0.25	0.52
Chloroform-acetic acid-water	(2:1:1)	0.45	0.20	0.27	0.43
Chloroform-acetic acid-water	(2:2:1)	0.76	0.33	0.43	0.75
n-Butanol-acetic acid-water	(4:1:1)	0.82	0.77	0.78	0.80
Benzene-dioxane-acetic acid	(90:25:4)	0.72	0.57	0.54	0.71
Benzene-methanol-acetic acid	(45:8:4)	0.78	0.51	0.68	0.78
Folin's Phenol Reagent		Blue**	Blue**	Blue**	Blue**
James Reagent		Grey-blue	Blue	Blue	Blue
Potassium Permanganate		Yellow	Yellow	Yellow	Yellow
Tetrazotised Benzidine		Grey-brown	Grey	Grey	Brown
p-Nitraniline		Grey	B.-yellow	B.-yellow	Grey
Diazotised Sulfanilic Acid		Grey	Br.-yellow	Br.-yellow	Grey
Ethylenediamine (viewed under U.V. light)		Green	Green	Green	Green

* C represents the dihydroxy spot formed in hydrolysed "vanillin-injected-extract."

**Blue with brown centre. (B = brown, Br = bright).

with an alkaline solution of ethylenediamine, one brilliant green spot was distinguished from hydrolysed "vanillin-injected-extract." No corresponding spot was visible in the unhydrolysed "vanillin-injected-extract," or in the hydrolysed control urine extract. Authentic catechol, co-chromatographed produced an identical fluorescent green spot with similar Rf.

Catechol was, thus, tentatively identified as one of the urinary metabolites of vanillin. It was apparently present predominantly as a conjugate, as Glusulase and acid hydrolyses intensified the spot on TLC plates considerably.

(viii) Vanillin Conjugate

So far, the conjugates of vanillic acid, vanillyl alcohol and catechol have been identified, either directly by the results obtained by acid or enzymatic hydrolyses, or indirectly by zone hydrolysis. The question of a vanillin conjugate arises especially when a dose of vanillin was administered. As mentioned before, vanillin could not be clearly distinguished by the Folin's reagent spray because of the faint coloration produced. This was further hindered by a background of countless other naturally occuring urinary phenolic constituents on the plate. Other phenolic reacting sprays mentioned produce very similar colored complexes for most of these phenolic constituents. Consequently, any vanillin, free or otherwise, would escape notification. We, therefore, resort to more specific sprays; the most natural consideration is to utilize the carbonyl and not the phenolic group of vanillin. Many aromatic aldehydes encountered as intermediary metabolites are readily attacked by aldehyde oxidase,

aldehyde dehydrogenase, or alcohol dehydrogenase. Because of this, aromatic aldehydes tend to be metabolically transient, and their presence in urine as end-products of natural metabolism has been considered to be negligible. On this consideration, if vanillin is excreted after an overload of it to the animal, it may be conveniently detected by carbonyl reagents, not interfered by other carbonyl compounds, which are presumably not found in normal urine.

The three spray reagents which condense with carbonyl groups to produce intense and characteristic colors are naphthoresorcinol, 2,4-dinitrophenylhydrazine and aniline-diphenylamine. All these three sprays are highly sensitive, 5 μg. or less of vanillin being readily visualized.

Using these three carbonyl spray reagents, it was found that no free vanillin was excreted in the urine. Vanillin was present only in the hydrolysed urine, indicating that its excretion occured exclusively as a conjugate (See Table IX). There was no indication of either free or conjugated vanillin in the control urine.

(ix) Enzymatic Hydrolysis of the Aqueous Fraction of urine after Ethyl Acetate Extraction at pH 1

Enzymatic hydrolysis had revealed the presence of the conjugates of VA, vanillyl alcohol and vanillin, in urine and yet zone hydrolysis showed only the presence of the first of these named conjugates. The discrepancy here seemed to suggest that the other two conjugates may have remained in the aqueous fraction, after ethyl acetate extraction at pH 1. Consequently, hydrolysis of the aqueous fraction was performed subsequently to the extraction with 6 vol. of ethyl acetate

at pH 1. Glusulase hydrolysis was carried out as before, followed by re-extraction of the neutral and acidic phenolic derivatives.

TABLE IX

Rf's of p-Vanillin in different Solvent Systems and its Color reactions

Solvent Systems		*A	**B
Benzene-propionic acid-water	(2:3:1)	0.78	0.78
Benzene-acetic acid-water	(2:3:1)	0.72	0.70
Chloroform-acetic acid-water	(2:2:1)	1.0	1.0
" " " "	(2:1:1)	0.96	0.95
n-Butanol-pyridine-water	(10:3:3)	0.72	0.71
n-Butanol-acetic acid-water	(4:1:1)	0.80	0.76
Benzene-methanol-acetic acid	(45:8:4)	0.89	0.87
Benzene-dioxane-water	(90:25:4)	0.73	0.72
Isopropyl alcohol-ammonia-water	(8:1:1)	0.59	0.60
Spray Reagents			
Napthoresorcinol		Red	Red
2,4-Dinitrophenylhydrazine		Orange	Orange
Aniline-diphenylamine		Bright Yellow	Bright Yellow

* A above represents Authentic/p-vanillin.

**B represents spot in Glusulase hydrolysate revealed by one of the three carbonyl spray reagents.

This experiment showed that indeed, both the vanillin and vanillyl alcohol conjugates were present in this aqueous fraction. Also, VA (possibly a fraction) was found in this aqueous phase. This may be due to incomplete extraction. Subsequently, more exhaustive extraction was carried out and the aqueous phase was tested for VA, to ensure its complete removal, before hydrolysis was carried out. By this way, it was confirmed that part of the VA conjugate was not extracted at pH 1.

The extractability of the various conjugates may or may not reflect the nature of the conjugates - either a sulfate or a glucuronide. Comparison of the quantitative result of the hydrolysates of whole urine, and that of the aqueous fraction alone should indicate the extent of extractability of each one of these conjugates. In addition, comparison of these results with those obtained by hydrolyses with β-glucuronidase, Mylase P, and Glusulase, may confirm these correlations.

(x) Nature of the Conjugates

The four metabolites of vanillin identified have all been shown to occur as conjugates. Two of these (vanillin and vanillyl alcohol) are excreted exclusively, and the other two (VA and catechol) partially as conjugates. As Glusulase had been used extensively in this study, and all these four conjugates revealed by this enzyme, they must be either glucuronide or sulfate conjugates. To differentiate the two, Mylase P (Nutritional Chemical Co.) and β-glucuronidase (Sigma) were used separately, and results obtained compared to that of Glusulase hydrolysis.

In the case of VA, no conclusion could be drawn owing to the very high concentration of free VA in the unhydrolysed urine. For

catechol, only a trace amount was present in the free state. As shown on the print in Fig.5 , both Mylase P and β-glucuronidase liberated free catechol. Thus, conjugation with sulfate and glucuronide might have occured with catechol.

Similarly, with vanillyl alcohol, both β-glucuronidase and Mylase P were found to be active. The former seemed to cleave the conjugate to a greater extent than the latter, as shown by the more intense and larger vanillyl alcohol spot produced by β-glucuronidase. In fact, the extent of hydrolysis by β-glucuronidase appeared to be as great as that of Glusulase. However, it is by no means a sufficient criterion to advocate the conjugation as glucuronide predominantly because of the lack of data with regard to the activities of the different enzyme preparations. Nevertheless, it appeared that vanillyl alcohol was conjugated to a greater extent as glucuronide than as sulfate. The discovery that β-glucuronidase, at a specific activity approximately equal to that of Glusulase (neglecting the differences in different preparations, as an excess of the enzymes was used in both cases) could produce an almost equally intense spot as that of Glusulase, was very encouraging. Unless the sulfatase activity in Glusulase is negligible, it was tempting to conclude from these results that vanillyl alcohol was primarily conjugated as glucuronide. A small fraction may be conjugated with sulfate though.

Qualitative results revealed that both Glusulase and β-glucuronidase hydrolysed the vanillin conjugates, as shown by the positive naphthoresorcinol, aniline-diphenylamine, and 2,4-dinitrophenyl-

hydrazine reactions. Mylase P, however, had no effect. This was further confirmed by quantitative analysis, to be discussed presently.

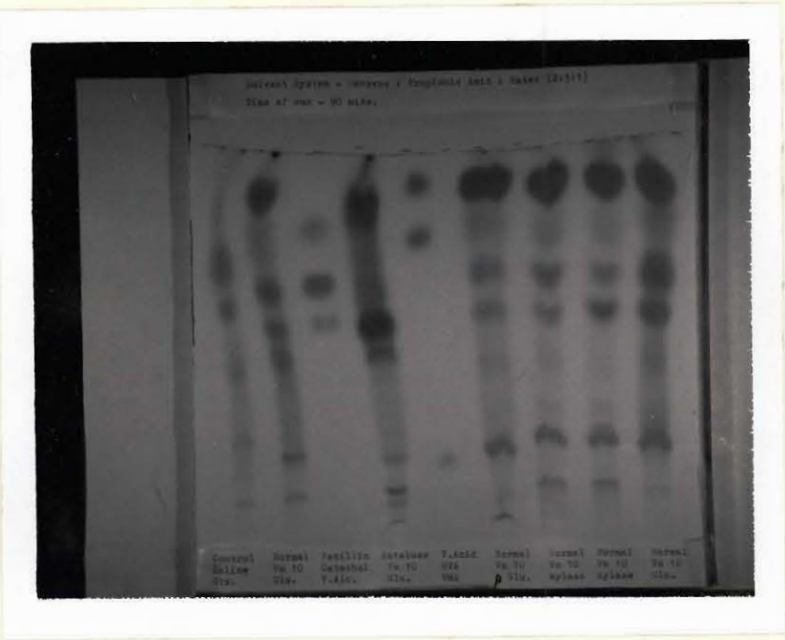


Fig. 5. TLC of the urinary metabolites of vanillin in normal, and antabuse-treated extracts.

2. QUANTITATIVE RESULTS

The four identified spots - vanillin, VA, vanillyl alcohol and catechol were analysed quantitatively. Three series of analyses were made. They are:-

(1) On the unhydrolysed urine. This represents the free fraction (F).

(2) On the hydrolysate of whole urine. This represents the total conjugates (Ct) plus the free fraction (F).

(3) On the hydrolysed aqueous fraction subsequent to preliminary exhaustive extraction at pH 1. This represents the conjugated

Metabolites	Total (Ct+F)	Free (F)	Conj. (Ca)	Conj. (Ce)
Vanillic Acid	41	17	8	16
Vanillin	6.5	-	6	0.5
Vanillyl Alcohol	10	-	10	-
Catechol	4	1	2	1
Total	61.5	18	26	17.5

TABLE X. Quantitative analysis of the urinary metabolites of vanillin. Results were expressed as % of the administered dose of vanillin of 100 mg / Kg.

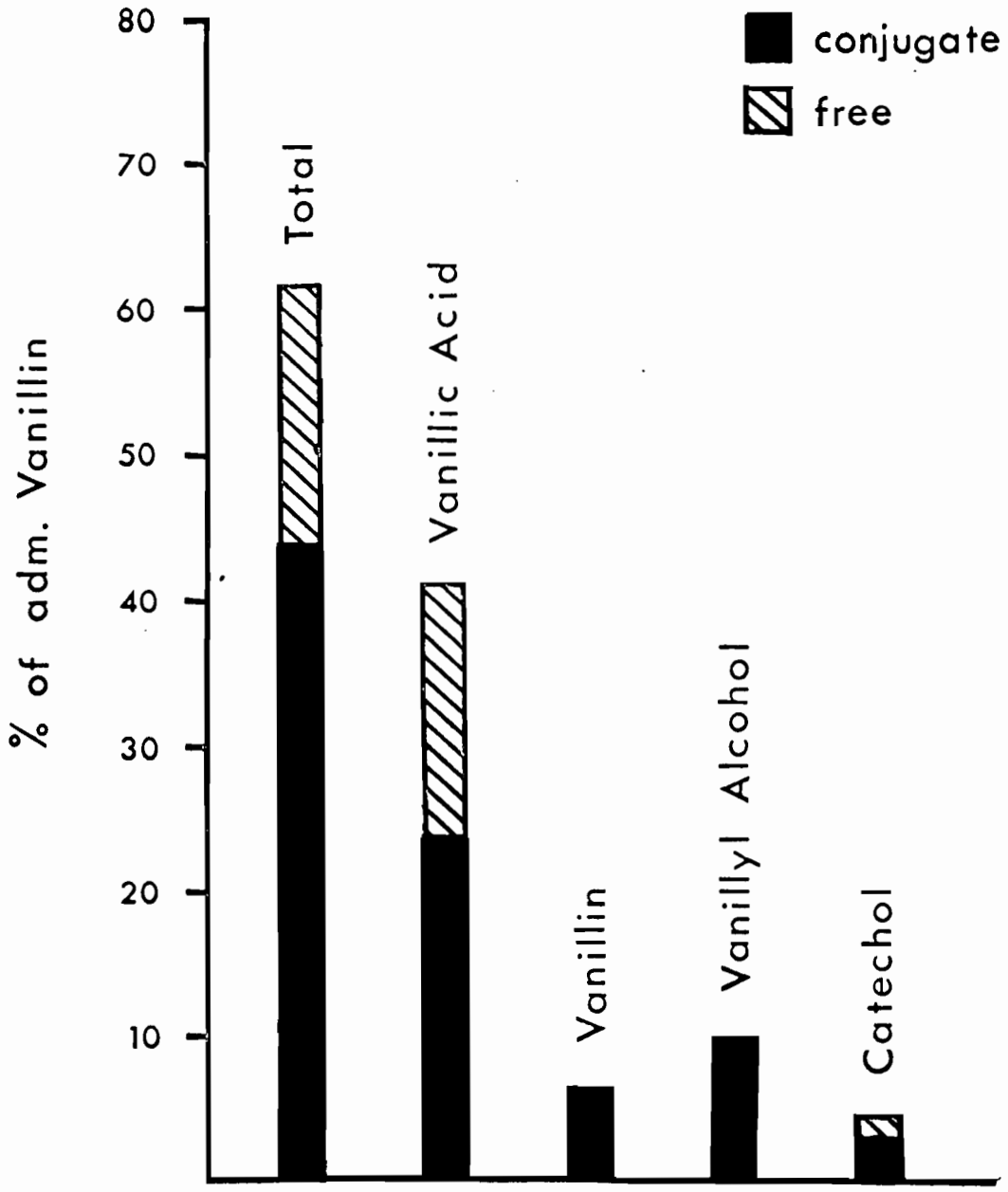


Fig. 6. Diagram illustrating quantitatively the distribution of the urinary metabolites after a dose of 100 mg / Kg of p-vanillin.

fraction (Ca), which was not extracted by ethyl acetate.

The difference between (2) and (3) represents the conjugate (Ce), which was extracted by ethyl acetate at pH 1. Table IX shows the amount of each of these metabolites formed, expressed as a percentage of the administered dose of vanillin. These results were obtained by Glusulase hydrolysis. Thus, both glucuronide and sulfate conjugates were included.

As seen from Table X and Fig. 6 , the major metabolite was VA, the free and conjugated forms of which account for 41% of the administered dose of vanillin (17% free and 24% conjugated). Both vanillin and vanillyl alcohol, present exclusively as conjugates, constitute 6.5% and 10% respectively, of the administered dose of vanillin. The results obtained here confirmed the suggestion that these conjugates were not extracted by ethyl acetate at pH 1, since in both these cases, the concentration of each present in the aqueous phase subsequent to ethyl acetate extraction, was identical to the amount present in total urine hydrolysate. This is interesting particularly in the light of the finding that both vanillin and vanillyl alcohol were conjugated predominantly as glucuronide. The correlation between the extent of extractability and the nature of the conjugates had not been investigated so far.

Catechol, though visually appeared to be a major metabolite, was found on quantitative analysis to constitute only 4% of the administered dose of vanillin, about 1% or less being free.

So far, a total of 62% of the administered dose of vanillin had been accounted for in the urine. Of this, two-third was represented

by VA (free and conjugated), one-sixth by vanillyl alcohol (conjugated only) and one-tenth by vanillin (conjugated only). About 70% of the total urinary metabolites accounted for was in the conjugated form.

(a) Hydrolyses by Glusulase, Mylase P and β-Glucuronidase

(i) Vanillin

As mentioned previously, the conjugate of vanillin appeared to resist the action of Mylase P. On the other hand, both Glusulase and β-glucuronidase hydrolyses yielded free vanillin.

Quantitative analysis confirmed the above results. The amount of free vanillin produced by Glusulase and by the 3 concentrations of β-glucuronidase were the same. This indicates that the lowest concentration of β-glucuronidase used (1000 units/ml. urine) was sufficient for complete hydrolysis of the vanillin conjugate, and that the sulfatase in the Glusulase had negligible action on the vanillin conjugate. Similarly, Mylase P had no activity on the vanillin conjugate.

Thus, it may be concluded that vanillin was excreted not only exclusively as a conjugate, but that the conjugate was specifically that of glucuronide. No conjugation with sulfate was indicated though Sammons and Williams (97) claimed the presence of both ethereal sulfate and glucuronide conjugates of vanillin.

(ii) Vanillyl Alcohol

Like vanillin, vanillyl alcohol was excreted exclusively as a conjugate, the nature of which could be analysed quantitatively with the 3 enzymes mentioned. As shown on Table XI, Glusulase, β-glucuroni-

dase as well as Mylase P liberated free vanillyl alcohol, but the amount differed quantitatively.

TABLE XI

Hydrolyses of Vanillin and Vanillyl Alcohol Conjugates by different Enzyme preparations

Urinary Metabolites	% of administered dose of vanillin				% of excreted urinary metabolites	
	Free	Glusulase	β-Glucuronidase	Mylase P	Glucuronide	Sulfate
Vanillin	Nil	6.5	6.3	Nil	100%	Nil
Vanillyl Alcohol	Nil	10.0	7.3	2.1	75-80	20-25
		10.0	8.0	1.5		

Using Glusulase, 10% of the administered dose of vanillin was liberated as vanillyl alcohol, while with β-glucuronidase, about 7-8%, and with Mylase P, 1.5 to 2% were obtained. This represents approximately 75-80% of vanillyl alcohol conjugation with glucuronide, and 20-25% with sulfate. It must be emphasized here that these results were obtained independently with β-glucuronidase and Mylase P hydrolyses. The fact that they were additive, and that their summated result corresponds closely to that obtained with Glusulase, inferred that the sulfatase in both Glusulase and Mylase P were active, or at least sufficiently so to hydrolyse the small amount of vanillyl alcohol sulfate.

(b) Effect of Antabuse on the Metabolism of Vanillin

Results so far obtained showed that vanillin, an aromatic al-

dehyde, undergoes both oxidation and reduction in vivo, to form VA and vanillyl alcohol respectively. In this case, oxidation is evidently a major pathway. An attempt was made in this study to inhibit the oxidation process, by the use of antabuse or disulfiram which is known to be an inhibitor of aldehyde oxidase or aldehyde dehydrogenase.

Antabuse was prepared as a suspension of 50 mg./ml. in propylene glycol:water (1:1). This forms a rather hetergenous suspension, which readily sediments on standing. To facilitate injection, it was subjected to sonification, which results in a more homogenous solution. It was then injected intraperitoneally daily for 3 days in a dose of 200 mg./kg. Six hours after the last injection, vanillin was injected (100 mg./kg.), also intraperitoneally as before. Control rats were subjected to the same treatment of antabuse. A third group of 3 rats served as "normal." These did not receive any antabuse, but were injected with vanillin in the same dosage simultaneously as the antabuse-treated rats. Both qualitative and quantitative analyses were made, and comparisons made with previously obtained results.

It was found that the antabuse-treated urine extract showed a distinct decrease in the VA spot both in the unhydrolysed and hydrolysed urines, compared to the corresponding (unhydrolysed and hydrolyased) normal "vanillin-injected-extract." There was a corresponding increase in the vanillyl alcohol spot in the antabuse-treated urine extract. This increase is very apparent on TLC plates both in the area and intensity of color of this spot. The vanillin spot, however, appeared to remain the same.

Another observation on the TLC plates, after antabuse treatment, was the disappearance of the catechol spot. This leads to the postulation that catechol may be formed via VA, as a diminuation in VA, as a result of antabuse treatment also brought about a decrease in catechol.

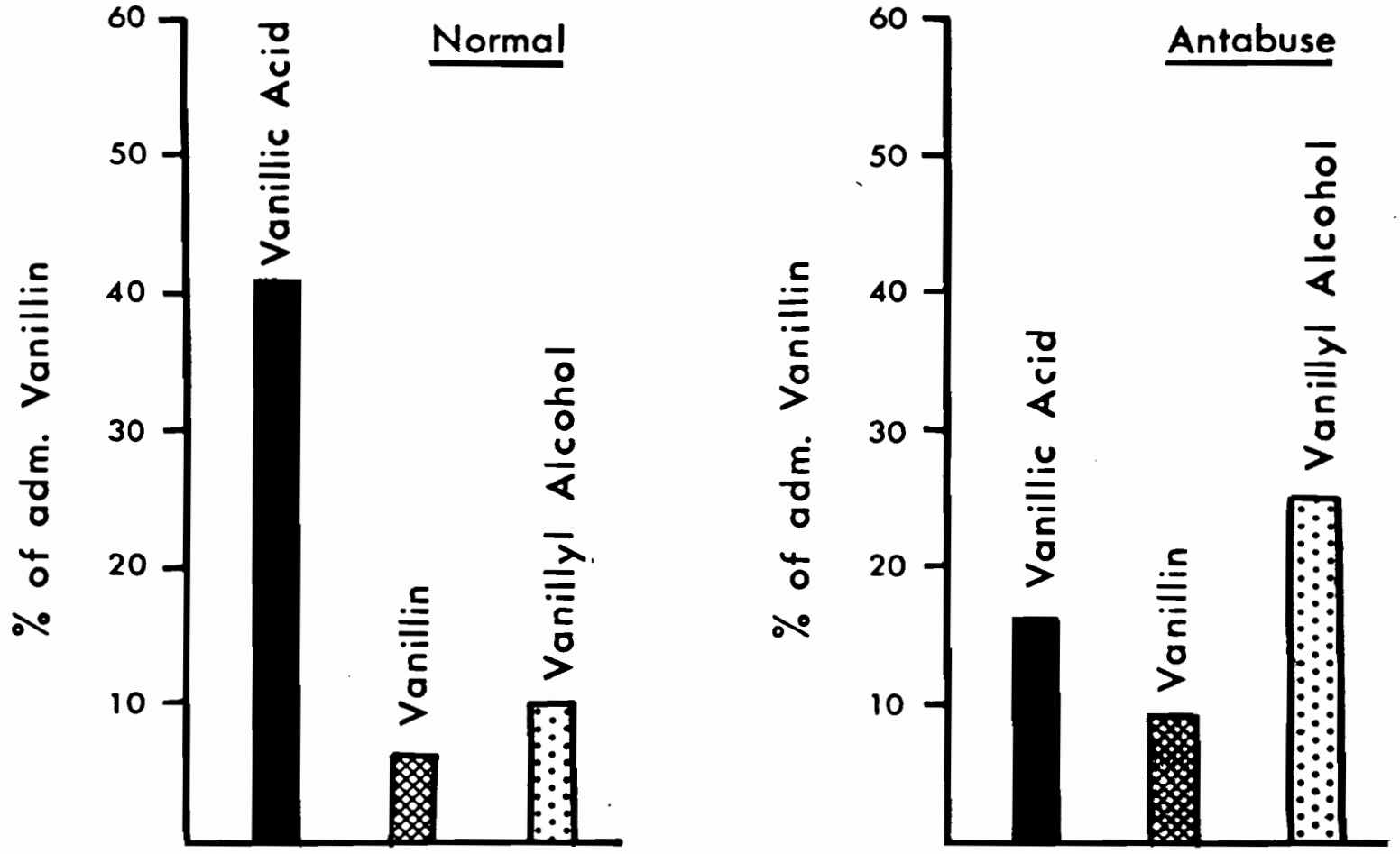
As shown in Fig. 7 , the total VA represents only 16% of the administered dose of vanillin after antabuse treatment, as compared to 41% obtained with normal rats. Of this 16%, half was conjugated and the other half free. This decrease, when expressed as a percentage decrease corresponds to 61%.

On the other hand, vanillyl alcohol has increased to 24% (of the administered dose of vanillin), after antabuse treatment. This 14% increase, when expressed as percentage of increase, again represents 61%. Thus, there seemed to be a direct proportionality between the two sets of results.

Vanillin on the other hand showed only a very slight elevation It now represents 7-10% of the administered vanillin.

The experiment with antabuse was repeated with 50 mg./kg. of vanillin, instead of 100 mg./kg. as previously used. It was hoped that with a reduction in the substrate concentration, but the same or presumably equal inhibition of the enzyme as in the previous experiment, a somewhat different result may be achieved. As shown on Table XII, the same picture of increased vanillyl alcohol and decreased VA was reproduced. However, the percentage of increase and decrease did not correspond exactly, though in both cases, the range is between 45 - 60%.

Fig. 7. Diagram illustrating the distribution of the urinary metabolites of vanillin in normal and antabuse-treated rats.



These results are of great significance, in that they further confirmed the vanillyl alcohol spot, which had hitherto been identified with a "standard" solution of vanillyl alcohol, obtained by sodium borohydride reduction of vanillin. This is convincing evidence that the spot identified as vanillyl alcohol must indeed be so, as no other compound would be increased after antabuse treatment, followed by vanillin injection, knowing that antabuse inhibits exclusively the aldehyde oxidase enzyme.

TABLE XII

Effect of Antabuse

Compounds	Normal	Antabuse Expt. I	Antabuse Expt. II	Percentage Change I	II
Vanillic Acid	30.0	16.5	13.0	45	57
Vanillyl Alcohol	7.3	14.4	17.3	49	58
Total accounted for as VA and Vanillyl Alcohol	37.3	30.9	30.3	-	-

All results were expressed as percentage of vanillin injected.

Dosage of Antabuse: 200 mg./kg. daily for 3 days.

Dosage of Vanillin: 50 mg./kg. 6 hrs. after the last dose of antabuse.

Period of urine collection: 24 hours.

3. EFFECTS OF NEOMYCIN AND STREPTOMYCIN

Various reports have indicated that a number of urinary phenolic acids, particularly m-hydroxyphenol acids, appear to be primarily bacterial metabolites of certain dietary precursors (121, 122). In this study of vanillin, catechol was formed which involved a process of demethylation, rarely observed in mammalian systems. However, metabolic studies suggest that demethylation occurs frequently, particularly in plants and in fungi. Thus, catechol may be microbial in origin. In order to eliminate the action of these intestinal microflora, neomycin and streptomycin were fed to rats prior to the injection of vanillin.

Neomycin sulfate (potency 675 mg. base/g neomycin sulfate) was obtained by courtesy of Dr. G. W. Bartlett of the Dept. of Bacteriology, McGill University. Streptomycin (in the form of streptomycin sulfate, containing the equivalent of 5 g. streptomycin base), was obtained from Unik Medical Labs. Inc., Montreal. These two antibiotics were dissolved in water, and were given to rats by means of a stomach tube. They were administered at a concentration of 10 mg. each/100 g. daily for a period of 3 days. Five hours after the last dose, the animals were injected with vanillin (100 mg./kg.) as before. The whole procedure of extraction, chromatography, and identification was repeated.

The urine obtained after the treatment of these antibiotics was strongly alkaline. Apart from this difference, the antibiotics did not in any way alter the chromatographic pattern of the urinary metabolites so far obtained. As before, VA, vanillin, and vanillyl alcohol were detected in the urine. The catechol spot persists.

Although bacterial counts were not made, it is presumed that most, if not all, the micro-organisms were eliminated from the rat's digestive tract by the comparatively large doses of neomycin and streptomycin. Yet results obtained were essentially the same as those of the untreated rats. Thus, these results probably reflect the actual metabolism of vanillin by the rat tissues.

4. PATHWAY FROM VANILLIN TO CATECHOL

Evidence so far obtained suggest that catechol was probably formed from vanillin by the rat tissue, and not the result of bacterial action of the gut. Two pathways may be postulated for the formation of catechol from vanillin. They are:-

(1) First demethylation to form protocatechuic aldehyde, which may be further oxidised to PA. Decarboxylation of the latter compound forms catechol. In this case, both protocatechuic aldehyde and PA would be the intermediates. This appears to be unlikely as neither of these compounds had been identified in the "vanillin-injected-extract."

(2) Alternatively, vanillin could be first oxidised to VA. This was clearly established by the previous results. This, if followed by decarboxylation, and then demethylation, would yield catechol. It is unlikely that demethylation precedes decarboxylation as such a sequence would again involve PA as an intermediate. On the other hand, decarboxylation followed by demethylation would produce o-methoxyphenol or guaiacol.

One piece of evidence which appeared to support this latter

pathway was the experiment with antabuse. It has been shown that antabuse treatment elicits a decrease in VA as well as catechol.

To establish the pathway from vanillin to catechol, four independent sets of experiments were performed, using protocatechuic aldehyde, PA, VA, and guaiacol. Each of these compounds was injected in a dose of 50 mg./kg. to rats. Acidic and neutral metabolites were analysed by the same procedure, both on the hydrolysed and unhydrolysed urine. No quantitative analysis was made.

(a) Protocatechuic Aldehyde

The metabolic pattern of protocatechuic aldehyde was found to be very similar to that of vanillin. Like vanillin, this aldehyde was excreted only as a conjugate. No free protocatechuic aldehyde was detected in the urine. Its oxidation product, PA, VA, and catechol were all found to be excreted after its injection. Of particular interest was the finding of the presence of vanillin as a urinary metabolite of protocatechuic aldehyde. Here again, only conjugated but no free vanillin was excreted. Apparently, methylation of the 3-hydroxy group of protocatechuic aldehyde had occured, producing vanillin, oxidation of which forms VA. The formation of VA may alternatively occur by methylation of PA, rather than via vanillin. This O-methylation has been demonstrated both *in vivo* (45) and *in vitro* (104), as PA is an excellent substrate for the COMT.

It must be mentioned here that methylation of protocatechuic aldehyde occurs, but the reverse process i.e. demethylation of vanillin to form protocatechuic aldehyde, was not observed. Besides

the catechol formed could arise directly from PA, by decarboxylation or via VA by decarboxylation plus demethylation, more probably by the former process.

(b) Protocatechuic Acid

The urinary metabolites of PA include free and possibly conjugated PA, VA, and catechol. Here again, methylation of the 3-hydroxy group was manifested. The amount of catechol formed appeared to be more than that formed by the injection of an equivalent concentration of VA. Apparently, it must be formed primarily by the direct route of decarboxylation of PA rather than from VA, though the latter would presumably form catechol, but to a smaller extent.

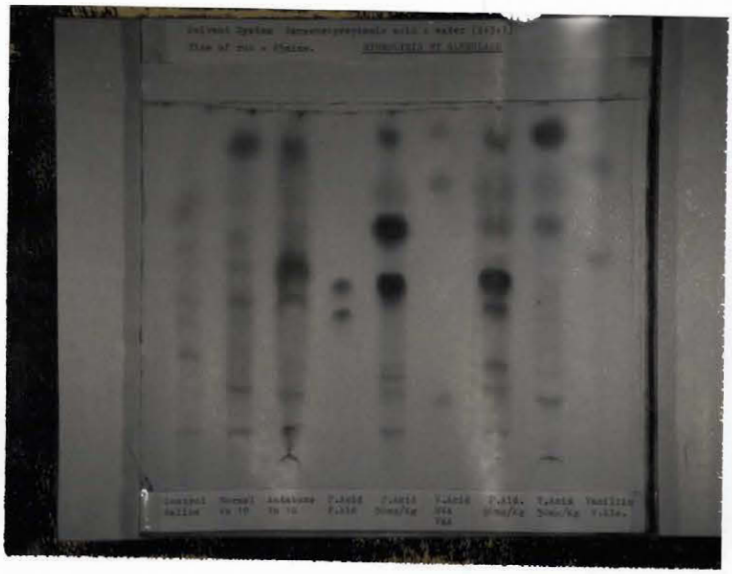


Fig. 8. TLC of the urinary metabolites of Protocatechuic aldehyde, Protocatechuic acid and Vanillic acid.

(c) Vanillic Acid

As shown on Fig.8 , VA appeared to be metabolically limited, as its injection gave rise to free and perhaps conjugated VA. Here again, catechol was identified, but no PA, the demethylated product of VA. Though both PA and VA formed catechol, there was no doubt that the former, which involves a single step of decarboxylation, occurs much more readily than the latter, which must first be decarboxylated and then demethylated. It has been suggested here that demethylation must occur subsequent to decarboxylation, since the reversal of these two processes should produce PA, but this was not found to be so. It appears that one of these two processes is limiting, as the amount of catechol formed from VA was much less than that formed by an equal amount of PA. Presumably, it is the demethylation step that was slow, as demethylation of both VA and vanillin were not manifested at all.

(d) Guaiacol

The injection of guaiacol also resulted in the excretion of catechol. No other urinary metabolite was identified.

5. COMPARATIVE STUDY OF URINARY METABOLITES OF ADRENALONE, NORADRENALONE AND ISOPRENALONE

Smith (37) has reported that oral administration of adrenalone to a human subject resulted in the excretion of grossly abnormal quantities of VA, isovanillin and VMA. In the guinea pig, he found that injection of either adrenaline or adrenalone yielded the same metabolite, believed to be protocatechuic aldehyde. This seems to suggest the existence of an alternative pathway for adrenaline and

noradrenaline through their β-keto derivatives. It is envisaged that this oxidation of the β-carbon is reversible so that these oxidation products, adrenalone and noradrenalone may be reduced to adrenaline and noradrenaline and metabolised as such. However, the possibility of the direct metabolism of these β-keto analogues cannot be ruled out.

Consequently, in this study, a comparative study of the metabolism of adrenalone, noradrenalone and isoprenalone was undertaken to evaluate the physiological significance of these 3 compounds as precursors of the vanillyl products claimed above, particularly of VMA and vanillic acid.

In our experiments, groups of 3 rats were given intraperitoneal injections of 40 mg./kg. of: (a) Adrenalone, (b) noradrenalone, and (c) isoprenalone. The acidic and neutral urinary metabolites were compared by TLC.

Results showed an apparent increase in the intensity of one spot over the control, the increase being greater in urine from noradrenalone injection than from adrenalone. With isoprenalone, there is hardly, if any increase at all. This spot had an Rf similar to that of VMA in at least 3 solvent systems as shown on Table XIII. Also, VMA added to the urine before extraction showed up in the chromatogram with the same Rf as this spot.

TABLE XIII

Rf's of VMA and Spot formed from Noradrenalone

Solvent Systems		Rf of auth.VMA	Rf of Spot
Benzene-propionic acid-water	(2:3:1)	0.25	0.26
Benzene-acetic acid-water	(2:3:1)	0.08	0.1
Chloroform-acetic acid-water	(2:1:1)	0.16	0.16
	(2:2:1)	0.24	0.25
	(8:8:3)	0.35	0.35

Elution and re-chromatography

Various attempts were made to identify this.spot (subsequently referred to as "Rf *0.25 spot"), by eluting, extracting and re-chromatographing it in different solvent systems. The authentic standard VMA spot was subjected to the same treatment. It was found that subsequent plates persistently gave more than one spot, not only from this "Rf 0.25 spot" but from the standard VMA spot, irrespective of the second solvent system used. This could probably be explained as a result of surface catalysis of atmospheric oxidation. Of the two major spots formed, one had an Rf of 0.80 in benzene:propionic acid:water (2:3:1), similar to the standard vanillin spot. This is consistent with the result of Rosenblatt et al. (79) who showed that successive chromatography, elution and re-chromatography of VMA in isopropanol-ammonia, and Butanol-acetic acid-water repeated gave in addition to VMA, small but significant amounts of vanillin, the yield

of which increased with exposure time.

The other spot had an Rf of 0.56 in this same solvent system. This is close to that of vanillyl alcohol (Rf = 0.56 - 0.6). As the conversion of VMA to vanillin involves decarboxylation and oxidation, it seems very probable that vanillyl alcohol could result by simple decarboxylation alone, the oxidation of which would produce vanillin. However, further confirmations are required before a tentative conclusion could be drawn.

As the artefactual multiple spot formation of VMA encountered so far may be eliminated by preventing aerial oxidation and/or decarboxylation of the spots while they were on TLC plates, it was thought that coating the gel surface should serve this purpose. Consequently, Quelspray (a commercial vinyl spray for surface protection) was tried and found to be relatively effective. However, the resulting formation of a thick hard coating renders difficult and thereby inefficient elution. Recovery was small. Nevertheless, when this treatment was applied, re-chromatography of the Rf 0.25 spot, standard VMA spot, as well as authentic VMA freshly applied on this second plate, all moved with the same Rf of 0.25 in benzene-propionic acid-water (2:3:1). The first solvent used in this experiment was choloform-acetic acid-water (2:2:1).

A preliminary quantitative determination of VMA formed from noradrenalone indicated that only about 4% of the administered noradrenalone could be accounted for as VMA. Because of this negligible conversion, no further work was pursued in this study.

6. STUDY OF VANILMANDELIC ACID

VMA has been established as an end-product of both adrenaline and noradrenaline. Our results showed that adrenalone and noradrenalone also serve as its precursors. However, there have been indications that VMA may be further metabolised to vanillic acid, but no quantitative significance has been established.

In our experiments, VMA was injected intraperitoneally in a dose of 100 mg./kg. to rats. As before, urinary metabolites were studied by TLC.

Injected VMA appeared to be metabolically limited as shown by the large amount of free VMA found in the urine after its injection. There was an increase in one spot with Rf 0.55 in Benzene-propionic acid-water (2:3:1). This is similar to the Rf of vanillyl alcohol. It may have arisen by simple decarboxylation of VMA. As free VMA was present in considerable amounts in the urine, its spontaneous decarboxylation would give rise to this result.

To determine whether this was an artefact or actual conversion of VMA to vanillyl alcohol, a control was set up. To 5 ml. of control urine was added an amount of VMA that would be present in this volume if all the injected VMA was excreted free. Results indicated that added VMA indeed gave rise to essentially the same kind of chromatographic pattern as that obtained with injection of VMA, showing that the new spot was undoubtedly an artefact. When the chromatogram was developed in Benzene-acetic acid-water (2:3:1), the new spot had an Rf of 0.38, different from that of authentic vanillyl alcohol (Rf = 0.32).

Glusulase hydrolysis did not show an increase of any spot over the unhydrolysed urine or the control. To test the possible participation of metal ions in the production of this artefact, Versene and Versenol (Versenes Inc.), both of which are potent chelating agents were added to the urine. Similar results were again obtained, suggesting that metallic ions were unlikely to be involved.

There appeared to be a very slight increase in VA after VMA injection. Zone hydrolysis of the band which had previously been located to contain VA conjugate also gave rise to VA. This was again insignificant. Thus, no quantitative determination of VA formed from VMA, was made.

Dirscherl and Brisse (106) have reported the formation of VA by incubating rat or human liver homogenate with DL-VMA. In addition, they also found VMA and a diazo positive compound. It is possible that the latter compound is analogous to the artefactual spot which was formed from exogenous VMA in our experiment. These workers considered VA as the final metabolites of both adrenaline and noradrenaline in human liver. The present study seemed to confirm their results that VMA was converted to VA. No great physiological significance could however be attached to such a minor conversion.

IV. DISCUSSION

Many aromatic aldehydes serve as intermediates in metabolism. As a rule, they are metabolised *in vivo* mainly oxidatively to form the corresponding acids, but variable proportions may be reduced to alcohols. This investigation began with the application of TLC to the problem of vanillin metabolism.

1. OXIDATION AND REDUCTION

In the present experiments with vanillin, both oxidation and reduction had occurred. Such a situation could arise via the aldehyde mutase system, which would account for the formation of both vanillic acid and vanillyl alcohol. This is analogous to the Cannizzaro reaction of aldehydes, brought about by the action of a solution of conc. KOH. In such a case, the aldehyde mutase system should theoretically produce one mole each of acid and alcohol from 2 moles of aldehyde.

This aldehyde mutase, a soluble enzyme from liver, was for a long time considered to be identical with aldehyde oxidase. In 1937, Dixon and Lutwak (141) obtained mutase preparations free from oxidase activity, and showed conclusively that the two enzymes were quite distinct. The mutase reaction was considered to couple the activity of two NAD-linked dehydrogenases. They are the alcohol dehydrogenase and aldehyde dehydrogenase. The reactions catalysed would be as follows:-

(i)	$RCHO + H_2O + NAD$	———	$RCOOH + NADH_2$
(ii)	$RCHO + NADH_2$	———	$RCH_2OH + NAD$
Sum	$2\ RCHO + H_2O$	———	$RCOOH + RCH_2OH$

AS can be seen from these two reactions, only a catalytic amount of the co-enzyme is required. Since the separate activities of these two enzymes could not be detected in their mutase preparations by reduction of methylene blue on addition of acetaldehyde or ethanol, Dixon and Lutwak concluded that the aldehyde mutase was a single enzyme. However, with the crystallization of the liver alcohol dehydrogenase by Bonnichsen and Wassen in 1948 (142), and the partial purification of a distinct NAD-linked aldehyde dehydrogenase from liver by Racker in 1949 (143), it seemed clear that the activity of the mutase of Dixon and Lutwak was entirely due to the activity of these two enzymes.

Recently, Dalziel and Dickinson (144) effected the complete separation of alcohol dehydrogenase and aldehyde dehydrogenase. Their results imply that the alcohol dehydrogenase also possesses aldehyde dehydrogenase activity. They showed that with small concentrations of NAD, 1 mole each of acid and alcohol are formed from 2 moles of aldehyde. Evidently, the co-enzyme undergoes successive oxidation and reduction. The reduced co-enzyme formed in the aldehyde dehydrogenase reaction being used directly in the succeeding reverse alcohol dehydro-

genase step. The dismutation will in fact proceed with only catalytic quantities of co-enzyme.

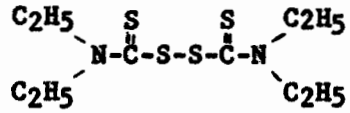
In our study of the metabolism of vanillin, no kinetic study of the enzymes involved in its metabolism was attempted. However, the fact that the oxidation process was predominant as suggested by the greater amount of urinary VA produced, seemed to suggest that the aldehyde oxidase has a greater affinity for vanillin than the alcohol dehydrogenase. Besides, the results of antabuse treatment which effectively channels the aldehyde into the reductive process, imply that two separate enzymes participate in this oxidation-reduction reaction. From the dismutase reaction, an almost equal amount of the acid and alcohol would be expected. Our experiments showed that the urinary VA was about four times that of vanillyl alcohol. However, measurements of urinary products do not necessarily reflect metabolism <u>in situ</u>. Though both VA and vanillyl alcohol are derived from vanillin, each one of these may undergo further degradations, so that the amount of each eventually excreted may differ considerably even if equal quantities were formed from vanillin. Nevertheless, the overwhelming predominance of VA suggests a major oxidative and a minor reductive pathway for vanillin.

According to Williams (98), the aldehyde mutase has no action on aromatic aldehydes, but causes dismutation of the aliphatic aldehydes. On the other hand, the aldehyde oxidase acts on both aromatic and aliphatic aldehydes, converting them to the corresponding acids. It seems

that aromatic aldehydes, without exception, undergo oxidation.

2. ACTION OF ANTABUSE

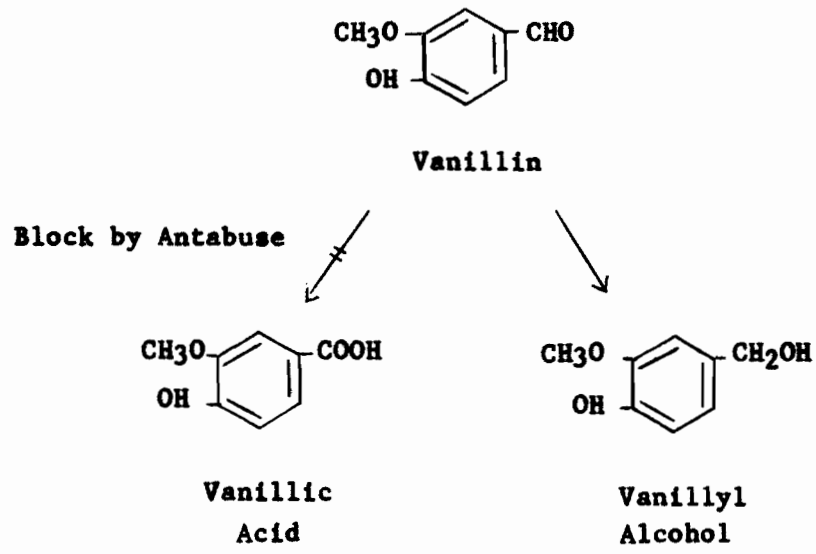
Antabuse or disulfiram, Bis (diethylthiocarbamoyl) disulfide, is an inhibitor of liver aldehyde oxidase. Its structure is:-



Asmussen and his co-workers (145) were the first to study the effect of antabuse and alcohol on the respiration and circulation in normal subjects. They concluded that the increase in ventilation, pulse rate, and asthma after antabuse treatment were due to the accumulation of acetaldehyde from ethanol. Next Hald and Larsen claimed that the metabolism of acetaldehyde was impaired in antabuse-treated animals (146), It was Kjeldgaard (147) who concluded that antabuse in concentration as low as 0.1 ug per ml. was sufficient to inhibit the oxidation of aldehyde to acid by liver aldehyde oxidase. There was an actual inhibition of the oxidative process. Inhibition appeared to be competitive, and is marked even with concentration as low as 1.3×10^{-7}M per ml.

Since then, Smith and Wortis have found its application effective in inhibiting the aldehyde oxidases which act <u>in vivo</u> on 3-methoxy-4-hydroxymandelic aldehyde (86), and on indole-3-acetaldehyde (87).

Our experiments with antabuse confirmed its action as an aldehyde oxidase inhibitor *in vivo*. The site of inhibition is shown below:-



SITE OF ACTION OF ANTABUSE

With the block in the oxidative process by antabuse, the excess of or accumulated vanillin was directed towards the reductive pathway, which is normally minor. Though Kjeldgaard suggested that inhibition may be competitive, our *in vivo* experiments did not bring any evidence to bear upon this suggestion.

In our experiments, an excess of inhibitor was used to ensure its effect. It was given over a period of 3 days. Six hours after the last dose, vanillin was given. Pharmacological studies have shown that in humans, the full effect occurs in 6 - 7 hr. (148). The absorption and elimination of this drug are very slow. We had ensured its action by administering 3 doses, and had performed the experiment at a time when

its action is presumably maximal.

3. CONJUGATION

Sherwin in 1922 put forward the generalisation that when a foreign compound finds its way into the body, the organism attempts to deal with it by direct oxidation (149). Alternatively, the organism may produce soluble and relatively non-toxic substances which are easily excreted. This is usually achieved by conjugation with glucuronic acid, sulfuric acid, or glycine.

When vanillin was injected, direct oxidation to form VA was found to occur. Conjugation, apparently secondary to oxidation (since free VA was found), must constitute an important process. Of the 62% of the administered dose of vanillin accounted for, more than two-thirds was conjugated with either glucuronic or sulfuric acids. The significance of conjugation therefore cannot be underestimated. The conjugation process has been studied fairly extensively in this research, as all the metabolites identified were excreted partially or completely conjugated.

Vanillin, on injection into rats, was not completely metabolised. A small fraction (approximately 6.5 % of the administered dose) was excreted in the urine as a conjugate of glucuronic acid. This conjugate was not extracted at pH 1 with ethyl acetate. Likewise, the conjugate of vanillyl alcohol is insoluble in ethyl acetate. Here again, conjugation with glucuronic acid predominated. A small fraction was however

conjugated with sulfate. Protocatechuic aldehyde, like vanillin, was excreted exclusively as a conjugate, the nature of which was not determined. PA and VA were found in the urine both in the free and conjugated forms, while catechol undoubtedly exists largely as a conjugate in the urine.

Harborne (150) reported that when catechol was given in a dose of 100 mg / Kg, about 70 % was excreted as glucuronide, and 18% as ethereal sulfate. It seems that in both these conjugates, only one OH group is conjugated. Possibly the utilization of one OH in conjugation renders the conjugated product sufficiently soluble to be excreted so that double conjugation was not essential. Alternatively, the presence of a glucuronide or sulfate moiety hinders the attachment of a second such group.

The nature of the conjugates of both vanillin and vanillyl alcohol had been elucidated from experiments with Glusulase, β-glucuronidase, and Mylase P. Both these conjugates were present in the urine in less than 10% of the administered dose of vanillin (or less than 0.3 mg per 5 ml. of urine). It is presumed that the activity of each of these enzymes was sufficient for complete splitting of the conjugated bonds in these two cases. This does not hold true for VA conjugate as no definite or complete hydrolysis could be ensured with either β-glucuronidase or Mylase P. An increasing concentration of either of these enzymes did not produce any consistent proportionate increase in hydrolysis as expected. This was particularly so with Mylase P, which contains many

other constituents which may have an inhibitory effect. In spite of this, convincing evidence has been obtained which showed that vanillin was conjugated exclusively and vanillyl alcohol predominantly with glucuronic acid.

With the conjugate of vanillin, it was demonstrated that the extent of hydrolysis by 1000, 2000 and 3000 units of β-glucuronidase / ml. urine and by Glusulase whose β-glucuronidase activity was 1000 units / ml. urine were the same. Obviously, the lowest activity of the β-glucuronidase mentioned above was sufficient for the complete hydrolysis of glucurono-vanillin. The negative result with Mylase P can be accounted for readily by assuming that the substrate i.e. vanillin ethereal sulfate, is not formed in metabolism. There was presumably no lack of enzyme activity, since the enzyme preparation manifests some activity towards the vanillyl alcohol conjugate.

It was found that 75 - 80% of the conjugation of vanillyl alcohol was with glucuronic acid, the remaining fraction being ethereal sulfate conjugate. Here again, though no absolutely controlled conditions were employed with regard to the enzyme concentration, results obtained with β-glucuronidase at 1000 units / ml. urine, and with Mylase P independently added up to those obtained with Glusulase. In two independent estimations, 20 and 25% of the vanillyl alcohol conjugated was accounted for as sulfate, and the corresponding values from β-glucuronidase hydrolysis were 80 and 75% respectively.

There have been reports which indicated that the rate of enzymic hydrolysis of different arylsulfates varies considerably, and indeed some substrates do not appear to be hydrolysed at all. Dodgson and Spencer (151, 152, 153) in their studies on sulfatases claimed that the sulfate and phosphate ions in urine have an inhibitory effect, sufficient to reduce the arylsulfatase activity of limpet to an inefficient level. Both these ions may be removed from urine by precipitation with barium chloride at pH 11.5.

Generally, the extent of the glucuronic acid conjugation of phenols considerably exceeds that of the sulfate conjugation. As far as foreign compounds are concerned, the sulfate conjugation is commonly encountered during the metabolism of phenols. The main site of conjugation appears to be the liver.

According to Williams (98), when the dose of phenol is relatively large (125 - 250 mg / Kg), the sulfate conjugate constitutes about 15 - 16%, and glucuronide 70%. With lower dose of phenol, the sulfate conjugate increases, while the glucuronide conjugate decreases. Results in this study more or less confirm this generalisation of preponderance of glucuronide conjugation. With a dose of vanillin of 100 mg / Kg, all the vanillin excreted, and 75 - 80% of the vanillyl alcohol formed were conjugated with glucuronic acid. Unfortunately, no consistent results have been obtained for VA conjugate.

4. EFFECT OF ANTIBIOTICS

As mentioned before, many urinary constituents, particularly the m-hydroxyphenyl acids, appear to originate from the actions of intestinal bacteria. It was therefore necessary to investigate whether the metabolites of vanillin so far identified are actually the result of metabolism by rat tissues, or are formed by the action of intestinal bacteria. Two antibiotics, neomycin and streptomycin were administered to rats. Urine of these rats and that of the untreated rats were compared chromatographically. The antibiotics were given by means of a stomach tube. This was preferred to oral feeding with the daily ration, as it ensures complete repression of the growth of the intestinal micro-organisms.

These antibiotic-treated rats produced urinary products similar to those of normal rats. Essentially the same chromatographic patterns were obtained. This evidence favours strongly the role of the rat tissues, and not the gut flora, in the metabolism of vanillin. The persistence of the catechol spot suggests that demethylation had indeed occurred, though the extent of this may be small.

5. DEMETHYLATION

The demonstration of catechol in urine after vanillin injection has led to the investigation of the demethylation process. Though demethylation occurs frequently in metabolic studies, it is confined more or less to fungi and plants. Few instances of demethylation in

mammalian systems have been reported. In the rabbit, Williams (98) claimed that demethylation of VA takes place to a slight extent, and a small amount of VA is excreted probably as PA. This is contrary to the results obtained in this study, where the injection of 50 mg / Kg of VA did not give rise to any trace of PA. The same author also reported that some 2% of administered veratric acid (3,4-dimethoxy-benzoic acid) undergoes double demethylation to a catechol derivative, presumably PA, which is excreted as an ethereal sulfate (128). Another instance of demethylation was the formation of 4-hydroxy-diphenyl ether from 4-methoxy-diphenyl ether (98). In all these instances, the extent of demethylation was very small indeed.

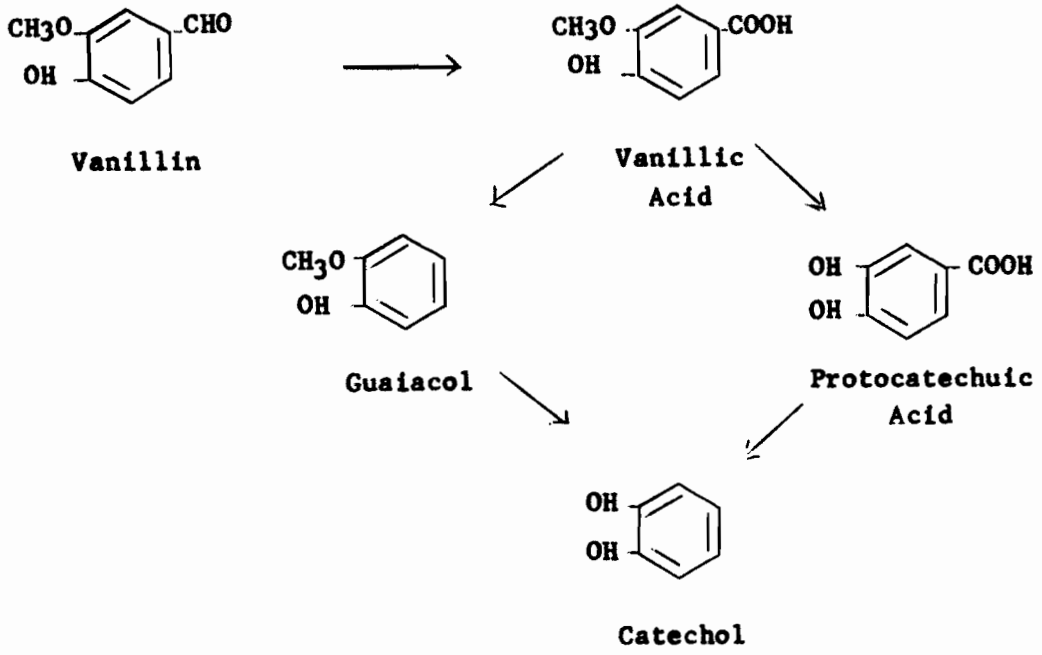
Similarly, in this study, the amount of catechol formed from vanillin, which inevitably involves demethylation, was only about 4% of the administered vanillin. Three metabolic processes are implicated in this conversion. They are oxidation, demethylation, and decarboxy-lation, the sequence of which has been established in this work. The two possible alternatives are as follows:-

(1) Oxidation to form VA. This has been clearly established. This when followed by

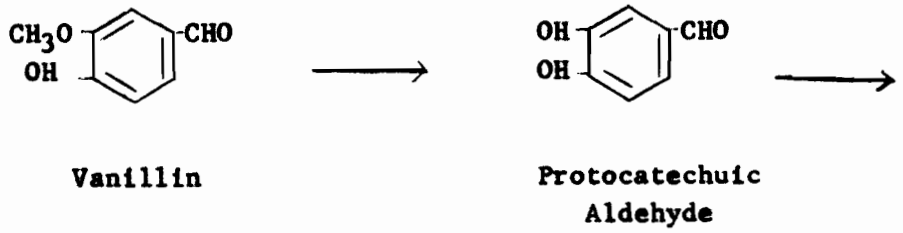
a. decarboxylation produces o-methoxy phenol or guaiacol.

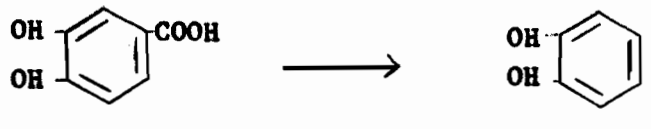
b. demethylation produces PA.

Demethylation of guaiacol, and decarboxylation of PA form catechol.



(2) The second possible pathway involves demethylation as the primary step, followed by oxidative decarboxylation. Here both protocatechuic aldehyde and PA would undoubtedly serve as intermediates.



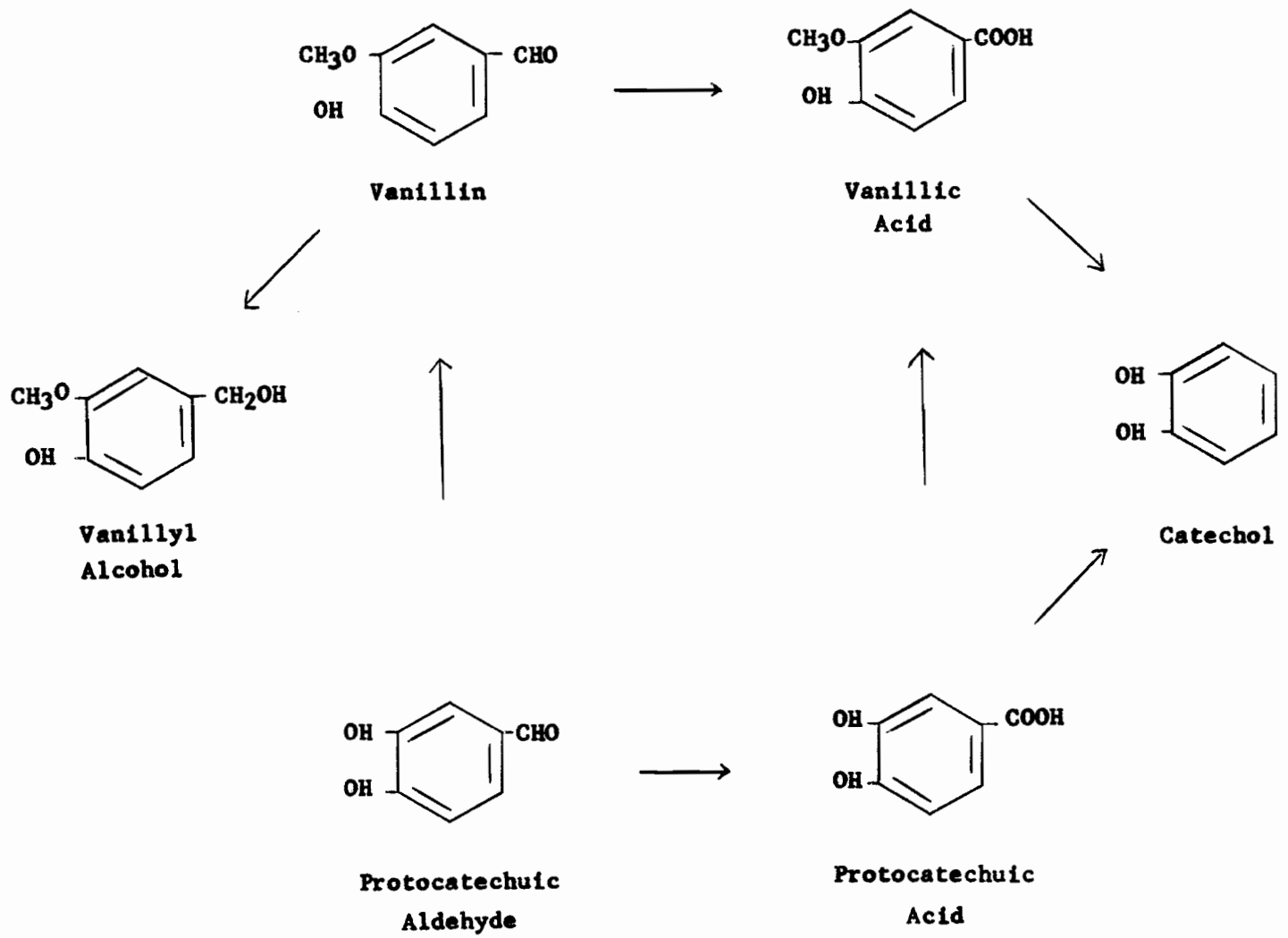


The experiments with vanillin and VA preclude pathways 1b and 2, since neither protocatechuic aldehyde nor PA was formed from their methylated derivatives. It would appear that the demethylation process is not possible when a substituent exists on the meta position. On the other hand, the reverse process i.e. methylation, occurs rapidly both *in vivo* and *in vitro*. Both protocatechuic aldehyde and PA were methylated to vanillin and VA respectively. Booth and his co-workers have studied the methylation of PA, caffeic acid and dopa, and have suggested that methylation of the 3-hydroxy group occurs only when the side chain has 1, 2 or 3 carbon atoms. On the contrary, this study suggests that demethylation occurs when there is no side chain meta to it, This could be related to the stereospecificity of the enzyme involved. Consequently, decarboxylation precedes, and thereby enhances demethylation.

On the basis of these experiments, the pathway from vanillin to catechol presumably occurs via VA, and *o*-methoxy phenol. This latter compound undergoes demethylation to yield catechol.

A scheme of the postulated pathway of vanillin metabolism is shown in Fig 9 . Vanillin is primarily oxidised to VA. It can also be reduced to vanillyl alcohol. The formation of catechol from protocatechuic aldehyde and PA probably involves the oxidative decarboxylation of the former, and direct decarboxylation of the latter, rather than via their methylated analogues, although these latter compounds have been shown to form catechol also.

Fig. 9. POSTULATED PATHWAY OF VANILLIN METABOLISM



Catechol, like other polyphenols, is an excellent methyl acceptor. In such a case, we would expect o-methoxy phenol to be formed from it if O-methylation occurs on one of its hydroxy groups. Yet catechol was excreted in the urine. Perhaps the site of its formation differs from the site of O-methylation. Nevertheless, it does not seem to be the result of microbial action.

6. OTHER LIKELY METABOLITES

(a) Veratraldehyde and Veratric Acid

These are the 3,4-dimethoxy analogues of vanillin and VA respectively. They represent other likely metabolites by a process of p-O-methylation. Veratric acid could be detected by its pale blue fluorescence under U.V. light, while veratraldehyde, like vanillin, could be distinguished by the carbonyl spray reagents. Neither veratric acid nor veratraldehyde had been found in rat urine after a dose of vanillin was given. Apparently p-O-methylation does not occur when there exists a methoxy group at the meta position. Masri, Booth and DeEDS (154) showed that with 3,4-dihydroxy phenolic acids, methylation in animals occurs at either the para or meta position. Thus only one of these positions can be methylated. The O-methyl transferase enzyme seems to have a discriminating action by the pattern of hydroxylation on the aromatic ring.

(b) Vanillylamine

The presence of this compound in urine has been reported (73, 74). However ethyl acetate at pH 8 - 10 failed to reveal its presence in the

"vanillin-injected-extract". The problem could probably be solved by Dowex column chromatography.

7. THE METABOLIC CONVERSION OF ADRENALONE AND NORADRENALONE TO VMA

A review of the literature on adrenalone and noradrenalone had been given. The metabolism of adrenaline and noradrenaline is well known, and VMA has been clearly established as the major metabolite of these two catecholamines. Experiments with injected adrenalone and noradrenalone showed that VMA was also derived from these β-keto analogues of adrenaline and noradrenaline. In the present instance, enzymatic reduction of the two β-keto analogues would bring them into these paths, and the formation of VMA would be readily understandable. In this respect, the stereoselectivity of the ketone reduction should be investigated, as only L-adrenaline is formed biosynthetically in the animal organism.

On the other hand, metabolism of adrenalone and noradrenalone could take place through a series of analogous steps – oxidative deamination, dehydrogenation of the resulting aldehyde, O-methylation, and β-reduction to the secondary alcoholic group. If a CA dehydrogenase does exist, such a series of reactions would constitute an alternative pathway of metabolism of these compounds. It is interesting that both these β-keto analogues are substrates of both COMT and MAO. This undoubtedly explains the formation of VMA from these compounds, as demonstrated by Smith (37), who found large amounts of VMA excreted in a human subject after oral administration of adrenalone. Our experiments confirmed his

results, except quantitatively, as we found only about 4% of the administered noradrenalone, and less in the case of adrenalone, excreted as VMA. Because of the relative insignificance of this pathway, no further investigation was carried out.

8. CONVERSION OF VMA TO VANILLIC ACID

With the discovery of VMA as a major end metabolite of adrenaline and noradrenaline by Armstrong, and the corollary establishment of O-methylation as the principal pathway of the CAs, the complete pathway of CA metabolism seemed clear. A large fraction, if not all the VMA was found to be excreted free in the urine. However, various reports indicated that VMA may be further metabolised to VA. It must be emphasized here that numerous artefacts have been reported to arise from VMA. Rosenblatt et al (79) reported the artefactual conversion of VMA to vanillin, a finding which we have confirmed in this study. Dirscherl and Brisse (106) found in addition to VMA, VA and a diazo-positive compound when DL- VMA was incubated with rat or human homogenate. We found that injected VMA appeared predominantly as free VMA in urine. Apparently, it is metabolically limited. In addition, there was a slight conversion to VA, which was regarded as of no biological importance. The artefactual spot, which had not been identified, probably corresponds to the diazo-positive compound reported by Dirscherl and Brisse. Gjessing (93) also reported the artefactual formation of many p-nitraniline positive spots, including HVA, VMA, p-hydroxyphenyl acetic acid, and p-hydroxymandelic acid from

VPA in alkaline solution. It is unlikely that VA formation from VMA is an artefact. However, its physiological importance as the end product of the CA metabolism, as compared to VMA is negligible. In fact its derivation from vanillin, a common dietary constituent appears to be more significant.

V. SUMMARY

Intraperitoneal injection of p-vanillin (3-methoxy-4-hydroxy-benzaldehyde) in a dose of 100 mg / Kg to rats, gave rise to a number of urinary metabolites, readily distinguished by thin-layer chromatography. Extraction of urine at pH 1 with ethyl acetate, followed by TLC was the principal method employed. The solvent system found to be most satisfactory was Benzene-propionic acid-water (2:3:1). Chromatographic characteristics in various solvent systems, U.V. fluorescence together with comparison of color reactions with authentic, or chemically prepared compounds form the basis of identification of most of these metabolites.

The major product obtained from vanillin was vanillic acid; it occurred both free as well as conjugated in the urine. Together they constitute about 41 % of the administered dose of vanillin (17 % free and 24 % conjugated). The nature of this conjugate has not been ascertained. Undoubtedly, a glucuronide or sulfate, or possibly both exist as indicated by hydrolysis with Glusulase.

The second major metabolite was vanillyl alcohol, which was excreted exclusively as a conjugate. This conjugate appeared to be insoluble in ethyl acetate at pH 1. Results of hydrolysis with β-glucuronidase and Mylase P indicated a predominance of glucuronide (75-80 %); the remaining fraction (20 - 25 %) being conjugated with sulfate. This metabolite represents 10 % of the injected dose of

vanillin. An attempt was also made in this study to obtain vanillyl alcohol by sodium borohydride reduction of vanillin. The vanillyl alcohol so formed was later shown to be chromatographically identical with authentic vanillyl alcohol.

A small amount of vanillin itself was excreted, also exclusively as a conjugate, after its intraperitoneal injection. Like the vanillyl alcohol conjugate, it was not extracted by ethyl acetate. It constitutes 6 - 7 % of the administered vanillin. Evidence was presented here that it exists entirely as a glucuronide conjugate.

The final metabolite identified was catechol, which formed about 4 % of the administered dose of vanillin. A trace amount of this was excreted free.

The effect of antabuse on the metabolism of vanillin was also investigated. Antabuse, or disulfiram, is an inhibitor of aldehyde oxidase or dehydrogenase. When it is injected prior to vanillin, it channels this aldehyde into the reductive pathway. Consequently VA excretion was reduced by about 60 %, with a corresponding and proportionate elevation of vanillyl alcohol. The vanillin excreted remained constant or slightly elevated, while no catechol was detected in the urine after this treatment.

To eliminate the intestinal bacterial action, neomycin and

streptomycin were administered to the rats daily for 3 days in a dose of 100 mg / Kg, prior to the experiment. The metabolic pattern as revealed by TLC was essentially unchanged. It was therefore concluded that these results reflect the actual metabolism by the rat tissues.

The formation of catechol from vanillin involves oxidative decarboxylation, as well as demethylation, the sequence of which has been discussed. To investigate which alternative pathway occurs, metabolic experiments were performed with protocatechuic aldehyde, protocatechuic acid, vanillic acid, and guaiacol, and the urinary products so formed were studied.

The metabolic pattern of protocatechuic aldehyde was found to be very similar to that of vanillin. It was also excreted only as a conjugate. Its oxidation product, protocatechuic acid, as well as vanillic acid and catechol were also excreted in the urine. In addition, its 3-O-methyl derivative, vanillin itself, was detected in the urine only after hydrolysis. It must be emphasized here that though methylation of protocatechuic aldehyde was manifested, no demethylation of vanillin was observed.

Both protocatechuic acid and vanillic acid appeared to be metabolically limited as few products were excreted in the urine after their injections. Like the corresponding aldehydes, O-methylation of protocatechuic acid to vanillic acid had occurred, but no demethylation.

of the latter compound. Catechol was the common product of these acids.

Based on the above results, it was established that the pathway from vanillin to catechol involves oxidation as the primary step to form vanillic acid, the decarboxylation and demethylation of which yields catechol. As decarboxylation of vanillic acid produces o-methoxy phenol, or guaiacol, this latter must also be metabolised to catechol. This was found to be the case when guaiacol was administered to rats.

Two additional studies were made in an attempt to evaluate the significance of VMA and VA as end products of the CAs, and the possibility of adrenalone, noradrenalone and isoprenalone as their precursors. Injected VMA appeared as free VMA in the urine. There seemed to be a slight conversion of VMA to VA, the extent of which was insignificant for quantitative determination. AN additional Folin-positive spot (unidentified) was found, which was apparently an artefact as VMA added to normal urine produced the same result.

Experiments with noradrenalone showed a urinary product which had chromatographic properties similar to authentic VMA in at least 3 solvent systems. This product accounts for only 4% of the administered noradrenalone. Hardly any VMA was formed from isoprenalone, while an intermediate amount was produced from adrenalone.

VI. BIBLIOGRAPHY

1. U.S. von Euler, Nature, 190, 170 (1961).

2. S. Udenfriend, W. Lovenberg, A. Sjoerdsma, Arch. Biochem. Biophys., 85, 487 (1959).

3. M. Goldenberg, M. Faber, E.J. Asltor, and E.C. Chargoff, Science 109, 534 (1949).

4. U.S. von Euler, Acta Physiol. Scand., 12, 73 (1946).

5. U.S. von Euler, Acta Physiol. Scand., 16, 63 (1948).

6. H. Blaschko, J. Physiol., 96, 50 P (1939).

7. H. Blaschko, J. Physiol., 101, 337 (1942).

8. P. Holtz and G. Kroneberg, Klin. Wochschr., 28, 605 (1948).

9. S. Gurin and A.M. Delluva, J. Biol. Chem., 170, 545 (1947).

10. S. Udenfriend and J.B. Wyngaarden, Biochem. et Biophys. Acta., 20, 48 (1956).

11. G. Rosenfeld, L.C. Leeper, and S. Udenfriend, Arch. Biochem. Biophys., 74, 252 (1958).

12. P. Holtz, K. Credner, and W. Koepp, Arch. Exptl. Pathol. Pharmakol., 200, 356 (1942).

13. C.G. Schmiterlow, Brit. J. Pharmacol., 61, 127 (1951).

14. H. Blaschko, Biochem. et Biophys. Acta, 4, 130 (1950).

15. C. Mitoma, H.S. Posner, D.F. Bogdanski, and S. Udenfriend, J. Pharmacol. Exptl. Therap., 120, 188 (1957).

16. M.D. Armstrong, K.N.F. Shaw, and P.E. Wall, J. Biol. Chem., 218, 293 (1956).

17. R.J. Boscott, and H. Bickers, Scand. J. Clin. Lab. Invest., 5, 380 (1953).

18. A. Sjoerdsma, W. Lovenberg, J.A. Oates, J.F. Crout, and S. Udenfriend, Science, 130, 225 (1957).

19. T.L. Sourkes, G.F. Murphy, A. Rabinovitch, Nature, 189, 577 (1961).

20. W.F. Bridges, Annals of Internal Medicine, 56, 963 (1962).

21. R.W. Schayer, R.T. Smiley, K.J. Davis, and Y. Kobayashi, Am. J. Physiol., 182, 285 (1955).

22. J. Axelrod, Science, 126, 400 (1957).

23. J. Pellerin, and A. D'Iorio, Rev. Can. Biol., 15, 371 (1957).

24. H. Blaschko, D. Richter, and H. Schlossmann, Biochem. J., 31, 2187 (1937).

25. D. Richter, J. Physiol., 98, 361 (1940).

26. Z.M. Bacq, Pharmacol. Revs., 1, 1 (1949).

27. H. Blaschko, Pharmacol. Revs., 4, 415 (1952).

28. T.L. Sourkes, Rev. Can. Biol., 17, 352 (1958).

29. R. Imaizumi, and K. Kawamoto, Med. J. Osaka Univ., 3, 269 (1952), as seen in C.A. 47, 8108f (1953).

30. R. Imaizumi, K. Kawamoto, T. Kitu, and H. Sato, Med. J. Osaka Univ., 3, 279 (1952),as seen in C.A. 47, 8108g (1953).

31. R. Imaizumi, H. Yoshida, K. Wataya, Y. Takaoka, T. Noda, M. Wada, Med. J. Osaka Univ., 5, 323 (1954).

32. K. Kawamoto, and T. Kita, Osaka Daigaku Igaku Zasshi, 7, 43, (1955),as seen in C.A. 49, 9127d (1955).

33. S. Kashiwagi, and N. Habu, Osaka Daigaku Igaku Zasshi, 6, 181 (1954),as seen in C.A. 48, 16, 9434b (1954).

34. H. Weil-Malherbe and A.D. Bone, Biochem. J., 67, 65 (1957).

35. K. Kawamoto, Symposia on Enzyme Chem., (Japan), 9, 52 (1954), as seen in C.A. 49, 9127 (1955).

36. T. Takaoka, N. Habu, M. Wada, Osaka Daigaku Igaku Zasshi, 7, 135 (1955),as seen in C.A. 49, 17, 11753i (1955).

37. P. Smith, Nature, 182, 1741 (1955).

38. M.D. Armstrong, and A. McMillan, Federation Proc., 16, 146 (1957).

39. D.W. Wylie, S. Archer, and A. Arnold, J. Pharmacol. Exptl. Therap., 130, 239 (1960).

40. J.W. Daly, J. Axelrod, and B. Witkop, J. Biol. Chem., 235, 1155 (1960).

41. K. Kawamoto, H. Sato, T. Kita, H. Kiramatsu, and R. Takana, Osaka Daigaku Igaku Zasshi, 6, 181 (1954), as seen in C.A. 48, 16, 9434c (1954).

42. M. Goldstein, J.M. Musacchio, M.C. Kenin, J.F. Contrera, M.D. Rice, Biochem. Pharmacol., 11, 809 (1962).

43. N.F. Maclagan, and J.H. Wilkinson, Nature, 168, 251 (1951).

44. A.N. Booth, C.W. Murray, F. DeEds, and F.T. Jones, Federation Proc., 14, 321 (1955).

45. F. DeEds, A.N. Booth, and F.T. Jones, J. Biol. Chem., 225, 615 (1957).

46. A.N. Booth, O.H. Emerson, F.T. Jones, and F. DeEds, J. Biol. Chem., 229, 51 (1957).

47. M.S. Masri, D.J. Robbins, O.H. Emerson, and F. DeEds, Nature, 202, 878 (1964).

48. J. Axelrod, J.K. Inscoe, S. Senoh, and B. Witkop, Biochem. et Biophys. Acta, 27, 211 (1958).

49. L.C. Leeper, H. Weissbach, and S. Udenfriend, Arch. Biochem. Biophys., 77, 417 (1958).

50. J. Axelrod, S. Senoh, and B. Witkop, J. Biol. Chem., 233, 697 (1958).

51. J. Axelrod, H. Weil-Malherbe, and R. Tomchick, J. Pharmacol. Exptl. Therap., 127, 251 (1959).

52. E.H. LaBrosse, J. Axelrod, and A. Sjoerdsma, Federation Proc. 17, 386 (1958).

53. C. Itoh, Kaorn Yoshinaga, T. Sato, N. Ishida, and Y. Wada, Nature, 193, 477 (1962).

54. I.J. Kopin, and J. Axelrod, Federation Proc., 19, 295 (1960).

55. I.J. Kopin, Science, 131, 1372 (1960).

56. E.H. LaBrosse, J. Axelrod, S. Kety, Science, 128, 593 (1958).

57. S. Udenfriend, C.R. Creveling, M. Ozaki, J.W. Daly, and B. Witkop, Arch. Biochem. Biophys., 84, 249 (1959).

58. Z.M. Bacq, Arch. Inter. Physiol., 42, 340 (1936).

59. Z.M. Bacq, Arch. Inter. Physiol., 44, 151 (1936).

60. D.W. Wylie, Nature, 189, 490 (1961).

61. J. Axelrod, and M.J. Laroche, Science, 130, 800 (1959).

62. I. Kopin, J. Axelrod, J. Gordon, J. Biol. Chem., 236, 2109 (1961).

63. J. Axelrod, and R. Tomchick, Nature, 184, 2027 (1959).

64. L.G. Whitby, J. Axelrod, and H. Weil-Malherbe, J. Pharmacol. Exptl. Therap., 132, 193 (1961).

65. P.A. Shore, J.A.R. Mead, R.G. Kuntzman, S. Spector, and B. Brodie, Science, 126, 1063 (1957).

66. McC Goodall, Pharmacol., 2, 416 (1959).

67. E.H. LaBrosse, and J.D. Mann, Nature, 185, 40 (1960).

68. J. Axelrod, Physiol. Revs., 39, 751 (1959).

69. L.G. Whitby, J. Axelrod, and H. Weil-Malherbe, J. Pharmacol. Exptl. Therap., 132, 193 (1961).

70. J. Axelrod, Biochem. Biophys. Acta, 45, 614 (1960).

71. G. Hertting, Biochem. Pharmacol., 13, 1119 (1964).

72. E.K. Nelson, J. Am. Chem. Soc., 41, 1118 (1919).

73. Y. Kakimoto, and M.D. Armstrong, J. Biol. Chem., 237, 208 (1962).

74. T.L. Perry, and W.A. Schroeder, J. Chromatog., 12, 358 (1963).

75. T.L. Perry, S. Hansen, M. Hestrin, and L. MacIntyre, Clin. Chem. Acta, 11, 24, (1965).

76. J. Axelrod, I.J. Kopin, J.D. Mann, Biochim. Biophys. Acta, 36, 516 (1959).

77. J. Axelrod, I.J. Kopin, and J.D. Mann, Arch. Biochem. Biophys., 89, 148 (1960).

78. A.A. Smith, Biochem. Biophys. Acta, 69, 203 (1963).

79. R. Fechner, J.D. Chanley, and S. Rosenblatt, Anal. Biochem., 9, 54 (1964).

80. M. Goldstein, A.J. Friedhoff, S. Pomerantz, and C. Simmons, Biochim. Biophys. Acta, 39, 187 (1960).

81. M. Goldstein, A.J. Friedhoff, S. Pomerantz, and J.F. Contrera, J. Biol. Chem., 236, 1816 (1961).

82. L.R. Gjessing, Scand. J. Clin. Lab. Invest., 15, 463 (1963).

83. L.R. Gjessing, and O. Bornd, The Lancet, Vol. II, No. 7363, 818 (1964).

84. A. Pletscher, and K.F. Grey, Biochem. J., 92, 300 (1964).

85. R.G. Bell, Can. Med. Ass. J., 74, 797 (1956).

86. A.A. Smith, and S.B. Wortis, Biochem. Pharmacol., 3, 333 (1960).

87. A.A. Smith, and S.B. Wortis, Biochim. Biophys. Acta, 40, 569 (1960).

88. M.D. Armstrong, A.McMillan, and K.N.F. Shaw, Biochim. Biophys. Acta, 25, 422 (1957).

89. K.N.F. Shaw, A. McMillan, and M.D. Armstrong, J. Biol. Chem., 226, 255 (1957).

90. K.N.F. Shaw, A. McMillan, and M.D. Armstrong, Federation Proc., 15, 353 (1956).

91. C.M. Williams, A.A. Babuscio, and R. Watson, Am. J. Physiol., 199, 722 (1960).

92. L.R. Gjessing, Scand. J. Clin. Lab. Invest., 15, 649 (1963).

93. L.R. Gjessing, and O. Bornd, Scand. J. Clin. Lab. Invest., 17, 80 (1964).

94. K.B. Choremis, N.S. Constantsas, C. Danelatos-Atharassiadis, and A.S. Agathopoulos, Clin. Chim. Acta, 10, 489 (1964).

95. H.G. Bray, W.V. Thorpe, and K. White, Biochem. J., 46, 273 (1950).

96. McC Goodall, H. Alton, and L. Rosen, Biochem. Pharmacol, 13, 703 (1964).

97. H.G. Sammons, and R.T. Williams, Biochem. J., 35, 1175 (1941).

98. R.T. Williams, Detoxication Mechanism,(1959).

99. A.N. Booth, D.J. Robbins, M.S. Masri, and F. DeEds, Nature, 187, 691 (1960).

100. I. Smith, and A.M.H. Bennett, Nature, 181, 709 (1958).

101. K.N.F. Shaw, and J. Trevarthen, Nature, 182, 797 (1958).

102. P. Smith, Nature, 182, 1741 (1958).

103. G.H. Hill, J. Ratcliffe, and P. Smith, Nature, 182, 1160 (1958).

104. J. Pellerin, and A. D'Iorio, Can. J. Biochem. Physiol., 36, 491 (1958).

105. W. Dirscherl, H. Thomas, and H. Schrieffers, 8, Symp-dtsch., Ges. Endokrin, (1961) (Berlin-Gottingen, Heidelberg, P. 18, 1962).

106. W. Dirscherl, and B. Brisse, Acta Endocrinologica, 45, 641 (1964).

107. A. Sturm, Jr., German Medical Monthly, 8, 234 (1963).

108. M.D. Armstrong, P.E. Wall, and V.J. Parker, J. Biol. Chem., 218, 921 (1956).

109. J.A. Anderson, M.R. Ziegler, D. Doeden, Science, 127, 237 (1957).

110. T.P. Waalkes, A. Sjoerdma, C.R. Creveling, H. Weissbach, and S. Udenfriend, Science, 127, 648 (1958).

111. G.B. West, J. of Pharm. Pharmacol., 10, 589 (1958).

112. A.N. Booth, C.W. Murray, F.T. Jones, and F. DeEds, J. Biol. Chem., 223, 251 (1956).

113. P.L. Petrakis, A.G. Kallianos, S.H. Wender, and M.R. Shetlar, Arch. Biochem. Biophys., 85, 264 (1959).

114. G. Curzon, and R.T. Pratt, Nature, 204, 383 (1964).

115. L. Gjessing, and M.D. Armstrong, P.S.E.B.M., 114, 226 (1963).

116. Schwartz and Tulipar, Occupational Diseases of the Skin, P. 440, (London, 1939).

117. White, The Dermatergoses, P. 355 (London, 1934).

118. Preusse, Hoppe-Seyl. Z. 4, 209 (1880).

119. Cohn, Hoppe-Seyl. Z. 17, 274 (1893).

120. Kotake, Hoppe-Seyl. Z. 45, 320 (1905).

121. Y. Nakagawa, M.R. Shetlar, and S.H. Wender, Biochim. Biophys. Acta, 97, 233 (1965).

122. K.N.F. Shaw, M. Gutenstein, and J.B. Jepsen, 5th International Congress of Biochemistry, Moscow, 1961, Abstracts P. 369.

123. A.N. Booth, and R.T. Williams, Nature, 198, 4881 (1961).

124. A.A. Smith, Nature, 190, 167 (1961).

125. U.S. von Euler, and F. Lishajka, Nature, 183, 1123 (1959).

126. G.A. Garton, and R.T. Williams, Biochem. J., 43, 206 (1948).

127. W.M. Azouz, D.V. Parke, and R.T. Williams, Biochem. J., 55, 146 (1953).

128. H.G. Sammons, and R.T. Williams, Biochem. J., 40, 223 (1946).

129. Irwin Sankoff, and T.L. Sourkes, Canadian Journal of Biochem. and Physiology, 41, 1381 (1963).

130. Nakajima, and Sano, Biochim. et Biophys. Acta, 90, 37 (1964).

131. Folin O, and Ciocalteu V., J. Biol. Chem., 73, 627 (1937).

132. Kurt Randerrath, Thin-layer Chromatography, 1963, Academic Press.

133. Bray, H.G., Thorpe, W.V., and White, K., Biochem. J., 46, 271 (1950).

134. Ivor Smith, Chromatographic and Electrophoretic Techniques, Vol. 1.

135. James, W.O., Nature, 161, 851 (1948).

136. A. Pearl, and F. McCoy, Analytical Chemistry, 32, 11 (1960).

137. K. Yagi, T. Nagatsu, I. Nagatsu, Nature, 186, 310 (1960).

138. T.L. Sourkes, and Boris D. Drujan, Can. J. of Biochem. Physiol., 712, 35 (1957).

139. Natelson, S., Lugovoy, J.K., and Pinus, J.B., Arch. Biochem., 23, 157 (1949).

140. H. Weil-Malherbe, and A.D. Bone, Biochem. J., 51, 311 (1952).

141. Dixon M, and Lutwak-Mann C. Biochem. J., 31, 1347, (1937).

142. Bonnichsen R.K. and Wassen A.M., Arch. Biochem., 18, 361, (1948)

143. Racker E., J. Biol. Chem., 177, 883, (1949).

144. Dalziel K., and Dickinson F.M., Nature 206, 255, (1965).

145. Asmussen E., Hald J., Jacobsen E., and Jorgensen G., Acta Pharmacol., 4, 297, (1948).

146. Hald J., and Larsen V., Acta Pharmacol., 5, 292, (1949).

147. Niels Ole Kjeldgaard, Acta Pharmacol, 5, 377, (1949).

148. Hunter F.E., and Lowry O.H., Pharmacol. Revs., 8, 89, (1956).

149. Sherwin C.P., Physiol. Revs., 2, 61, (1922).

150. Harborne J.B., Biochemistry of Phenolic Compounds, Academic Press, P. 221, (1964).

151. Dodgson K.S., Lewis J.I.M., and Spencer B., Biochem. J., 55, 253, (1953).

152. Dodgson K.S., and Spencer B., Biochem. J., 55, 315, (1953).

153. Dodgson K.S., and Spencer B., Biochem. J., 55, 436, (1953).

154. Masri M.S., Booth A.N., and DeEDS F., Biochim. Biophys. Acta 65, 495, (1962).