Measurement of Homovanillic Acid in Human Urine

by

Irwin Sankoff, B.Sc.

A thesis,
submitted in partial requirement for
the degree of Master of Science
in the Biochemistry Department
at McGill University.

Allan Memorial Institute of Psychiatry,
McGill University,
Montreal 2, Quebec.

April, 1963.

ACKNOWLEDGEMENT

I am deeply indebted to Dr. T. L. Sourkes for his counsel, encouragement, and guidance during the time I spent in his laboratory, particularly throughout the course of this study. The valuable scientific, technical, and philosophical discussions we held proved to be both educational and stimulating.

I am grateful to my fellow laboratory companions at the Allan Memorial Institute - students and technicians alike - for their theoretical and practical help, and for providing a friendly and stimulating atmosphere in which to work.

I should like to express my gratitude to the staff members of the Biochemistry Department at McGill for their kindness and help with course work.

To my wife, Marie, my special thanks for assistance in the laboratory with some of the experiments, and for proofreading this manuscript.

LIST OF ABBREVIATIONS USED IN THIS THESIS

Abbreviations used in this thesis for the names of compounds, techniques, and chemical data are listed below:

Aldomet®	α-methyl-L-dopa
dopa	3,4-dihydroxyphenylalanine
dopac	3,4-dihydroxyphenylacetic acid; homoprotocatechuic acid
dopamine	3-hydroxytyramine; 3,4-dihydroxyphenylethylamine; 4-(2-aminoethyl)pyrocatechol
5-HIAA	5-hydroxyindoleacetic acid
m-HPAA	meta-hydroxyphenylacetic acid
HVA	homovanillic acid; 4-hydroxy-3-methoxyphenylacetic acid
MAO	monoamine oxidase
3-Medopamine	3-methoxytyramine; 4-hydroxy-3-methoxyphenylethylamine
m.p.	melting point
phloretic acid	4-hydroxydihydrocinnamic acid
popac	para-hydroxyphenylacetic acid
TLC	thin-layer chromatography
vanillic acid	4-hydroxy-3-methoxybenzoic acid
VMA	vanilmandelic acid; 4-hydroxy-3-methoxymandelic acid

TABLE OF CONTENTS

PREFACE

Homovanillic acid has been shown by several investigators to be a constituent of normal human urine. In certain disease states, the urinary level of homovanillic acid is altered. This fact may be used as a criterion for clinical diagnosis of these diseases. Several methods are available for measuring homovanillic acid, but they are relatively involved and costly procedures.

The purpose of this study was to determine a rapid method which could be used routinely for the clinical measurement of homovanillic acid.

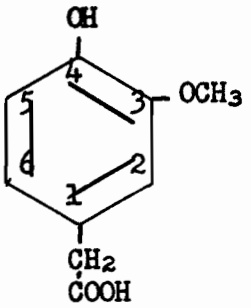
Dopamine and its metabolite, 3,4-dihydroxyphenylacetic acid, have been shown in Dr. Sourkes' laboratory to be abnormal in certain diseases. Another metabolite of these substances, homovanillic acid, has also been found in elevated amounts in some diseases. A rapid method for measuring homovanillic acid would serve as further diagnostic aid in these disease states and might conceivably be used at some time as a simple method to diagnose these diseases.

Methods involving the use of paper chromatography and silica gel columns were rejected after preliminary trials as being either too time-consuming, or for not giving satisfactory separations of homovanillic acid from other compounds. Thin-layer chromatography proved to be extremely useful. The final procedure can be completed in less than ten hours for twenty samples, is relatively inexpensive, and lends itself to an assembly-line type of operation.

I. INTRODUCTION

A. NOMENCLATURE OF HOMOVANILLIC ACID

Homovanillic acid has the empirical formula $C_9H_{10}O_4$. The commonly used generic name is 4-hydroxy-3-methoxyphenylacetic acid. It is also called homoprotocatechuic acid 3-methyl ether and 4-hydroxy-3-methoxy-α-toluic acid (1). Its structural formula is:



Homovanillic acid

Homovanillic acid is commonly abbreviated to HVA and this form will be used throughout the thesis.

In German HVA is homovanillinsäure or 3-methoxy-4-hydroxyphenyl-essigsäure(2,3,4). These names are abbreviated to HVS and MHPES respectively.

B. NATURAL OCCURENCE OF HOMOVANILLIC ACID

HVA occurs naturally in plants and animals. Mitchell, Evans, and Hibbert noted its presence in plants and showed its relation to lignin structure (5). Fisher and Hibbert found HVA (m.p. 142-3°) to be a product of the breakdown of lignin in 1947 (6). Armstrong, Shaw, and Wall first reported HVA in normal human urine (7).

HVA has since been found in abnormal amounts in the urines of people with various diseases (3,4,8-13). HVA is also present in certain plant foods used in human dietaries (14,15).

C. PHYSICAL AND CHEMICAL PROPERTIES OF HOMOVANILLIC ACID

The molecular weight of HVA is 182. It has a melting point of 142°, although values as low as 139° have been reported. It is soluble in hot water and many organic solvents (e.g. methanol, ethanol, and ether).

The 4-acetyl derivative has a melting point of 140° and the 4-carbomethoxyl derivative a melting point of 140-1° (1).

HVA is fluorescent (16,17). At pH 7, it is activated at 270 mμ and fluoresces maximally at 315 mμ. Practical sensitivity is 0.2 μgm./ml. (16). It does not fluoresce below pH 1 or above pH 10. It fluoresces less strongly than 3,4-dihydroxyphenylacetic acid (dopac) because ionization of the phenolic group suppresses fluorescence (17).

DeEds et al. have recorded the optical and crystallographic characteristics of HVA isolated from urine and found them to be comparable with synthetic HVA (18). The properties of the synthetic HVA were observed on crystals grown from water, methylene chloride, or from fusion (See Table I). The crystals are tabular to platy. The plates are about twice as long as they are wide and have an end angle of 130°. HVA crystallizes in the orthorhombic system.

TABLE I

Optical and Crystallographic Properties of Homovanillic Acid
(After DeEds et al. (18))

Refractive Indices (5893 A)	$\alpha = 1.568$ $\beta = 1.583$ $\gamma = 1.697$
Axial angle	(+) $2E = 68.5^{\circ}$ observed $2V = 42^{\circ}$ calculated
Dispersion	($v > r$) slight
Optic orientation	Tablets (end angle = 130°) show centered Bx_a figure, α lengthwise
Fusion data	M.p. $143\text{-}4^{\circ}$; scales or plates showing same interference figure as tablets from solution.

Smith et al. (19) and McGeer et al. (20) have listed the paper chromatographic characteristics of HVA, along with many related compounds.

HVA reacts with $FeCl_3$ to give a faint green colour and with phenol to give a blue colour. Distillation of the calcium salt with $Ca(OH)_2$ gives creosol while the action of hot dilute HCl on HVA yields dopac and CH_3Cl (1).

D. SYNTHESIS OF HOMOVANILLIC ACID

Before the occurrence of HVA was observed in animals, chemists had prepared the compound, made derivatives of it, and used it as an intermediate in the preparation of other compounds.

First mention of HVA in the literature occurs in 1877. F.Tieman and N. Nagai (2) were working on the degradation of aceteugenol.

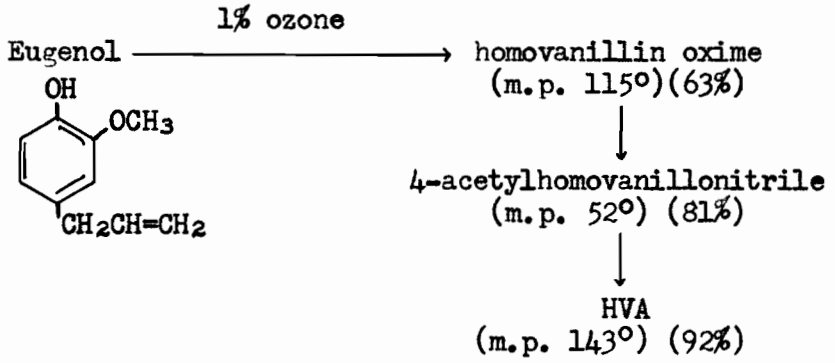
They attempted to isolate vanillic acid (m.p. 140°). However the substance they obtained had a melting point of 142° and they called it "homovanillinsäure". Acetylhomovanillic acid was an intermediate in the preparation.

Some 30 years later F. Mauthner, at the University of Budapest's Chemical Institute, synthesized a compound identical to that made by Tiemann (21). This was the first synthesis of HVA and was described in Justus Liebig's Annalen der Chemie in 1909.

Pschorr, at the University of Berlin, brominated HVA to obtain 6-bromo-3-methoxy-4-hydroxyphenylacetic acid (m.p. 180-1°)(22). In the same year (1912) Alfons Klemenc , at the University of Vienna, reacted HVA with diazomethane to obtain 5-nitrohomoveratrumic acid, a yellow compound (m.p. 113-4°)(23). Kitasato prepared ethylcarbonatohomovanillic acid (m.p. 138-9°) in 1927 by the action of ethyl chlorocarbonate on HVA (m.p. 140-1°)(24).

In 1931 Gulland was working on the synthesis of alkaloids related to aporphine (25). During the preparation of intermediates, and while he was trying to obtain the nitro derivative of dopac, he made 2-nitro-3-methoxy-4-hydroxyphenylacetic acid . Douglas and Gulland formed 6-nitro-3-methoxy-4-hydroxyphenylacetic acid (m.p. 184°) as an intermediate in the preparation of laurotetanine in 1931 (26).

Hahn and Schales formed HVA by saponification in 1934 (27). Their method essentially involved obtaining β-(hydroxyphenyl)ethylamines and the corresponding acetic acids from allyl compounds. They used natural allyl compounds as starting materials, submitted them to ozonization to form oximes, boiled them with acetic anhydride to get the nitrile derivative, and then saponified to get the corresponding acetic acid.



Conversion of eugenol to homovanillic acid

In 1947 Challis described the preparation of 0.7 grams of HVA from ten grams of O-carbethoxyeugenol (28). The starting compound was converted to O-carbethoxyhomovanillic acid (m.p. 125-6°). HVA (m.p. 139°) was obtained by heating the O-carbethoxyhomovanillic acid with 10% sodium hydroxide.

Berlin et al. isolated HVA (m.p. 138-41°) during the preparation of derivatives of zingerone (29). Pearl and Beyer obtained the hydrazides of various carboxylic acids related to vanillic acid and tested them for antibacterial activity against pathogenic microorganisms. The hydrazide of HVA had a melting point of 158-9° (30).

Shaw et al. synthesized HVA and 3-methoxytyramine (3-Medopamine) from vanillin and acetylaminoacetic acid in 1958 (31). Sweeley and Williams converted the urinary aromatic acids to methyl esters and utilized gas chromatography to measure the resulting HVA and other compounds (32,33). Ruthven and Sandler developed a method to measure HVA during which the HVA is demethylated to dopac (9). The latter two methods are described in detail on pages 19 and 22 of this thesis.

E. BIOCHEMISTRY OF HOMOVANILLIC ACID AND METHODS OF DETERMINATION

1. Formation of homovanillic acid in vivo from flavonoids

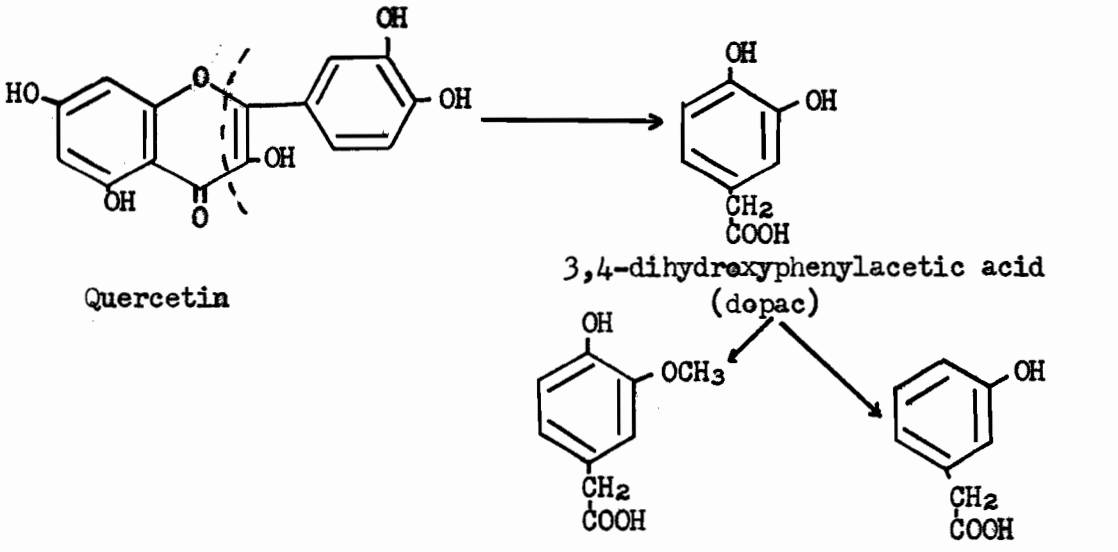
Floyd DeEds, Albert Booth, Francis Jones, and Charles Murray, working with the United States Department of Agriculture at Albany, California, (18,34,35,36) conducted experiments in the mid-1950's showing that HVA is a product of dopac and of the flavonoids, rutin and quercetin. Another group in Utah, Marvin Armstrong, Kenneth Shaw, Armand McMillan, and Patricia Wall, showed at the same time that HVA is a normal constituent of human urine (7,14,37).

Booth et al. fed rutin and its aglycone, quercetin, orally to rabbits and rats (34,36). The urines were collected in acid and extracted for five to six hours in a continuous liquid-liquid extractor. The ether extract was evaporated under a vacuum with nitrogen and the residue dissolved in acetone. Small amounts of the acetone solution (0.02 to 0.1 ml.) were applied to Whatman #1 filter paper. Ascending two-dimensional chromatography was used to develop the chromatograms. The solvent system in the first direction was the lower phase of chloroform:acetic acid: water (2:2:1); 20% aqueous KCl was used to develop them in the second direction. Running time was 16 and three hours for the first and second directions, respectively. Diazotized sulphanilic acid, followed by 20% sodium carbonate was sprayed on the papers to visualize the spots.

HVA could also be obtained from the ether extract by evaporating the ether and extracting the solids with hot xylene. Plates of impure HVA were obtained as the solution cooled. These were purified by partitioning between water and chloroform, thus taking advantage of the fact that HVA is more soluble in chloroform than is dopac (36).

The feeding of rutin and quercetin results in an increase in the amount of certain normal urinary constituents, meta-hydroxyphenylacetic acid (m-HPAA), HVA, and dopac, both of which were not found at the time in the normal control urines. It was also shown in these experiments that administration of dopac causes excretion of urinary HVA and m-HPAA. Dopac had previously been shown by Murray et al.(38) to be a metabolic product of rutin and quercetin.

Booth and his co-workers believed that the dopac was methylated in the meta position to give HVA and dehydroxylated in the para position to give m-HPAA.



Quercetin

3,4-dihydroxyphenylacetic acid (dopac)

homovanillic acid (HVA)

m-hydroxyphenylacetic acid (m-HPAA)

Conversion of quercetin to homovanillic acid and m-hydroxyphenylacetic acid.

Further experiments proved that dopac was the precursor of HVA and m-HPAA (18,35). Other 3,4-dihydroxy compounds were administered orally to animals and were methylated in the 3-position. 3,4-dihydroxybenzoic (protocatechuic) acid was metabolized to 3-methoxy-4-hydroxybenzoic (vanillic) acid; 3,4-dihydroxycinnamic (caffeic) acid was metabolized to 3-methoxy-4-hydroxycinnamic (ferulic) acid, 3-methoxy-4-hydroxyphenylpropionic (dihydroferulic) acid, 3,4-dihydroxyphenylpropionic (dihydrocaffeic) acid, and meta-hydroxyphenylpropionic acid; 3,4-dihydroxyphenylalanine (dopa) was converted to HVA, dopac, and m-HPAA.

The results with dopa presented new evidence on the metabolic fate of tyrosine and phenylalanine. This was the first time that HVA (from the methylation of dopac) and m-HPAA (from the dehydroxylation of dopac) were shown to be metabolites of dopa. These results were confirmed shortly after by Shaw et al.(14,37).

The pathway presented by DeEds et al. at this time (18) consisted of the conversion of dopa by dopa decarboxylase to 3,4-dihydroxyphenylethylamine (dopamine) (39,40); oxidation of dopamine by amine oxidase to 3,4-dihydroxyphenylacetaldehyde; oxidation of the aldehyde to dopac; and methylation of this compound to form HVA.

Work with quercetin-C^{14}, administered orally to rats, confirmed the above results, for radioactive HVA, dopac, m-HPAA, and m-HPAA glucuronide were recovered in the urine (41).

2. Formation of Homovanillic Acid from Amino Acids

At about the same time the California group were conducting their initial studies on HVA, Armstrong et al. showed that HVA is a normal constituent of human urine (7, 14, 37). Armstrong, Shaw, and Wall described the chromatographic behavior of 49 phenolic acids found in urine and gave a preliminary identification of 23 of them, including HVA (7). They estimated that most of the acids were excreted daily in amounts of 2 to 25 mg.

In 1956, Shaw et al. obtained a normal value of 3-8 mg. of HVA per day in human urine. They suggested that only a small amount of this was conjugated, most of the HVA being excreted in the free form (See Table II).

Shaw's method involved acidifying the urines, saturating with NaCl, and extracting with ethyl acetate four times. The pooled ethyl acetate extracts were mixed with 1 N sodium bicarbonate until the pH of the aqueous phase was 7.5-8. This phase was acidified, saturated with NaCl, and extracted four times with ethyl acetate. The combined ethyl acetate fractions were diluted until 1 ml. corresponded to 10-15 mg. creatinine in the original urine. Two-dimensional chromatography was used for the separation of the various compounds. The solvents were isopropyl alcohol: ammonia:water (8:1:1) followed by benzene:propionic acid: water (2:2:1) (organic phase). HVA gave a rose spot when the papers were sprayed with diazotized sulphanilic acid. The results of the experiments are summarized in Table II.

TABLE II
Metabolism of HVA, Dopac, L-Dopa, D-Dopa, and 3-Methoxy-DL-tyrosine in Man.
(After Shaw et al. (14))

Compound Ingested*	Dopac in Urine			HVA in Urine		
	Free mg.	Conjugated mg.	Recovery %	Free mg.	Conjugated mg.	Recovery %
HVA	0	0	0	377	43	84
Dopac	123	55	36	209	28	44
L-Dopa	103	38	33	153	53	45
D-Dopa	37	0	9	21	2	5**
3-Methoxy-tyrosine	0	0	0	15	0	4***

* 500 mg. of each compound was administered orally.
** 27% D-Dopa was excreted in the urine unchanged.
*** 13% 3-Methoxy-DL-tyrosine was excreted in the urine unchanged.

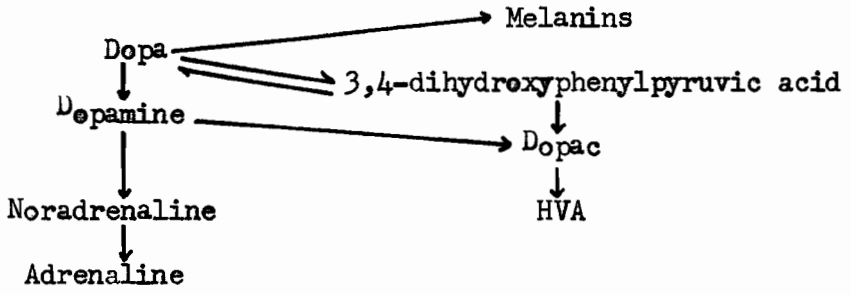
In all instances where 50-500 mg. of HVA, dopac, and L-dopa were administered, a greater amount of HVA was recovered than dopac. When HVA was given, no dopac was recovered. Table II also shows that smaller amounts of HVA and dopac are recovered from the D-isomer than from the L-isomer. The data indicated that HVA is probably a natural terminal metabolite of endogenous L-dopa formed by the methylation of the intermediate dopac.

During the experimental period, the intake of certain foods - tea, coffee, fruits, vegetables, and spices - was restricted. After ingestion of the test compounds, excretion of the HVA was essentially complete in eight hours. Recovery from the urine during the second eight-hour period was less than one per cent of the ingested compound.

When known amounts of the metabolites are added to normal urine, all the HVA was recovered by the extraction procedure. HVA was autoclaved at acid pH with normal urine, from which the free phenolic acids had been extracted; no significant destruction of the added compounds was evident.

Experiments with rats (14) showed that when dopac was administered intraperitoneally, 61% was recovered as dopac and 33% as HVA. If L-dopa was injected into rats, 25% was recovered as HVA and 16% as dopac.

Shaw et al. (14) summarized the probable metabolic pathway of dopa at the time as follows:



Metabolic pathway of dopa according to Shaw

Further confirmation of the work in the California and Utah laboratories was published by Pellerin and D'Iorio at the University of Montreal (42). In 1955 they had shown that when DL-dopa-C^{14} was given to albino rats, 80% of the radioactivity occurred in the urine and 2% in the respiratory CO_2 (43). Among the metabolites identified by descending one-dimension chromatography were dopa, dopamine, dopac, and 3,4-dihydroxyphenylpyruvic acid. They did not find HVA, but suggested that phenolic O-methylation may nevertheless occur.

Shaw et al. suggested that Pellerin and D'Iorio's Compound No. 7, tentatively characterized as 5,6-dihydroxyindole, may be HVA (14). Shaw's recovery of HVA after L-dopa injections is similar to that of Pellerin and D'Iorio. Furthermore, Shaw's R_f values for HVA are similar to those obtained by Pellerin and D'Iorio's unknown substance.

Pellerin and D'Iorio repeated their experiment three years later (42); among the metabolites identified by paper and ion exchange chromatography was HVA. Of the 80% radioactivity in the urine, 16% was due to HVA and 3% to dopac.

In 1958 Booth and DeEds observed that dihydroquercetin is metabolized in a similar way to quercetin and dopa; HVA, dopac, and m-HPAA are metabolites (44). Shaw and Trevarthen showed that urinary HVA levels increased, although they did not exceed the normal range, after ingestion of bananas (15). Excretion of extra HVA also occurred after ingestion of coffee, caffeic, or ferulic acids.

Williams and Babuscio injected DL-tyrosine-2-C^{14} into rats and collected urine for 24 hours (45). After chromatography, no radioactivity was found in the spot corresponding to HVA. Duchon and Gregora obtained similar results with unlabelled tyrosine fed to normal humans; they failed to find an increase in HVA in the 24 hour urine collections (8).

3. Alternate Pathways in the Formation of Homovanillic Acid

That catecholamines could undergo O-methylation was shown in three laboratories (46, 47, 48) at about the same time.

Armstrong, McMillan, and Shaw (46) administered noradrenaline parentally and DL-3,4-dihydroxymandelic acid orally. They found increased amounts of 4-hydroxy-3-methoxymandelic acid (VMA) in the urine in both instances. They also suggested that VMA was a metabolite of adrenaline.

Pellerin and D'Iorio (47) showed that several phenolic acids could undergo O-methylation *in vitro*. Protocatechuic acid was methylated to vanillic acid, dopac to HVA, 3,4-dihydroxymandelic acid to VMA, caffeic acid to ferulic acid, and 3-hydroxybenzoic acid to 3-methoxybenzoic acid.

In 1958 Axelrod *et al.* (48) demonstrated that catecholamines could undergo O-methylation *in vivo*, i.e. they showed 1) the normal occurrence of O-methyl metabolites of catecholamines in urine and 2) the O-methylation of administered catecholamines. The enzyme required was O-methyltransferase; S-adenosyl methionine and divalent cations (Mg^{++}, Mn^{++}, and Co^{++}) were also needed. By this mechanism dopamine could be converted to 3-Medopamine which is then oxidized to give HVA. Thus HVA can be formed by a pathway by-passing dopac.

It was of interest to determine whether under physiological conditions O-methylation preceeded deamination, deamination preceeded O-methylation, or if both routes were concurrent. The experiments established that O-methylation occured before oxidative deamination for the principal metabolic route to noradrenaline and adrenaline.

Figure 1 shows Axelrod's scheme for the metabolism of catecholamines (48).

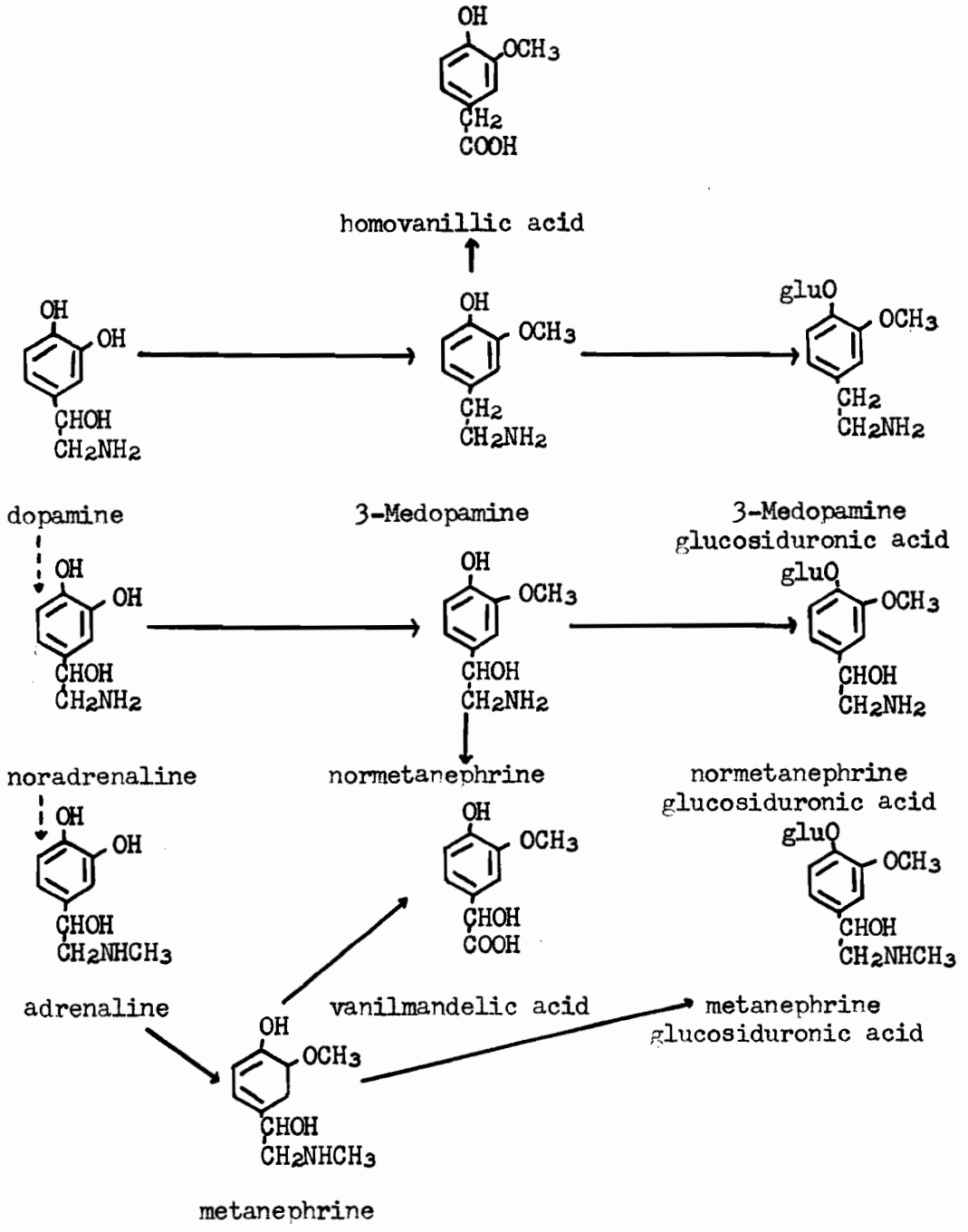


Figure 1. Metabolism of catecholamines

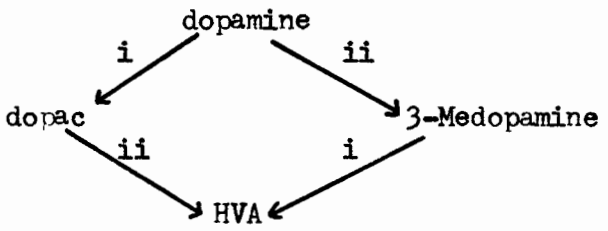
Experimentally, the first step was to obtain 3-Medopamine from dopamine. Rats were injected intraperitoneally with dopamine and the urine was collected for 24 hours. The urine was incubated with β-glucuronidase and the 3-Medopamine extracted and chromatographed. The 3-Medopamine isolated from urine had UV fluorescence (activation 285 mμ, fluorescence 335 mμ), colour reactions, and chromatographic mobility similar to the authentic compound. Three per cent of the administered dopamine went to 3-Medopamine , half of it being conjugated with glucuronic acid. If the animals were pretreated with iproniazid, five times more 3-Medopamine was excreted.

For HVA, the urines were treated with β-glucuronidase, acidified with HCl, and extracted with n-butanol. The organic phase was shaken with 3% sodium bicarbonate solution, and the aqueous layer re-extracted with n-butanol at pH 1. The solution was reduced in volume and chromatographed. After spraying, a spot with the same R_f and colour reaction as the HVA listed by Armstrong et al. (7) appeared.

Pellerin and D'Iorio's work in 1958 involved methylation of the meta-hydroxy position of catechol acids using rat liver and kidney preparations (47). Dopac was incubated (37° C. for two hours) in Warburg vessels with L-methionine-methyl-C^{14}, the rat liver or kidney homogenate, adenosine triphosphate, Mg^{++}, phosphate buffer (pH 7.4), and reduced glutathione. To stop the reaction 0.2 ml. concentrated HCl was added. Centrifuging removed the protein and NaCl was added to the supernatant. The supernatant was made acid (pH 1) and extracted three times with ethyl acetate. Quantitative measurement of homovanillic acid was done by measuring its absorbance at 279 mμ.

The enzymatic activity was found mostly in the supernatant, and the liver homogenate proved to be more active than the kidney homogenate.

Thus two routes for the formation of HVA were now established (Figure 2).



i. monoamine oxidase; aldehyde dehydrogenase.
ii. O-methyltransferase (S-adenosyl methionine; Mg++).

Figure 2. Metabolism of dopamine to HVA.

The metabolic pathway of dopamine was further investigated in 1959 by Goldstein et al. (49). Rats were given dopamine-1-C^{14} and 24 hour urines collected. Of the administered dose of dopamine, 60.5% was excreted as HVA, 2.6% as 3-Medopamine, and 6.4% as dopamine. The conclusions were that dopamine undergoes methylation and deamination and is excreted mainly as HVA. If the rats were treated with the monoamine oxidase (MAO) inhibitor iproniazid four hours prior to the dopamine injection , 25.5% was excreted as HVA, 1.25% as dopac, 31.5% as 3-Medopamine, and 19.25% as dopamine. Thus amine oxidase functions in dopamine metabolism.

Goldstein et al. also injected a mixture of dopamine-β,β-H_2^3 and β-C^{14} into rats and recovered from the urine radioactive dopac, HVA, and a previously unknown compound, 3-methoxy-4-hydroxyphenylethanol (50).

The urines were hydrolyzed at pH 2, extracted with ethyl acetate, and chromatographed two-dimensionally. Iproniazid enhanced the formation of 3-methoxy-4-hydroxyphenylethanol, but lessened the formation of HVA.

Williams, Babuscio, and Watson altered the amounts of dopamine metabolites by pretreating rats with MAO inhibitors (51). For controls they injected dopamine-C^{14} intraperitoneally into rats which were then fasted for 24 hours. They recovered 39% of the radioactivity as unconjugated HVA, 3.4% as dopac, 6.5% as 3-Medopamine (80% of this as the glucuronide) and 6.1% as dopamine. Pretreatment with MAO inhibitors gave a ten-fold reduction in HVA and dopac, and a three-fold increase in 3-Medopamine. There was no effect on the dopamine excretion. If the animal was pretreated with an inhibitor of catechol O-methyltransferase, 3-Medopamine was reduced to negligible amounts, dopac excretion was increased five times, while there was no significant effect on HVA or dopamine excretion. Williams concluded that HVA is the main urinary metabolite of exogenous dopamine and probably of the endogenous amine also. Williams calculated that 21% of the HVA excreted after a dopamine load test was derived from 3-Medopamine and 79% from dopac.

Williams also found normal amounts of HVA in the urine of patients with Huntington's chorea (11). He concluded that the metabolism of dopamine is not altered in this disease. For these experiments Williams used two-dimensional chromatography and a spray of diazotized sulphanilic acid. The spots were eluted with phosphate buffer (pH 7) and their absorbance at 508 mμ was determined. Accuracy was ± 10% and recovery 94%.

Normal urine (7 determinations) gave a mean of 9.6 mg./24 hours of HVA (5.5 mg./gram creatinine. The range was 2.4 to 17.1 mg./24 hours (2.0 to 8.6 mg./gram creatinine). In Huntington's chorea (9 determinations) mean excretion of HVA was 5.4 mg./24 hours (6.2 mg./gram creatinine). The range was 1.9 to 8.2 mg./24 hours (2.3 to 14.2 mg./gram creatinine).

Later, Sweeley and Williams (32) extracted aromatic acids from urine using Armstrong's method (7, 14) and converted them to methyl esters by a reaction with diazomethane in anhydrous ether. The ether was then evaporated and the residue dissolved in absolute ethanol for gas-liquid partition chromatography to identify the HVA. At the same time they identified para-hydroxyphenylacetic acid (popac), hippuric acid, VMA, indolacetic acid, and 5-hydroxyindolacetic acid (5-HIAA). They also found HVA excretion in a carcinoid patient to be within the normal range (33). Indolacetic acid was also normal. Hippuric acid was reduced to 1/10 the normal value, but popac and 5-HIAA were above normal. In 1962 (52) several more urinary aromatic acids, including HVA, were further investigated using gas chromatography.

4. Variations in urinary concentrations of homovanillic acid in health and disease.

Thus, by 1960 several workers had identified HVA as a normal urinary constituent and had developed different methods to measure it. There were indications that the determination of urinary HVA, along with other metabolites of tyrosine and phenylalanine, would be useful in diagnosing certain diseases.

Robinson and Smith tried to show an effect of stress (spinal shock, operations, accidents, burns) on urinary phenols (53). They found an increase in VMA (from 1.0-2.5 μg./minute to 4-10 μg./minute) in stress, but they claimed that the increase in HVA excretion in stress could not be as well defined owing to the presence of large amounts of HVA in normal urine.

Von Studnitz showed a rise of HVA in cases of neuroblastoma (3,4). In 1960 he demonstrated unequivocally for the first time the presence of dopa and 3-methoxy-4-hydroxyphenylalanine in urine. He found, using high voltage electrophoresis to separate the organic acids, that VMA was increased in six cases of neuroblastoma while in two of the cases, HVA, dopamine, dopac, and 3-Medopamine values were above normal. Further work in 1962 showed HVA increases in 17 of 25 neuroblastoma cases in children (4). The range in the disease cases was from 10-1626 μg./mg. creatinine. Von Studnitz established the normal limit of HVA at 40 μg./mg. creatinine with a mean of 16.4 ± 6.4 μg./mg. creatinine. He also obtained high values of VMA (10-770 μg./mg. creatinine in disease states as against 1.2-9.5 μg./mg. creatinine in normals) in 24 of the cases. In five other children with neural tumors (paraganglioma, neurinoma, and ganglioneuroma) the excretion of HVA and VMA was normal. In several children with neuroblastoma, the urinary excretion of HVA and VMA declined to normal levels following removal or x-irradiation of tumors, and in one case rose again with recurrence of the tumor.

Greenberg and Gardner found increases in HVA and dopamine in a 2 1/2 year old patient suffering from posterior mediastinal ganglioneuroma (10). Von Studnitz measured the HVA in this case.

Tompsett measured HVA using two-dimensional paper chromatography, but his values were much lower than those previously reported in the literature (54). His method consisted of refluxing urine for 90 minutes with 10 N HCl and then taking the ether extract up in ethanol (1 ml. equivalent to 50 ml. urine). The solvent systems for the two-dimensional ascending paper chromatography were isopropanol:ammonia: water (8:1:1) and benzene:propionic acid:water (2:2:1). The papers were sprayed with 1) diazotized p-nitroaniline followed by 20% sodium carbonate or 2) diazotized sulphanilic acid. The spots were cut out and eluted for two hours with 10 ml. 50% aqueous methanol. HVA was measured at 500 mμ. The average values were 1.2 mg./24 hours with a range of 0.8-1.6 mg./day.

Zeisel, using paper electrophoresis and chromatography, showed that HVA is a breakdown product of dopamine occuring in children's urine (55).

Smellie and Sandler were the first to correlate the enormous amounts of HVA and dopamine formed with the clinical picture in a patient with a secreting intrathoracic ganglioneuroma (12). The tumor itself contained dopa, dopamine, and noradrenaline. Before the operation there were large amounts of HVA and VMA in the urine, but post-operative values returned to normal. Smellie and Sandler suggested that most of the dopamine produced by the tumor was probably metabolized and represented as HVA. A schematic pathway of dopa metabolism with representative assay figures (mg./24 hours) of metabolites in urine of their case is shown in Figure 3 (12). Normal values for children the same age as the case patient (i.e. two years) are shown in parenthesis.

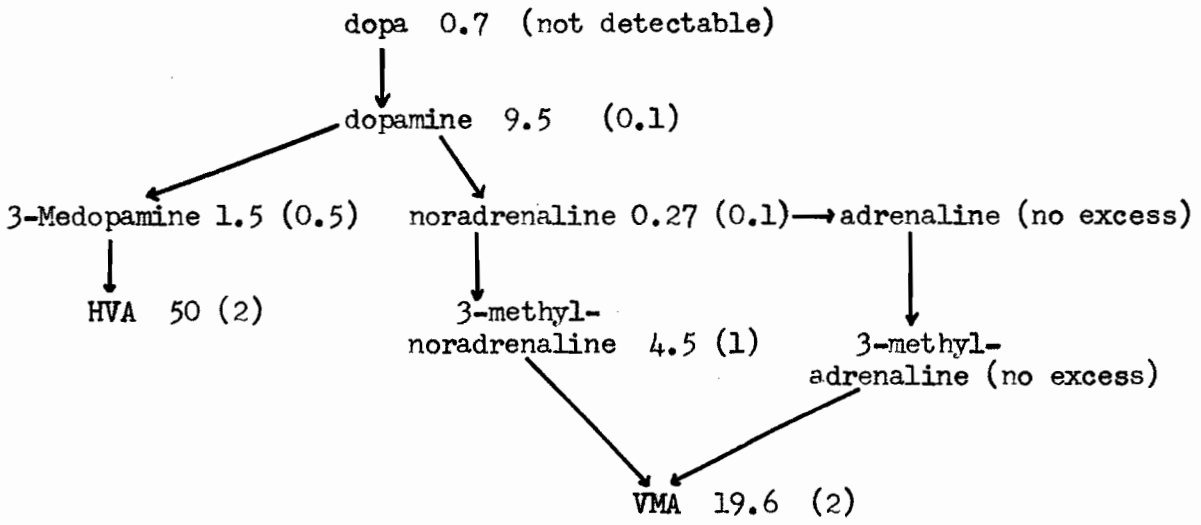


Figure 3. Disposition of dopa according to Smellie and Sandler.

Evidence that HVA values may not be normal in diseases of the basal ganglia or extrapyramidal system has come from studies on metabolic precursors of HVA. Ehringer and Hornykiewicz reported (56) that dopamine is decreased in the neostriatum, and noradrenaline in the hypothalamus, in Parkinson's disease. Barbeau et al. recorded lower excretion of dopamine in Parkinsonism, and raised dopamine values in striatial syndrome cases (57).

Ruthven and Sandler demethylated HVA to dopac and measured the latter compound by fluorescence of its ethylenediamine derivative (9). Their HVA values ranged from 2.5-9 mg./24 hours with a mean excretion of 6.0 ± 1.1 mg. (standard deviation) in 24 hours. Raised levels were also reported in dopamine-secreting tumors. They extracted acidified urine with methylene chloride and passed the organic phase through a column of silica gel , eluting with 3% methanol in methylene chloride.

The eluate was evaporated in a stream of nitrogen at 40° and the residue autoclaved with glacial acetic acid, cysteine, and HBr. Versene and ascorbic acid were added to the digest (pH 8.3-8.4), which was passed through a column of neutral alumina. The column was washed with water and eluted with 0.2 N sulphuric acid. Colour was developed with aqueous sodium molybdate-sodium nitrite and N NaOH and read at 510 mμ.

Carlsson and Hillarp noted large amounts of HVA and dopac accumulating in the brain stem after administering L-dopa (58). The HVA was measured semi-quantitatively on chromatograms. Two and four hours after the dopa load, HVA was the predominant metabolite. Normal brain HVA, dopa, and dopac were too low to measure. Other findings were as follows: 1) accumulated phenolic acids did not escape easily from the brain to the blood, but passage in the other direction occured; 2) phenolic acids did not seem to accumulate in granules, in contrast to catecholamines; 3) MAO is close to the site of synthesis of the amines, possibly within the amine-producing cell, but the O-methyltansferase is localized outside the amine-producing cell. After administration of dopa there was a rapid accumulation first of dopac and then of HVA. This suggested to the authors that MAO is important in the regulation of amine formation and that transferase is important for the inactivation of amines released to the receptors.

Experimentally, the phenolic acids were extracted from the homogenized and treated brain tissue (pH < 2) with ethyl acetate.

A small volume of the extract was chromatographed two-dimensionally on paper using benzene:propionic acid: water (20:14:1) and n-butanol: N HCl (4:1) as the solvent systems. The phenolic acids were seen after spraying the chromatograms with diazotized p-nitroaniline (58).

Duchon and Gregora found normal values of HVA to be 4-7 mg. per 24 hours in urine. The levels were increased to 10-20 mg./24 hours in melanoma cases (8). Their method was essentially that of Armstrong et al. (7), except that the ethyl acetate fractions are taken to dryness and the residue dissolved in ethanol. The two-dimensional ascending paper chromatography technique was used to analyse the ethanolic solution using isopropanol:ammonia:water (8:1:1) and benzene:propionic acid:water (2:2:1) systems. The papers were sprayed with diazotized p-nitroaniline and the coloured spots eluted with tert.-butanol-water-10% sodium carbonate system. The colour was read at 556 mμ.

Melanoma patients fed 3.5 grams of tyrosine showed an increase in HVA excretion. Normal subjects, given the same dose of tyrosine orally, showed practically no change in urinary HVA excretion. Similar results were obtained after loads of seven grams of tyrosine. In the melanoma patients, after tyrosine loads for three consecutive days, there was an increase in HVA for the first two days and a decrease after the third dose, "as though the organism had adapted itself". These results correspond with the findings of Williams and Babuscio (45) who failed to find radioactivity in spots corresponding to HVA after injections of labelled tyrosine (See page 13).

Robinson and Smith found high HVA values in neuroblastomas and in one of six cases of pheochromocytoma (suggesting release of dopamine by tumors) (13).

Williams and Greer (59) found elevated HVA values (360 µg./mg. creatinine) in a three-month old child with neuroblastoma. The control value for a four-month old child was 9 µg./mg. creatinine.

5. Summary of methods for the determination of homovanillic acid

A summary of methods used for determining urinary HVA is set up in Table III (page 26).

TABLE III

Excretion of Homovanillic Acid by Healthy Subjects
(After Sankoff and Sourkes (60)).

Method	Mg. per 24 hours		Mg. /gm. creatinine		Reference
	Range	Mean	Range	Mean	
Paper chromatography	3-8				14
Paper chromatography	0.8-1.6	1.2			54
Paper chromatography	2.4-17.1	9.6±1.9 (SD)	2.0-8.6	5.5±0.8 (SD)	24
Determination as dopac after demethylation	2.5-9.0	6.0±1.1 (SD)			9
Gas chromatography		5.0*	2.6-4.8	3.6*	33
High-voltage electrophoresis			3.9-39.9	16.8±6.4 (SD)**	4
Thin-layer chromatography (Children)	4.3-10.0	6.6	7.3-24.5	14.6	60
(Adults)	4.5-15.6	8.63	3.05-12.36	6.71	

* Based on four values, assuming an average excretion of 1.4 gm. creatinine/24 hours.
** Value for 12 healthy children, age 1 1/2 - 12 years.

II. DEVELOPMENT OF METHODS

A. INTRODUCTION

The purpose of these experiments was to separate HVA from other components in urine and to measure the amounts of HVA present. Among the methods used were: extraction into organic solvents; separation of HVA by column, paper, and thin-layer chromatography; and identification and measurement of HVA with colour reactions.

After extraction of HVA, attempts were made to separate it from other compounds that were extracted along with it. Silica gel columns, similar to those used by Goldstein (61), proved unsatisfactory. Single dimension paper chromatography did not give a sharp enough separation. In the summer of 1962 we tried thin-layer chromatography. After several trials results were obtained which were in general agreement with those obtained by diverse methods (See Table III, page 26).

Several colorimetric reactions with diazo compounds were tested before the Folin-Ciocalteu phenol reaction (62,63) was accepted as a reliable method for quantitative determination of HVA. Colours were unstable and faded quickly from thin-layer chromatography (TLC) plates when diazotized amines were used (64). Diazotized sulphanilic acid (65) reacted well when sprayed on TLC plates, but several problems were encountered while trying to elute HVA from silica gel and then performing colour reactions with the eluted HVA.

A schematic representation of the methods used in the extraction of urine is presented in Figure 4.

In this section, the data are not necessarily presented in chronological order.

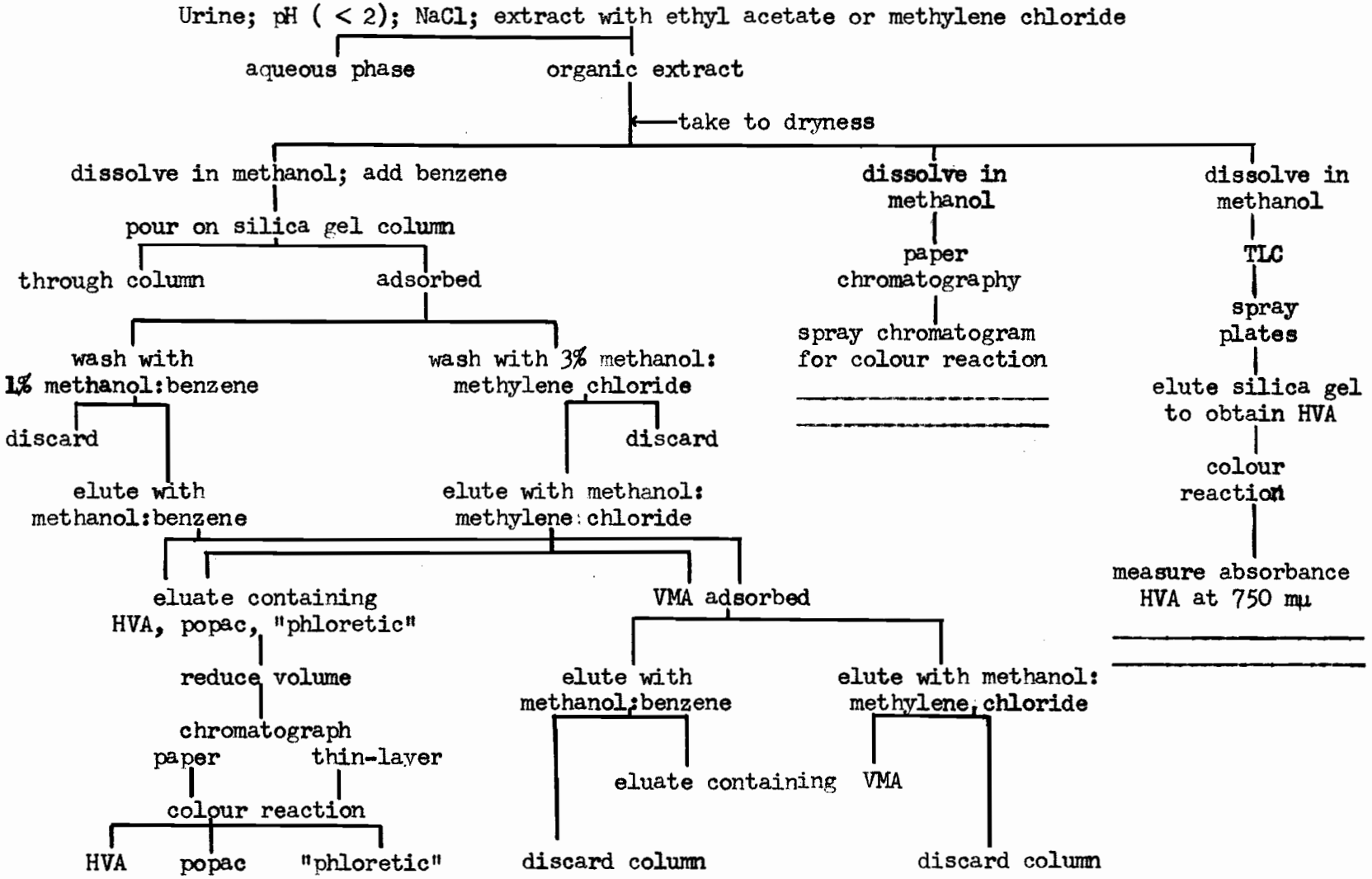


Figure 4. Schematic representation of methods for extraction of urine.

B. EXTRACTION OF HOMOVANILLIC ACID FROM URINE

Two methods were studied: those of Armstrong et al. (7,14), using ethyl acetate, and of Ruthven and Sandler (9), using methylene chloride. The latter solvent extracted fewer components from the urine than ethyl acetate did. As we wished to assess the mobilities and relative amounts of certain other compounds present in urine with HVA, especially during the TLC studies, the ethyl acetate extraction was more thoroughly evaluated.

1. Ethyl acetate extraction

Five ml. of urine was adjusted to an acidic pH (< 2) with 18% HCl and saturated with NaCl. The urine was extracted with six ml. ethyl acetate and the mixture centrifuged to obtain two layers. Five ml. of the upper organic layer was transferred with a pipette to another test tube and the aqueous portion again extracted with five ml. of fresh ethyl acetate. After centrifuging, five ml. of the organic phase was removed and the aqueous lower layer discarded. It is essential to have a water-free extract. The pooled volumes of solvent were then evaporated under reduced pressure using a flash evaporator (Labline, Chicago, Cat. No. 5100). In some early experiments, urine was extracted with three successive portions of ethyl acetate: six, five, and five ml., and 15 ml. of the pooled extracts was evaporated. However, there was no advantage in using three, instead of two, extractions. Smaller portions of urine (2 ml.) were also extracted with aliquots of ethyl acetate (3 ml. and 2 ml.), but this method was neither faster nor more accurate.

The residue was treated in a different manner depending upon whether column, paper, or thin-layer chromatography was to be used.

For column chromatography it was necessary to wash the walls of the vessel containing the residue with small volumes (1-3 ml.) of acetone. The acetone was collected in the bottom of the vessel and evaporated by placing the vessel in warm water. The resulting residue was dissolved in 0.2 ml. methanol. Benzene was added gradually to a volume of 10 ml. This material was added to the silica gel columns. In certain instances the dried extracts were dissolved in 5 ml. methylene chloride before being added to the columns.

For paper chromatographic use, the residue was treated with acetone as for column chromatography. The acetone was evaporated and the residue dissolved in 3 ml. methanol. One ml. of the methanol was applied to filter paper.

For TLC, the ethyl acetate residues were dissolved in 0.5 ml. methanol and 1/20th of this was applied to the thin-layer plates.

2. Methylene chloride extraction

Five ml. of urine was acidified and saturated with NaCl. The urine was extracted with 5 ml. of methylene chloride and the mixture centrifuged to obtain two layers. The upper aqueous phase was discarded and the organic layer added directly to the silica gel columns. Emulsions, which formed between the layers, were exceedingly difficult to remove in some instances and were also added to the columns. It is not believed that the emulsions added to the columns had an effect on the final results, other than slowing the flow of the eluting solvent.

For TLC, two successive methylene chloride extractions of 6 and 5 ml. were used. The first organic fraction was removed by passing a pipette through the upper aqueous layer and removing 5 ml. of the methylene chloride. After extracting with methylene chloride a second time and centrifuging, the aqueous layer was discarded and the methylene chloride pooled with the previous organic phase. The dried residues were treated in the same way as were those obtained with the ethyl acetate extraction.

The procedure for blanks and standards was the same for both types of extraction: water, urine, water containing 0.1, 0.2, and 0.3 mg. HVA, and urine to which these amounts were added were carried through the entire procedure.

3. Back extractions

Back extractions were carried out in an effort to improve the TLC results, by eliminating some of the compounds extracted along with HVA. The ethyl acetate extracts of urine were extracted into alkaline solutions (sodium carbonate or sodium tetraborate decahydrate). The alkaline aqueous phases were then acidified and re-extracted with ethyl acetate. Results did not differ from the single extraction procedure.

C. SEPARATION OF COMPOUNDS

Three methods of chromatography were tried: column, paper, and thin-layer.

1. Column chromatography

Goldstein separated HVA from dopac and VMA using silica gel columns (61). His procedure was followed in setting up our columns. Thirty grams of silica gel (Davison, Grade 922) was washed with successive 225 ml. portions of methanol, acetone, and benzene and dried in an oven. The washed silica gel was stored in a tightly covered glass jar.

a. Columns prepared with benzene

The columns were prepared by placing a small piece of absorbent cotton in the narrow end of a 27 mm. x 1 1/2 mm. glass tube. The cotton was covered with about 0.25 inches of fine-grained sea sand. At this stage the columns should be free flowing to allow quick passage of any liquid that is added. Two grams of silica gel, for each column, were weighed on aluminium foil. The glass columns were filled with benzene and the slow addition of silica gel was begun at once. The sides of the column had to be continually tapped as the silica was added, as any lumps or dry silica at the bottom of the column impedes the rate of flow of eluting solvents. More benzene is added as necessary. The addition of benzene and silica gel was continued until the required amount of silica was in the columns. The benzene used in this part of the process may be saved and used again for preparing other columns. The silica gel was discarded after each experiment. Columns must be used immediately after they are prepared or they will dry out and be useless. Eight columns were found to be the maximum that can be handled at one time.

The solution (5 or 10 ml.) containing the substances to be separated was added to the column and the liquid which runs through discarded. The column, containing the compounds, was washed with 10 ml. of a 1% methanol:benzene (V/V) solution and the eluate rejected. HVA was eluted with successive 5 ml. portions of 5% methanol:benzene (V/V). Usually 25-30 ml. of eluate was collected. After the last portion had passed through the column, elution with 20%, and then 50%, methanol:benzene (V/V) was begun. These fractions were submitted to a colour reaction or chromatographed.

Standards were made in 1% methanol:benzene and added directly to the column. Washing the column with 1% methanol:benzene did not remove the standards from the column. Known amounts of compounds in 5% methanol: benzene were compared colorimetrically with the eluates of test samples.

Initially HVA appeared to be eluted from the column in the second 5% methanol:benzene fraction. VMA was eluted with the 50% solution while it was uncertain just when the dopac was eluted. Colour reactions in the test tubes containing the eluates were the basis for identification of the eluted compounds. Colour reactions with HVA were often poor; VMA formed a pink colour. Under these conditions, it was extremely difficult to set up a standard curve by eluting known amounts of standards from the columns.

It was noted that complete elution of HVA did not occur in any single 5 ml. fraction. For instance, in one experiment seven different samples were added to seven different silica gel columns and eluates collected.

Five 5% methanol:benzene fractions and one 20% fraction were collected. Table IV (page 35) shows the degree of fluorescence (activation 290 mμ, fluorescence 325 mμ) in the various eluates from the columns.

In samples 1 and 7, where fluorescence should be due only to HVA, 32% and 40% of the fluorescence respectively was found in fractions other than the second 5% methanol:benzene fraction. Paper chromatography, in equal volumes of n-butanol:2% aqueous ammonia, of several of the fractions which showed fluorescence gave mixed results. No distinct separations of HVA from other compounds were obtained. High fluorescence in samples 1, 2, and 7 with added HVA was verified by distinct spots on the chromatograms, but other compounds were seen to be running together with the HVA in sample 2. On chromatographing several fractions showing fluorescence (e.g. fraction 3 of samples 2, 3, and 4; fraction 5 of sample 4), spots corresponding to HVA could not be detected by our method. It is assumed in fractions 3-5, samples 2-6, that the fluorescence is due mainly to many interfering substances that are collected on the column and eluted at this time. HVA may occur in these samples, but our chromatographic separations were poor and not distinct enough to identify HVA. Most of the fluorescence in the 20% methanol:benzene fraction (samples 2-6) is due to other substances extracted from urine, possibly including some residual HVA from the columns. There is also a possibility some of the fluorescence is due to VMA, even though previous experiments in this laboratory and Goldstein's results (61) show VMA not being eluted until 50% methanol: benzene washings were passed over the column. Spots on the chromatograms, corresponding in colour and R_f values to VMA, lead us to believe that some VMA is eluted in the 20% methanol:benzene fraction.

TABLE IV

Fluorescence of Eluates Collected from Silica Gel Columns

Sample Number	Amount Extracted ml.	Constituents of sample	Fluorescence* Fraction number. Collected in: 5% methanol:benzene					20%
			1	2	3	4	5	1
1.	10	0.1 mg. HVA in water	0	17	3	1	0	4
2.	10	0.1 mg. HVA in urine	5	16	11	19	60	60
3.	10	normal urine	0	5	14	10	24	49
4.	10	sample urine patient with neuroblastoma	0	13	27	62	77	51
5.	10	sample urine patient with neurofibroma	0	0	24	12	36	38
6.	10	sample urine	4	12	23	17	35	73
7.	10	0.05 mg. HVA in 5% methanol:benzene	29	50	5	0	0	0

* Arbitrary units. A 5% methanol:benzene blank, eluted from the columns, showed 10 units of fluorescence. This value has been subtracted from the above results. The blanks had a high, although uniform from fraction to fraction, fluorescence.

It was concluded, in the light of the above experiment and others, that if the above column separations were used, then paper chromatography would eventually be needed in addition to separate HVA satisfactorily from other compounds.

b. Columns prepared with methylene chloride

Columns were also prepared with methylene chloride. The residues of extracts dissolved in methylene chloride were added to the columns which were eluted with various proportions of methanol in methylene chloride (9).

The columns were prepared in the same way as the "benzene" columns except methylene chloride was substituted for benzene and only one gram of silica gel was used. In the final procedure, methylene chloride containing the dissolved compounds was added to the columns which were then washed with 3% methanol:methylene chloride (V/V). The columns were eluted with 4-10% methanol:methylene chloride (V/V) solutions. The best colour development occured in the 4% methanol:methylene chloride fraction. A small amount of colour was observed in the 5% fraction and a trace in the 6% fraction. On repeating the experiment, the best colour development occured in the 5% fraction. The eluates were chromatographed on paper. HVA, popac, and an unidentified compound appeared on the chromatogram corresponding to the 5% fraction.

In a further experiment, colour development was again best in the 5% fraction with some colour showing in the 4% fraction. HVA and popac were eluted in both fractions, the results being verified by paper chromatography.

An attempt was made to separate standard solutions of VMA and dopac using methylene chloride prepared columns and eluting with solutions of 25-50% methanol:methylene chloride (V/V). Paper chromatography of the eluates showed most VMA appearing in the 25% fraction, some in the 20% fraction, and a trace in the 30% fraction. All the HVA was eluted in the 20% fraction. No spots corresponding to dopac standards could be discerned on the chromatograms.

In another experiment, in which chromatography was used to substantiate the results, urines plus known amounts of HVA, popac, and VMA were extracted and the extracts added to columns. The columns were eluted with 3,4,5,25,30, and 35% methanol:methylene chloride solutions. HVA was eluted in the 4% fraction with popac appearing in the 4 and 5% fractions. The unknown compound, which corresponded in chromatographic mobility to phloretic acid, was found in the 4% fraction. VMA was eluted with 25% methanol:methylene chloride.

c. Other column chromatography experiments

Urine extracts and standard solutions of HVA were added to silica gel columns prepared with benzene and methylene chloride. The columns were eluted, and the eluates chromatographed on TLC plates. The results did not show any advantage over the direct application of the urine extracts to the plates. Recoveries were low and erratic. Best results (35% recovery of HVA) were obtained with ethyl acetate extractions added to columns prepared with methylene chloride, and eluted with 6 and 25% methanol:methylene chloride solutions.

Columns were prepared with Florisil (60/100 mesh, obtained from the Floridin Company, Tallahassee, Florida) (66). Urines were added directly to the columns and eluted with water without specific results. Ethyl acetate extractions added to Florisil columns also failed to give specific results.

Since one could not differentiate, during these experiments, between the elution of HVA, popac, and "phloretic acid" in different fractions without paper chromatography, it was decided that column chromatography was not advantageous for our use and the same results might be achieved with paper chromatography alone.

2. Paper chromatography

The results with column chromatography showed that other substances were being eluted from silica gel columns in the same fraction as HVA. Neither dopac nor VMA was among these compounds; they are eluted with higher concentrations of methanol. Goldstein states that HVA does not separate from an interfering substance that he thought may be popac (personal communication to Dr. Sourkes). We also found another compound, with the chromatographic characteristics of phloretic acid. It was eluted in the same fraction as HVA and popac. It became apparent that a separation between HVA and popac would have to be attained. Furthermore, if phloretic acid were to occur in mammalian metabolism, it also would have to be separated.

a. n-Butanol:ammonia solvent system

A solution of equal volumes of n-butanol and 2% aqueous ammonia (67) was the first solvent system tried in these experiments.

Whatman # 1 filter paper (46 cms. x 20 cms.) cut across the fibres was used. It was drawn through a saturated solution of H_3BO_4 (pH 4.8) and dried before use. The chromatographic chambers were equilibrated for three hours with the stationary phase. The mobile phase was placed in troughs in top of the chamber and descending chromatography developed for 16 hours. The papers were sprayed with Reagent 5 (See Table XI, page 60) followed by sodium carbonate. HVA gave a light purple colour and popac a light pink. The separation between popac (R_f 0.30)and HVA (R_f 0.27) was not distinct.

The experiment was repeated, using buffered and unbuffered Whatman # 3 paper. The standard compounds failed to move as far on the unbuffered paper (popac R_f 0.17, HVA R_f 0.14) and there was not a distinct separation. On buffered paper, the spots ran together. VMA ran half as far as HVA and gave a reddish-purple colour.

b. Butanol:propionic acid:water solvent system

These reagents were mixed in a proportion of 7:1:2. Whatman # 1 paper (50 cms. x 20 cms.) running in the machine direction of fibres was used. The running time was 25 hours. Popac and HVA did not separate from one another; VMA ran much slower.

c. Benzene:propionic acid:water solvent system

For the remainder of the paper chromatography experiments, Whatman # 1 paper, 51 cms. x 20 cms., cut in the machine direction was used. The solvent system was the organic phase of benzene:propionic acid:water (2:2:1) (7, 14, 45). The aqueous phase of this system was placed in the bottom of the chromatographic chambers for equilibration purposes.

Fresh solvent was prepared for each experiment. The average developing time was 6.25 hours. The papers were dried overnight and sprayed with Reagent 5 followed by sodium carbonate. Any deviations from the above method will be noted in the text.

Whatman # 1 and # 3 papers were developed as above. On Whatman # 1 papers, the R_f's were : VMA 0.18, popac 0.54, HVA 0.69. On Whatman # 3 papers, the R_f's were: VMA 0.16, popac 0.49, HVA 0.65. Spraying gave a bluish-purple spot for HVA, pink for popac, and reddish-purple for VMA.

Dopa barely moved from the starting line while dopamine had a trailing spot from the starting line to a position corresponding to an R_f of 0.10. Dopac (R_f 0.24) ran just behind VMA (R_f 0.27), but the two compounds could not be separated on the same chromatogram. Dopa, dopamine, and dopac all gave a purple colour on spraying.

Although pure compounds were separated rather well in this system, chromatography of urine extracts containing HVA and popac showed that these compounds run together, or that there is another compound between them. Urine also seemed to contribute a retarding factor, as the R_f of HVA was slightly lower in urine (R_f standard 0.68, R_f urine 0.65). There was also a difference in the colour between popac standards and what we believe to be endogenous urinary popac. This may be due to an unknown interfering compound.

From the silica gel column eluates ("methylene chloride preparation") similar results were obtained. The R_f of HVA was 0.71 and that of popac 0.57.

Generally the R_f's for the methylene chloride extractions were the same as those of the ethyl acetate extractions, or possibly slightly higher. From these eluates it appeared that there was a compound between HVA and popac with a colour that was slightly darker than that of the popac standards.

d. Two-dimensional chromatography

In an effort to resolve the problem of the unknown compound, we ran two-dimensional chromatograms through the courtesy of Dr. G.H.N. Towers of the Botany Department of McGill. The papers (Whatman # 1) were first run for six hours in a benzene:acetic acid:water (6:7:3) system. The papers were dried, turned in a 90° angle, and run for three hours in a 2% formic acid solution (68), in the second direction.

The chromatograms were sprayed with Reagent 5 and diazotized sulphanilic acid (See page 62). The separation between popac and HVA was not improved by two-dimensional chromatography over that achieved with benzene:propionic acid:water unidimensionally. The unknown compound found between HVA and popac was distinctly separated (Table V). Dr. Towers suggested that the compound may be phloretic acid or dihydroferulic acid.

TABLE V
R_f's of Compounds after Two-dimensional Chromatography

Compound	R_f's of Standards		R_f's in Urine	
	Benzene:acetic acid:water	2% formic acid	Benzene:acetic acid:water	2% formic acid
VMA	0.025	0.290	-	-
Popac	0.100	0.275	0.120	0.270
HVA	0.190	0.260	0.200	0.260
Unknown	-	-	0.160	0.275

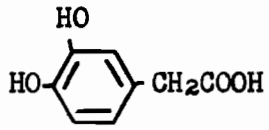
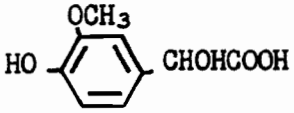
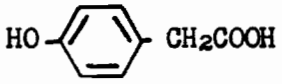
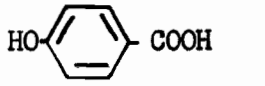
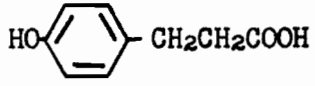
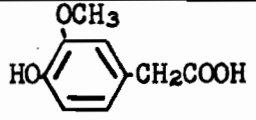
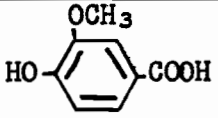
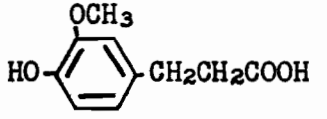
e. Chromatography of other compounds

Several compounds were chromatographed in benzene:propionic acid:water (2:2:1) in an effort to identify the unknown compounds. The results of one experiment are set out in Table VI, page 43.

Over the series of experiments, the average R_f's obtained are shown in Table VII, page 44, where they are compared with those of Armstrong et al. (7).

TABLE VI

Characteristics of Certain Phenolic Compounds

Name	Structure	R_f	Colour	Remarks
Dopac		0.27	Brownish	
VMA		0.28	Reddish-purple	Runs with dopac
Popac		0.57	Pink	
p-hydroxybenzoic acid		0.63	Orange	
Phloretic acid		0.65	Purplish-pink	Between HVA and popac, trails into both
HVA		0.70	Bluish-purple	
Vanillic acid		0.72	Reddish-purple not as dark as VMA	Runs above HVA
Dihydroferulic acid		0.76	Bluish-purple same as HVA	Trails into HVA and vanillic acid

In experiments with pure solutions, vanillic and dihydroferulic acids run almost together, but the colour of vanillic acid stands out. Phloretic acid is difficult to distinguish, but its presence alters the colour of popac.

TABLE VII

Comparison of Paper Chromatographic R_f Values of Phenolic Acids

Compound	Number of determinations	Average R_f	R_f values of Armstrong *et al.*
VMA	20	0.24	0.16
Dopac	14	0.27	-
Popac	47	0.56	0.49
p-hydroxybenzoic acid	5	0.65	0.55
Phloretic acid	7	0.66	0.59
HVA	65	0.70	0.68
Vanillic acid	15	0.73	0.78
Dihydroferulic acid	5	0.78	0.74

f. Structure and R_f relationships

Vanillic acid series. Certain relationships can be drawn between the structures and R_f values of the compounds in Tables VI and VII. Vanillic acid, HVA, and dihydroferulic acid have the same ring structure with increasing length of side chain. Adding a CH_2 group (HVA) lowers the R_f while adding two CH_2 groups (dihydroferulic acid) increases the R_f. In another comparison, between HVA and VMA, if we replace an H on the β-carbon of HVA with an OH, we will have VMA with a lower R_f than HVA.

p-Hydroxybenzoic acid series. In another homologous series, p-hydroxybenzoic acid, popac, and phloretic acid, similar results can be shown. All have the phenolic ring with increasing length of side chain. Adding a CH_2 group (popac) lowers the R_f, adding two CH_2 groups (phloretic acid) increases the R_f. The decreases and increases in the R_f values in this group are almost of the same order as those in the vanillic acid series.

Effect of m-methoxy groups. Adding a m-methoxy group to p-hydroxybenzoic acid, popac, and phloretic acid will give vanillic acid, HVA, and dihydroferulic acid respectively. As expected this decreases the polarity of the latter group and the compounds of this group have higher R_f values.

Effect of ring-OH groups. If an OH is added to the phenol ring of popac, dopac results with a lower R_f. Similarly if an OH is added to the ring of phenylpyruvic acid to give p-hydroxyphenylpyruvic acid, the latter compound will have the lower R_f value (See Table X, page 57).

It is interesting to note that the R_f of dopac is about half that of popac while the R_f of p-hydroxyphenylpyruvic acid is also half that of phenylpyruvic acid.

Methylation of catechols. Adding a m-methyl group to dopac lowers the polarity and gives HVA with a higher R_f. Similarly, adding methyl groups to the ring structures of 3,4-dihydroxymandelic and caffeic acids gives VMA and ferulic acid, both of which have higher R_f values than the compounds from which they were formed (See Table X, page 57).

The neutral or basic nature of dopa and dopamine probably results in the failure of these compounds to move from the starting line (See Table X, page 57).

The above discussion shows the relationship between structure and R_f. Adding a CH_2 group to a carboxyl side chain on a phenol ring decreases the R_f of the compound thus formed. Adding two CH_2 groups tends to increase the R_f of the compound formed. Adding a m-methoxy or m-methyl group to the ring structure lowers the polarity of the molecule and increases the R_f of the homologue. Adding an OH to the benzene ring increases the polarity of the molecule and it does not move as far. These results are valid for the compounds tested in a benzene:propionic acid: water (2:2:1) solvent system on paper chromatography and for a benzene: acetic acid:water (2:3:1) solvent system with TLC. These relationships of structure and chromatographic mobility agree with expectations for variations in polarity of the series discussed.

g. Benzene:acetic acid:water solvent system

These solvents were used in the proportions of 6:7:3 . The lower aqueous phase was used to equilibrate the chambers and the upper organic phase to develop the chromatograms in a descending direction. The developing time was five hours. The solvent front was allowed to run off the chromatogram (a distance of 44 cms.) for better separations in certain experiments.

TABLE VIII

R_f's of Phenolic Compounds Using Benzene:acetic acid:water (6:7:3)

Compound	Number of Cms. compound moved from starting line	R_f
Dopac	3.5	-
VMA	3.5	0.05
Popac	11	0.23
p-Hydroxybenzoic acid	13	0.26
Phloretic acid	17	0.35
HVA	21	0.50
Vanillic acid	25	-

A separation satisfactory for qualitative identification could be obtained with paper chromatography, but the separations were not satisfactory for quantitative work.

h. Elution of homovanillic acid from chromatograms

Elution of the spots containg HVA proved to be a difficult task. Four different eluants were tried: sodium carbonate, sodium bicarbonate, water, and ammonia in water. Fluorescence was determined at 280/315 mμ and absorbance of coloured solutions (Reagent 5 and sodium carbonate) at 530 mμ.

Standard curves using pure solutions were obtained with sodium bicarbonate and aqueous ammonia in the spectrophotofluorometer. Only water and pure solutions gave a satisfactory curve on the spectrophotometer.

Test solutions of unsprayed spots gave poor fluorometric and colorimetric results. Water eluates gave the best results, but less than 50% HVA was recovered. No logical results were obtained if the chromatograms were sprayed with the colour reagent first and the spots then eluted in any of the four solvents mentioned above.

3. Thin-layer chromatography

The method of thin-layer chromatography (TLC) is a relatively new advance in chemical technology (69). Since our results with paper chromatography left much to be desired, we turned our attention to TLC. Various solvent systems were tested, the final choice resting upon the ability of the system to separate HVA from closely related urinary constituents that would interfere in the ultimate estimation.

a. Procedure for preparing thin-layer chromatography plates

Glass plates, 20 cms. x 20 cms., are rinsed with distilled water, swabbed dry, then rinsed and swabbed with acetone. One volume of Silica Gel G (Merck, Darmstadt) is mixed with two volumes of water until a slightly viscous slurry is obtained. The slurry is poured into a DeSaga applicator (Canadian Laboratory Supplies, Montreal) and with a quick, easy motion moved over the plates to coat them with a layer of silica gel 0.25 mm. thick. This operation should be performed quickly so as not to allow the silica gel to harden before it is spread over the glass plates.

Plates matched in thickness should be used to avoid having ridges formed when the applicator moves from one plate to another. Seven plates can be made at once using 30 grams silica gel and 60 ml. water. The plates are air dried and then placed in an oven for 90 minutes for final drying. It is best to use the plates as soon as possible after preparing them because plates tend to absorb moisture from the atmosphere. Used plates should be washed carefully with soap and rinsed with distilled water immediately after use.

Solutions are applied to the silica gel with a micropipette, about 1.5 cms. from the base of the plate and the spots are dried with a stream of hot air. Twenty spots can safely be put on one plate at a time, if small amounts of standard solutions are used (five µg. dissolved in five µl.). Standard solutions of HVA and other compounds are prepared fresh weekly. For quantitative work, fewer spots must be applied to the plates. For urine extracts, nine to eleven samples can be spotted (25 µl. per spot) on the plates without danger of overlapping samples laterally. Some positions on the plates should be reserved for 1) similar extracts prepared after adding 100, 200, and 300 µg. of HVA, respectively, to 5 ml. of urine; and 2) methanolic solutions of HVA (5, 10, 15, and 20 µl. containing one µg./µl. HVA) that serve for estimation of recovery and for construction of a calibration curve.

For best results the material to be spotted should be dissolved in organic solvents. Methanol was used for our experiments. Added water tends to spread on the plates and the desired small size (0.5 cms.) of the spot cannot be obtained. Furthermore water spots tend to spread thus causing an overlapping of samples, or limiting the number of samples that can be applied to a plate.

The plates are run in the direction of application of the silica gel, i.e. spots are usually put on the leading edge of the plate. The plates were placed in battery jars containing about 100 ml. of the solvent system. The chambers were allowed to equilibrate and then two or three plates were placed in each jar.

In early experiments, the walls were lined with solvent-saturated filter paper. However, if solvent was always kept in the jars, using fresh solvent every time plates are to be developed, and discarding the old solvent, the atmosphere within the jars was always saturated with vapour. Filter paper was then not needed. Care must be taken in removing and adding the solvent so as not to disturb the conditions inside the chamber.

The plates were run until the solvent had risen to within an inch of the top of the plate. This involves a longer developing time (80-120 minutes) than is recommended in most literature, but for quantitative work we find it gives a better separation. Faster movement is observed with higher ambient room temperatures. There is usually a dip in the solvent front, i.e. at the edges of the plate the solvent is about half a centimeter higher than the center of the plate. This dip is not always regular. It appears that the dip cannot be compensated for (due to its non-regularity), and thus R_f values from experiment to experiment, and even from plate to plate, are not reliable. The plates are dried in a current of air in a fume-hood for 30 minutes and then sprayed with a colour reagent. It is advisable to spray the plates first in order to detect HVA rather than guess, or estimate, at its position on the plates.

The spots can be scraped off the plates with a straightedged plastic ruler into small test tubes (13 mm. x 100 mm.). The silica gel is eluted with water in the tubes and the Folin-Ciocalteu colour reaction run. The absorbance of the solutions at 750 mμ is determined in the Beckman DU Spectrophotometer.

b. Miscellaneous solvent systems

In order to assure the separations of HVA from known and some potential interfering constituents of the urine, a solvent system was sought that would separate HVA from popac, and from phloretic, vanillic, and dihydroferulic acids. HVA did not migrate with chloroform, methylene chloride, benzene, benzene containing 5% or 20% methanol, or benzene: formic acid:water (2:2:1) as solvent systems. There was movement of HVA, but poor separation of it from other compounds in n-butanol:acetic acid: water (4:1:1), isopropanol:ammonia:water (8:1:1), and n-butanol: acetic acid:water (6:1:2). The developing time for the last four solvent systems mentioned was 83, 221, 222, and 100 minutes respectively

Various proportions of benzene:propionic acid: water (upper organic phase) were tried, but they did not give adequate separations. The proportions 2:3:1 gave a fairly good separation in 94 minutes, but the compounds didn't run as far nor as fast and the separation was not as good as that obtained with the solvent system finally chosen. Rf values with pure solutions used in this solvent system were: VMA 0.24; popac 0.72; p-hydroxybenzoic acid 0.79; phloretic acid 0.79; HVA 0.83; dihydroferulic acid 0.86; and vanillic acid 0.90. The proportions 10:9:1 as a solvent system produced poor separation between HVA and popac in 115 minutes.

A fair separation was obtained with chloroform:acetic acid:water (2:2:1) run for 125 minutes. R_f values obtained with pure solutions were: VMA 0.18; popac 0.65; phloretic acid 0.68; p-hydroxybenzoic acid 0.76; HVA 0.79; vanillic acid 0.86; and dihydroferulic acid 0.89.

c. Benzene:acetic acid:water solvent systems

Good separations of the compounds were achieved with the upper phase of various mixtures of benzene:acetic acid:water, and the ratio of these liquids 2:3:1 was finally selected. The average developing time was about 90 minutes.

Any change in the proportion of acetic acid decreased the efficiency of the separations. The proportions 6:8:3 gave good separations as did the proportions 6:7:3, but they were not as distinct as the separation with 2:3:1. A good separation was obtained with the proportions 6:10:3, but the spots were closer together than they were with the other solvent systems.

Table IX lists the R_f values of various compounds run in the benzene: acetic acid:water (2:3:1) solvent system on several occasions (See page 53).

From Table IX we can see that the mobility of the compounds, after their addition to urine and their extraction therefrom, was slightly reduced. It can also be seen that the specific R_f values on the plates are not consistant from day to day and, in fact, can vary quite widely. In an effort to reduce the developing time, a set of plates was run in benzene: acetic acid:water (2:3:1) for 45 minutes. The solvent front was 11 cms. from the starting line (compared with 16-18 cms. for full developing time of 90 minutes). The dip in the solvent front was 0.5 cms. at the center of the plate. The mobility of HVA at the center of the plate was 8 cms.

TABLE IX

R_f Values of Phenolic Compounds With
Benzene:acetic acid:water (2:3:1) Solvent System

	July 24	July 27		September 24		Dec. 21
Solvent front distance, cms.	15	15		16		17
Compound	R_f2*	R_f1**	R_f2	R_f1	R_f2	R_f1
VMA	0.10	0.10	0.07	0.07	0.08	0.11
Dopac	-	-	-	-	-	0.29
Popac	0.53	0.40	0.37	0.44	0.41	0.56
p-Hydroxybenzoic acid	-	0.43	-	-	-	0.61
Phloretic acid	0.57	0.50	0.43+	0.54	0.50+	0.62
HVA	0.66	0.60	0.57	0.64	0.59	0.67
Vanillic acid	-	0.66	-	0.72	-	0.69
Dihydroferulic acid	-	0.63	-	0.78	-	0.72

* R_f1, R_f value for the pure solution.
** R_f2, R_f value for the compound extracted from urine.

For July 24th and 27th, and September 24th, HVA, popac, and VMA were dissolved in methanol; phloretic acid was dissolved in ethanol; vanillic, dihydroferulic, and p-hydroxybenzoic acids were dissolved in water.

For December 21st, all of the compounds were dissolved in methanol, except phloretic acid which was dissolved in ethanol.

For July 24th and 27th, urine extracts (ethyl acetate) were applied directly to the TLC plates.
For September 4th, the ethyl acetate residue of the urine extract was dissolved in methanol and an aliquot of the methanol was applied to the TLC plates.

+ This urinary compound has not been shown to be identical with phloretic acid.

At the edges of the plate, the mobility of the HVA was 8.5 and 9 cms. The R_f of HVA was 0.73, higher than for full development of the plates. A good qualitative separation of HVA from other compounds was achieved, but the separation was not good enough for quantitative studies.

d. Spraying of plates

In order to detect HVA, the plates were sprayed with several reagents. (The colour reactions and plate sprays are described in more detail on pages 58 - 63). The first spray tried was Reagent 5 followed by sodium carbonate. HVA gave a purple colour, popac and phloretic acid gave yellow. These colours were not stable and faded quickly. Spraying with Reagent 1 produced clearer spots, but the colour was not as deep as that with Reagent 5 and again the spots faded. Diazotized sulphanilic acid produced good colour development, but after a while the spots faded slightly. Bright colours could be regenerated by placing the plates in jars saturated with ammonia vapour. The Folin-Ciocalteu phenol reagent followed by 10% sodium carbonate produced an easily distinguishable blue spot for HVA that was sharply defined and did not fade. At this stage, the plate can be photocopied in the same manner as a printed page or photograph (Apeco, Model No. 125, American Photocopy Equipment Co., Chicago).

It is advisable not to allow the plates to stand overnight before spraying. The plates may react with atmospheric components and give a misleading colour reaction. Should the spots develop spontaneously in the atmosphere, quantitative determinations cannot be done.

e. Elution of homovanillic acid from silica gel and colour reaction

The HVA-containing spots were isolated from other compounds by outlining them carefully with a wooden pick; and these areas were scraped from the plate, using a flexible plastic straightedged ruler, into a 13 mm. x 100 mm. test tube. The first attempts to elute the HVA from the silica gel were made with methanol. Both sprayed (with phenol reagent) and unsprayed spots were eluted in methanol and the colour reaction performed. The colour was virtually the same in both instances, but when sodium carbonate was added to complete the reaction, a large amount of precipitate formed with a great deal of the colour product remaining in it.

Water was found to be excellent for eluting HVA from silica gel. The silica gel was scraped off the plate and three ml. of water added to the test tube. The tubes were allowed to stand overnight, during which time the HVA was eluted from the powder. In the morning, the tubes were agitated briefly and then centrifuged for a few minutes to precipitate the silica gel. The Folin-Ciocalteu colour reaction was carried out and the absorbtion of the solutions at 750 mμ recorded.

f. Ultraviolet irradiation of homovanillic acid on thin-layer chromatography plates

HVA, in methanol, was applied to the plate and irradiated with ultraviolet light for 25 minutes. HVA was then spotted at the other end of the plate (not irradiated) and the plate was developed in benzene: acetic acid: water (2:3:1). The plate was sprayed with 1 N phenol reagent followed by 10% sodium carbonate. The following spots were visible.

Plate treatment	Spot	Cms. from starting line
Untreated	HVA	10.5 - 11
Irradiated	HVA	11.5
Irradiated	very faint spot	6
Irradiated	faint spot	4
Irradiated	Circle where spot applied visible	0

Shepherd and West (70) have shown that noradrenaline can be formed by the irradiation of p-norsynephrine (octopamine; p-hydroxyphenyl-ethanolamine) and m-norsynephrine with ultraviolet radiation. By analogy the results with the irradiation of HVA may represent oxidation of the ring to a dihydroxy-monomethoxy compound. Oxidation of the ring to give other hydroxyl groups may thus occur during the extraction of urine, causing the breakdown of certain compounds, the formation of other compounds, and possibly affecting the amounts of substances being extracted. As a precaution, the plates should be protected from direct sunlight during the procedure. By analogy with West, if we are getting hydroxylation of the ring, the hydroxyl group could go onto any of three positions. If the hydroxyl went onto the 5 position, we would obtain a compound similar to that of one of the metabolites of mescaline (71).

g. R_f values and colour reactions with several compounds

Several compounds were applied to plates which were developed in benzene:acetic acid:water (2:3:1). Experiments 2 and 3 were run on a different day than experiment 1. R_f values and colours are recorded in Table X, page 57.

TABLE X

R_f Values and Colours of Several Compounds on TLC

Experiment	1	2	3	1	2	3
Compounds	R_f*	R_f*	R_f*	Spray Reagents Folin-Ciocalteu Phenol Reagent**	Diazotized Sulphanilic Acid	Ethylenediamine
Dopa	-	0	0	Blue	Light brown	Light brown
Dopamine	-	0	0	Blue	Brownish	Purple
3,4-Dihydroxymandelic acid	0	0.01	0.01	Blue, brownish center	Brown	Purplish-red
VMA	-	0.12	0.10	Blue	Reddish-orange	Orange
Homogentisic acid	0.16	0.22	0.22	Blue	Light pink	Pink
Dopac	-	0.29	0.29	Blue	Reddish-brown	Reddish-brown
p-Hydroxyphenylpyruvic acid	0.23	0.35	0.33	Blue	Yellow	Yellow
Caffeic acid	0.31	0.43	0.42	Blue, brownish center	Brown	Brown
Phenylpyruvic acid	0.48	-	-	Light blue	-	-
Popac	-	0.57	0.55	Blue	Light yellow	Light orange
p-Hydroxybenzoic acid	-	0.62	0.61	Blue	Light yellow	Yellow
Phloretic acid	-	0.62	0.62	Blue	Yellow	Orange
HVA	-	0.68	0.67	Blue	Brownish-purple	Purple
Vanillic acid	0.69	0.69	0.68	Blue	Light orange	Reddish-orange
Dihydroferulic acid	-	0.72	0.72	Blue	Orange-brown	Reddish-purple
Ferulic acid	0.75	0.74	0.73	Dark blue	Light orange	Lt. reddish-orange

All of the solutions were made in methanol except phloretic acid which was dissolved in ethanol.
* Solvent fronts: Experiment 1, 16 cms.; Experiment 2, 17 cms.; Experiment 3, 16.75 cms.
** Colours recorded for compounds without R_f values in Experiment 1 are the colours observed over a series of experiments.

h. Conclusions

Of the three types of chromatography tried (column, paper, and thin-layer) thin-layer chromatography gave the most useful separation of HVA from other urinary compounds for quantitative studies. The TLC method enjoys all the facilities we were searching for - it is rapid, it can be used routinely, and it is relatively inexpensive. A complete determination - twenty samples (including standards) can be extracted, chromatographed in duplicate (five TLC plates), sprayed, eluted, the colour reaction developed and the absorption at 750 mμ determined, and the calculations performed in less than ten hours. Using an assembly-line type of technique, we have done 80 samples in a five-day working week. One drawback is that specific R_f values on the plates are not as consistent from day to day as those observed with paper chromatograms. However, this is compensated for by the advantage that the spots are more compact and do not spread as on paper.

D. COLOUR REACTIONS

Three different sets of reactions were used during the course of this study. (1) Stable diazo salts were used with eluates from column chromatography and as a spray for paper chromatography. They were not suitable for use with TLC. (2) Freshly prepared diazotized sulphanilic acid was used as a spray for TLC. (3) Folin's phenol reaction was utilized as a TLC spray and as the colour reaction to measure HVA, the absorbance at 750 mμ in the spectrophotometer being related to the amount of HVA present in the urine.

1. Stable diazo salts

The stable diazo salts (64) were a gift from Chemical Developments of Canada, Irwin Dyestuff Division, Montreal. The six salts that were used in this study are described in Table XI, page 60. The coloured solutions themselves were not stable for any length of time and fresh solutions were prepared for each use.

The reaction (with solutions) was carried out with the compound to be measured, 0.1 ml. of a 0.05% solution of the colour reaction (this was increased to 0.08% in later experiments for stronger colour development), and 0.5 ml. of 20% sodium carbonate. For colour development on chromatograms, a 0.1% solution of the colour reagent was sprayed on the chromatograms followed by a 10% sodium carbonate solution. Several trials showed that the best colour development in test tubes and on the chromatograms was given with Reagent 5. The order of decreasing effectiveness of the other reagents was 1, 2, 3, 6, and 4.

The colours produced by reaction of the diazotized amines with various compounds are listed in Table XII, page 61.

On TLC plates, Reagent 1 gave the best colour development, although Reagent 5 also produced good colour. The colours formed on the silica gel with these reagents were not stable and faded quickly. They could not be used reliably for quantitative determinations, but they were useful for qualitative identification.

TABLE XI

Stable Fast Diazo Salts

Reagent Number*	Commercial name	Stabilized diazo salt of:	Colour of fresh solution	Colour of solution after standing 60 hours
1.	Red Salt GG	p-nitroaniline	Colourless	Yellow
2.	Red Salt RL	2-amino-3-nitrotoluene	Faint yellow	Yellow
3.	Yellow Salt GC	o-chloroaniline	Colourless	Dark reddish-orange
4.	Black Salt K	4-amino-2,5-dimethoxy-4'-nitroazobenzene	Reddish-orange	Dark orange
5.	Bordeaux Salt BD	4-amino-2,5-dimethoxy-benzonitrile	Bright yellow	Light yellow
6.	Corinth Salt V	4-amino-2,4'-dimethyl-5-methoxy-2'-nitroazo-benzene	Orange	Dark orange

* The compounds will be referred to in the thesis by these numbers.

TABLE XII

Colours of Diazotized Compounds

Compounds	Reagent Number					
	1.	2.	3.	4.	5.	6.
HVA*	lt.+ purple	beige	lt. orange	gray-purple	lt. purple	brown
VMA*	purple	purple	orange	gray	rose	lt. brown
HVA**	dk.++ purple	brown	red	purple	purple	brown
VMA**	purple	yellow	yellow	purple	red	brown
HVA***	greenish-blue	greenish-blue	lt. pink	pale blue	lt. purple	blue-green
VMA***	purple	purple	orange	blue	reddish-purple	blue
Popac***	lt. purple	lt. pink-purple	lt. orange	faint blue	lt. pink	purple
Vanillic acid***	purple	purple	orange	pale blue	purple-red	blue
p-hydroxy-benzoic acid***	pink	purple	-	purple	lt. orange	brownish-orange
Dopac***	dk. purple	lt. brownish	lt. brown-orange	lt. gray-brown	lt. brown-purple	lt. brown-orange

* Compounds spotted on filter paper, but not developed in a solvent system.
** Reaction carried on with compounds in spot plate.
***Compounds spotted on chromatography paper, developed in benzene:propionic acid:water (2:2:1) solvent system.
\+ Light
++ Dark

2. Diazotized sulphanilic acid

Diazotized sulphanilic acid was prepared by dissolving 4.5 grams of sulphanilic acid in 45 ml. of 12 N HCl, with warming, and the solution was diluted to 500 ml. with water (65). Equal volumes of this solution and 4.5% sodium nitrite solution were mixed in an ice bath and this mixture was added to an equal amount of 10% sodium carbonate. The resulting solution was sprayed on TLC plates. This reagent gave very good colour development with TLC plates.

However, the colour reaction in the test tubes was not suitable to merit continued use for quantitative measurements. Areas of silica gel containing HVA were sprayed with diazotized sulphanilic acid and then eluted with water. The eluates were reacted with 1) diazotized sulphanilic acid again and 2) Folin's phenol reagent. Colour development was erratic and a logical sequence of results could not be obtained. For colour development of various compounds with diazotized sulphanilic acid sprays, see Table X, page 57.

3. Folin-Ciocalteu phenol reagent

The Folin phenol reagent (62, 63) proved to be the best reagent for developing the colour reaction in test tubes. The colour reaction was carried out by adding 0.25 ml. of 1 N phenol reagent and 1 ml. of 20% sodium carbonate to the solution containing HVA, heating in a water bath at 95° for one minute, and finally centrifuging to remove a slight precipitate that forms. The absorbance of the blue solutions is measured in the Beckman DU Spectrophotometer at 750 mμ. The colour is stable for at least 90 minutes.

Plates were sprayed with a 1 N solution of the phenol reagent followed by a 10% solution of sodium carbonate. The colours appeared to be quite stable. If these colours are eluted, they are not dense enough for the quantitative determination of HVA. This is probably because only a small portion of the HVA on the silica gel reacts with the spray. The combination of spraying the plates with the phenol reagent and then carrying out the Folin reaction with the eluates proved to be the best combination of the several tried.

4. Nitrosonapthol

The nitrosonapthol colour reagent (72) gave a purple colour with HVA and a good calibration curve with small amounts (less than 15 µg.) of HVA. However, the colour was much less intense than that achieved with the Folin reaction and similar concentrations of HVA (e.g. 15 µg. of HVA under certain conditions had an optical density of 0.275 with the Folin's reagent for phenols and 0.058 with the nitrosonapthol reagent).

The reaction was carried out by adding 0.5 ml. of the 1-nitroso-2-napthol reagent (0.1% 1-nitroso-2-napthol in 95% ethyl alcohol) and 0.5 ml. freshly prepared nitrous acid reagent (0.2 ml. 2.5% sodium nitrite in 5 ml. 2 N H_2SO_4) to a test tube containing the substance to be measured. The components were mixed and allowed to stand for ten minutes at room temperature. Five ml. of ethyl acetate was then added and the mixture shaken. The mixture separated into two phases, with a purple colour in the bottom layer indicating the presence of HVA.

E. FLUORESCENCE

Pure solutions of HVA (pH 5.9) fluoresced maximally at 315 mμ with maximal activation at 270 mμ using the Aminco Bowman Spectrophotofluorometer uncorrected for instrument variation. VMA exhibits greater fluorescence at the same wave lengths. We were able to obtain an excellent calibration curve for 5-20 μg. of HVA in water with the instrument set at maximum sensitivity. Greater amounts of HVA proved to be beyond the range of the instrument. The curves were quite erratic if the HVA was dissolved in 5% methanol:benzene. HVA also fluoresced well in 5% sodium carbonate (pH 8.1). Curves with VMA, in water, were not as reliable as those obtained with HVA, especially with more than 15 μg. of VMA.

After eluting compounds from silica gel not sprayed with a colour reagent, it was difficult to obtain a fluorescent (activation 280 mμ, fluorescence 315 mμ) calibration curve, even after centrifuging the eluate. It is assumed that minute amounts of silica gel remained in suspension thus causing the erratic results.

F. REFERENCE COMPOUNDS

The following compounds were purchased: HVA, popac, dopac, dopamine, ferulic acid, caffeic acid, and 3,4-dihydroxymandelic acid, from California Corporation for Biochemical Research; homogentisic acid and dopa from Mann Research Laboratories; VMA from Chemicals Procurement Laboratories Inc.; and p-hydroxyphenylpyruvic acid from Nutritional Biochemicals Corporation. Dr. G. H. N. Towers kindly gave us samples of phloretic, vanillic, dihydroferulic, and p-hydroxybenzoic acids.

III. RESULTS

A. INTRODUCTION

The following section contains results for most of the determinations of urinary HVA during this study. It can be seen that there is good agreement between our results for normal urine and the results obtained by other authors (See Table III, page 26).

The recovery of HVA from urine was determined on every occasion by comparing the colorimetric value obtained when a given amount of HVA was carried through the entire procedure with the value obtained when the same amount of the compound was directly applied, without extraction and concentration, to the silica gel. Recoveries consistently ranged between 85 and 95%. In stating values for urines, such losses were always accounted for.

Urine was collected from 18 healthy men and women ranging in age from 18 to 67 years, from six healthy children 1 - 12 years, and from patients with various diseases as described in this section. The urine was collected in bottles containing 10 ml. of 6 N HCl. No dietary restrictions were instituted. In most cases the total urine volume was collected over a 24 hour period. Where this is not so, the time for the total volume to be collected is stated.

In the tables throughout this section, the amount of HVA is measured in mg. per total volume of urine and mg. per gram of creatinine. I should like to thank Mrs. H. Berger of the Allan Memorial Institute's clinical laboratory for performing the urinary creatinine determinations.

The urine reference numbers refer to the numbers assigned consecutively to different urines as they are received in Dr. Sourkes' laboratory for measurements of catecholamines, HVA, 5-HIAA, and other compounds. The case histories are recorded in Dr. Sourkes' laboratory.

Adrenaline, noradrenaline, dopac, and dopamine were determined in most of these urines in Dr. Sourkes' laboratory before studies on HVA were started. The HVA values represent further study of these samples, after they had been stored in the frozen state. During the discussion of the results, frequent references are made to data obtained by other personnel in the laboratory.

B. EXCRETION OF HOMOVANILLIC ACID BY HEALTHY PERSONS

The normal adult and children's urines were collected from staff members of the Allan Memorial Institute and their families.

1. Adults

The excretion of HVA in adults was 8.23 ± 2.96 mg./24 hours (mean ± Standard Deviation), or 6.42 ± 2.28 mg./gm. creatinine.

Table XIII gives a comparison of 18 urine samples extracted with ethyl acetate and methylene chloride. The mean values found were (mean ± Standard Error): 9.00 ± 0.20 and 9.20 ± 0.29 respectively. The similarity of results indicates that either solvent can be used. The procedure followed to determine the HVA in these urines was that of TLC followed by the Folin-Ciocalteu colour reaction, performed on eluted material. Other urine values (noted in the tables) were obtained in earlier experiments by TLC followed by a diazotized sulphanilic acid spray and the Folin-Ciocalteu colour reaction. Some of the values may be over-estimated for reasons outlined on page 90.

TABLE XIII

Excretion of Homovanillic Acid in Urines of Normal Adults
Comparison of Ethyl Acetate and Methylene Chloride Extractions

Number*	Ethyl Acetate			Methylene Chloride	
	Folin's Reagent		Diazot'n.**	Folin's Reagent	
	mg.	mg./gm. creatinine	mg.	mg.	mg./gm. creatinine
Males					
7669	12.91	6.78	11.24	13.17	7.02
7676	13.61	7.24	27.30	14.30	7.80
7677	5.56	4.75	9.78	5.75	5.00
7679	9.55	6.36	9.79	9.44	6.79
7680	8.29	5.69	10.32	8.61	6.06
7681	7.55	7.67	8.26	5.90	5.83
7682	9.07	8.09	10.20	8.50	7.35
7685	5.64	3.94	-	5.67	3.91
7687	8.50	5.26	-	9.18	6.11
7689	5.39	3.22	-	5.94	3.65
7641	5.40	4.85	15.60	-	-
Females					
7673	10.58	8.82	6.90	10.58	8.82
7675	13.38	11.33	23.70	14.65	13.41
7678	9.07	6.83	13.44	8.07	6.00
7683	5.64	6.17	13.80	5.25	5.33
7684	8.68	9.70	-	7.42	8.23
7640	4.70	4.99	19.91	-	-
7643	4.56	3.83	13.68	-	-

The values with ethyl acetate are means of 3-5 determinations.
The values with methylene chloride are means of two determinations.
* 24-hour urine collections
** With diazotized sulphanilic acid (single determination).

2. Children

In six healthy children, the excretion was 6.60 mg./24 hours, or 14.60 mg./gm. creatinine. It may be noted that creatinine output is relatively low in children. The creatinine values are included in Table XIV.

TABLE XIV

Excretion of Homovanillic Acid in Urines of Normal Children

Number	Sex	Age (years)	mg.	mg./gm. creatinine	grams creatinine	Remarks
7592	M	1	1.35	24.54	0.055	24 hour collection (incomplete)
7601	M	7	4.25	8.64	0.492	24 hours
7600	F	8.5	8.16	13.37	0.606	24 hours
7594	F	9	4.77	18.35	0.260	12 hours
7599	M	11	8.30	7.35	1.130	24 hours
7589	F	12	8.70	13.60	0.640	24 hours

C. EXCRETION OF HOMOVANILLIC ACID IN DISEASE STATES

The excretion of HVA in the urine of patients with various diseases was also studied. The results in three diseases of the basal ganglia: Huntington's chorea, Wilson's disease, and Parkinson's disease, and in four diseases with accessory catechol-producing tissue: neuroblastoma, ganglioneuroma, pheochromocytoma, and malignant melanoma are presented in the following section. The urines of children with hyperkinetic syndrome were also studied to see if this condition led to the abnormal excretion of HVA.

1. Diseases of the basal ganglia

These diseases are of special interest because the basal ganglia of the brain contain high concentrations of dopamine (1-8 µg./ gm. wet weight) (73). A disease affecting these organs might also be reflected in the metabolism of dopamine.

a. Wilson's disease (hepatolenticular degeneration)

Patient A was a patient at the Royal Victoria Hospital in Montreal where urines were obtained and made available for this work. The urines had been preserved in the frozen state for nearly three years, at which time they were thawed and the HVA determinations performed.

Patient B has been on penicillamine therapy for several years, attended by outstanding clinical improvement; this patient works once again professionally.

Cases C, D, E, and F are under the care of Dr. Anne C. Carter, Kings County Hospital, Brooklyn, N.Y., and have been investigated in regard to their copper metabolism by Dr. I. Sternlieb, Albert Einstein College of Medicine, New York 61. Patient C has clinical symptoms of Wilson's disease, but patients D, E, and F (siblings, and children of a patient with the disease) show only disorder of copper metabolism.

TABLE XV

Excretion of Homovanillic Acid in Urines of Patients with Wilson's Disease

Patient	Number	Sex	Age	mg.	mg./gm. creatinine	Creatinine gms.
A	4886	F	48	16.10	-	-
A	4891	F	48	20.00	-	-
B*	7603	M	47	5.04	3.36	1.499
B*	7604	M	47	6.00	4.07	1.476
C	7757	M	16	10.70	13.43	0.797
D	7758	M	8	4.50	10.51	0.428
E	8001	F	10	4.69	16.01	0.293
F	8002	F	6	3.31	11.91	0.278

* Treated with penicillamine.

The values obtained for patient A are well above the normal range. Because these urines have been kept for a long time, it is uncertain whether these are true values. There is a possibility of hydrolysis occuring in the urines and the repeated freezing and thawing of the urines may also have had an effect.

The output of HVA by patient B was in the normal range.

High values are also observed in patient C while D, E, and F have values falling in the normal range for mg. per total urine volume. However, the mg./gm. of creatinine values are above the normal range.

b. Parkinson's disease

The urines for patients A and B were collected at the Allan Memorial Institute. Patient A has been an outpatient at the Institute while patient B has been suffering from traumatic Parkinsonism as a result of an automobile accident.

TABLE XVI

Excretion of Homovanillic Acid in Urines of Patients with Parkinson's Disease

Patient	Number	Sex	Age	mg.	mg./gm. creatinine
A	7651	M	41	3.30	2.36
A	7656	M	41	4.80	3.25
B	7652	M		6.30	6.50

Although these values are in the lower range of normal values, it appears that there is not a change in the excretion of HVA in Parkinsonism. Patient B's condition is a result of an accident and any change in his condition would probably be due to physical disturbance of the basal ganglia rather than direct disturbance of metabolic pathways. It would be an advantage to study more cases with this disease in order to be able to draw a more definite conclusion as to whether or not there is a metabolic abnormality in this disease.

c. Huntington's chorea

Urines, from the patients listed in Tables XVII and XVIII were collected at the Allan Memorial Institute and at Wakefield, Quebec. Patients A, B, and C suffered from Huntington's chorea. Patient B's father also suffered from Huntington's chorea and it is probable that she too has the disease. In addition she is suffering from some degree of mental deficiency.

Patients D and E are unaffected relatives of patients A, B, and C.

TABLE XVII

Excretion of Homovanillic Acid in Urines of Patients with Huntington's Chorea.

Patient	Number	Sex	Age	mg.	mg./gm. creatinine
A	7621	M	50	6.69	4.65
A	7623	M	50	4.88	3.63
A	7625	M	50	6.30	4.65
A	7664	M	50	6.90	4.26
B	7605	F	44	11.83	10.17
B	7630	F	44	2.96	4.25
B	7653	F	44	5.25	5.11
B	7655	F	44	5.85	5.82
C	7608	M	54	8.30	9.40
C	7627	M	54	7.04	5.00
D*	7633	M		9.48	10.01
E*	7634	F		5.83	3.97

* Unaffected relatives of patients A, B, and C.

From the above results it can be seen that there is no difference in urinary HVA values of Huntington's chorea patients and normal persons. The values are also similar to those of two unaffected relatives from the same locality.

Reserpine and certain other tranquilizers have been used to control some symptoms of Huntington's chorea. It is well known that reserpine will release brain amines. α-Methyldopa (Aldomet®) is another compound that releases brain amines (74). Aldomet was available and tested for action on some of the Huntington's chorea patients.

Aldomet is also an inhibitor of dopa decarboxylase (75) and it was thought if the action of dopa decarboxylase is important in these patients, the results might be reflected in lower HVA values.

TABLE XVIII

Excretion of Homovanillic Acid in Urines of Patients with Huntington's Chorea after Receiving Aldomet*

Patient	Number	Sex	Age	mg.	mg./gm. creatinine
A	8054	M	50	6.24	8.00
C	7655	M	54	5.80	5.49
C	8055	M	54	8.10	8.00
F**	7631	M	46	3.33	3.88
F**	7666	M	46	6.76	8.79
F**	8053	M	46	5.68	11.36

* α-methyl-L-dopa, taken chronically as medication, 1-2 gm./ day.
** 12 hour urine collections from patient F.

It can be seen from the above results that the range of HVA during prolonged treatment with Aldomet is not different from what was observed in patients before receiving Aldomet.

It was thought that the conversion of exogenous dopa to dopamine and HVA may be affected if there is a defect in amine metabolism in Huntington's chorea. Therefore D- and L-dopa load tests were tried.

Urines from the patients described in Table XIX were collected at the Verdun Protestant Hospital. I would like to extend my thanks to Dr. G. Peterfy and Miss Arthurs, R.N. for making the urines available.

All of the patients had been at the hospital for long terms. Six of the patients (G, H, I, J, K, and L) were suffering from psychotic disturbances (schizophrenia) and were used as controls for the Huntington's chorea patients (M and N). Six hour urines were collected from these patients after the dopa load tests. Most of the metabolites of dopa are excreted during this period (unpublished results). "Loads" consisted of 200 mg. of the respective isomers and were taken orally in a capsule.

Both D- and L-dopa have been shown to be converted to dopamine (76). Dopamine is a precursor of HVA and if dopa is administered, one would expect a rise in the urinary HVA levels.

D-dopa administered orally gave a slight excess of HVA in some cases over the control period. L-dopa gave rise to large amounts in most cases. D-dopa must travel through more intermediate steps until it is completely metabolized, perhaps accounting for the lower HVA values after its administration.

The two patients with Huntington's chorea gave HVA values below the range observed in others. But the urines were collected for six hours and are subject to error due to the difficulties in obtaining properly timed specimens from these patients. When mg./gm. creatinine were calculated, the HVA values for the Huntington's chorea patients were within the range of the other patients. Although one can question the attempts to relate exogenous metabolites to endogenous substances such as creatinine, the calculations are made as a check against the irregular collections of urine. It is better to have two measurements than one in these cases.

TABLE XIX

Excretion of Homovanillic Acid in Urines of Patients with Huntington's Chorea, and after Receiving 200 mg. D-dopa or 200 mg. L-dopa

Patient	Control test			200 mg. D-dopa				200 mg. L-dopa			
	No.	mg.	mg./gr. creatinine	No,	mg.	mg./gr. creatinine	% HVA excreted*	No.	mg.	mg./gr. creatinine	% HVA excreted
Males				Psychiatric Patients							
G	7691	1.00	8.54	8005	1.63	18.95	0.32	8017	13.56	118.94	6.28
H	7692	2.40	11.16	8004	2.44	16.83	0.02	8016	17.33	76.34	7.47
	7699	3.27	10.00					7705	13.83	49.93	5.28
I	7693	3.86	8.35					7706	15.83	42.90	5.99
	7700	3.47	9.30								
J				8003	3.68	43.29		7707	20.92	67.48	
								8015	24.00	83.33	
Females											
K	7715	4.34	9.18	8006	2.20	15.83		8018	26.78	101.83	11.22
L	7716	1.10	8.53	8007	2.56	26.67	0.73	8019	13.20	220.00	6.05
				Huntington's Chorea Patients							
Females											
M	7717	1.54	6.91	8008	2.77	20.52	0.62	8020	4.51	92.04	1.49
N	7721	2.30	11.92	8009	2.27	19.74		8021	12.36	112.36	5.03

* HVA excreted (in excess of control) as a per cent of the dose.

2. Diseases with catechol-producing tumors

a. Pheochromocytoma

Three cases are presented here. The urines from patient A were obtained from Mexico through the courtesy of Dr. P. Serrano. At the time of the sample collection, the patient was being treated with hydergine.

The urines from patient B, a 47-year old woman, were obtained from her physician, Dr. B. J. S. Harley, Corner Brook, Newfoundland. In 1958 this lady had a pheochromocytoma removed and was thereafter asymptomatic until 1962. The urines 6494, 6525, 6526, 6543, and 6545 were collected following recurrence of symptoms. Urines 6543 and 6545 were collected after an exploratory laparotomy revealed multiple metastases.

Patient C's urines were collected at the Royal Victoria Hospital. The patient was a 34-year old woman. Urine 8048 was a pre-operative collection while 8060 was collected two weeks after the removal of a bilateral pheochromocytoma.

TABLE XX
Excretion of Homovanillic Acid in Urines of Patients with Pheochromocytoma

Patient	Number	HVA(mg.)	Dopamine(mg.)*
A	4847	7.44	0.38
B	6494	13.51	2.13
B	6525	15.12	4.02
B	6526	12.83	2.31
B	6543	26.00	6.93
B	6545	38.70	3.27
C	8048	10.76	0.38
C	8060	10.53	0.27

* Mean normal values of dopamine are 0.30 mg., the range is 0.20 - 0.41 mg. per day. The dopamine values are included in this table for comparative purposes.

Patient A's HVA values were within the normal range as were his values for dopamine. The values for patient B were determined using the diazotized sulphanilic acid reaction and consequently they may be overestimated. These values are included here because the phenol reaction was not in use at the time. However, the dopamine values were also very high. It is interesting to note that the values after the operation were higher than those before surgery. This could be due to disturbance of the tumor causing it to excrete excess amounts of catechols, or to metastasize further. In patient C, both HVA and dopamine values were within the normal range. After removal of the bilateral pheochromocytoma, there was a marked clinical improvement and a decrease in the urinary dopamine. The HVA value remained within the normal range.

b. Neuroblastoma

HVA was measured in several cases of neuroblastoma. Table XXI gives the results of these determinations. Several of the cases - A, B, and D - were followed through treatments administered to these children.

In the case of patient A, a 10-year old girl, diffuse skeletal metastases were diagnosed when the urine samples were first collected. Sample 6439 was collected during radiation therapy, which resulted in clinical improvement. Samples 6452 and 6470 were collected while the patient was being fed a protein-free diet. In both cases the urinary HVA values were lower in the collections made during the diet. This is important to explore further as other metabolites, e.g. dopamine and dopac (77) did not show a constant decrease. Samples 6451, 6550, and 7567 were collected while the patient was being treated with cyclophosphamide and actinomycin D.

The dopamine values are also listed in the table to show a comparison between the two values. When the dopamine values are high, the HVA levels are usually high too. The dopamine was measured by a modification of the Carlsson and Waldeck method (78). The HVA measurements were made with the diazotized sulphanilic acid reaction for this case. The excretion by this patient of other metabolites present is discussed by Sourkes et al. (77). It can be seen from the HVA values of this patient that there was a period of partial remission, even though the patient was still excreting tremendous amounts of catechols and their metabolites.

Patient B was a 15-month old girl with a thoracic neuroblastoma compressing the spinal cord and causing quadriplegia. Urines 6484 and 6500 were collected after a laminectomy to relieve the pressure on the spinal cord. However, the tumor recurred in the original site and the gradual increase in HVA in urine 7563 at this time can be noted. The values in this patient appear to be low, but this was a very young child. It is unfortunate that we have not had an adequate number of controls in this age group.

Sample 6471 (patient C, 30-month old male) was collected after the patient was found to have developed metastases. The HVA value is very high. This patient was unsuccessfully treated with irradiation.

Patient D was a 9.5-year old boy. After irradiation he had symptomatic relief, corresponding to the low HVA values in urines 6413, 6423, and 6432. However, eventually he developed uncontrolable multiple metastases.

Urine 7570 was collected after the patient had received Aldomet therapy for four days. However, the results did not show any change as the HVA values remained very high. The HVA measurements in this case were made with the diazotized sulphanilic acid reaction.

Patient E, a 3.5-year old girl, had a cystic neuroblastoma of the left kidney in 1960. The condition remitted spontaneously, possibly by ischemia of the tumor. The tumor was removed later that year. The recorded values fall within the normal range.

Patient F, a four-year old girl, was treated at the Montreal Children's Hospital for a neuroblastoma with diffuse metastases. Table XXII lists the amounts of HVA and dopamine excreted in her urines and gives a summary of her treatment throughout her stay in the hospital, where she was treated with drugs and radiation.

The effect of a diet deficient in phenylalanine and tyrosine (Lofenalac® with tyrosine omitted, Mead Johnson) was tested on the patient. Such a diet deprives the patient of exogenous precursors of catechols, and was tested here in the attempt to reduce production of these compounds. There was a decrease in the HVA excretion on the first day only with a rise in the HVA values the following day. This may have arisen through conversion of endogenously turned-over phenylalanine and tyrosine to catechol products. During the next period, after her return to a normal hospital diet, she was given Aldomet. The HVA values on three of the four days she received the drug were somewhat lower than the two succeeding non-Aldomet days.

TABLE XXI
Excretion of Homovanillic Acid in Urines of Patients with Neuroblastoma

Patient	Number	HVA(mg.)	Dopamine*(mg.)	Remarks
A	6430	149.16	82.70	At beginning of radiation therapy.
A	6439	41.76	2.50	
A	6451	86.73	2.73	Cylophosphamide, actinomycin D. (continuing treatment)
A	6452	53.55	2.04	Patient on protein-free diet.
A	6453	78.08	2.55	
A	6469	97.92	1.40	
A	6470	79.95	1.14	Patient on protein-free diet.
A	6474	103.95	1.12	
A	6550	113.96	12.23	
A	7567	65.74	12.29	
B	6484	2.64	0.14	After laminectomy.
B	6500	3.04	0.17	
B	7563	4.10	0.12	Recurrence of symptoms.
B	7565	2.17	0.14	
B	7566	2.30	0.20	
C	6471	56.00	0.18	
D	6413	35.26	0.49	Remission of symptoms
D	6423	29.53	0.60	
D	6432	37.44	0.34	
D	6540	65.41	1.03	Recurrence of symptoms; metastases.
D	7568	50.75	1.33	
D	7570	83.84	3.60	
E	7619	5.30	0.14	

* Mean value for normal children, 0.16 mg.
The dopamine values are included in this table for comparative purposes.

TABLE XXII

Excretion of Homovanillic Acid in Patient F
Suffering from Neuroblastoma

Number	HVA		Dopamine+ (mg.)	Treatment and remarks
	mg.	mg./gr. creatinine		
7708	9.70	68.31	3.97	
7723	21.50	104.36	6.53	
7725	31.59	190.30	11.03	
7726	16.17	107.09	10.25	
7727	24.54	153.83	11.27	
7728	40.48	183.17	7.60	
7729	37.80	121.94	15.38	
7733	25.88	85.98	12.59	
7734	14.98	116.12	7.90	
7738	14.56	151.67	5.73	
7741	18.16	96.60	6.70	
7742	16.00	114.29	13.66	
7743	19.58	90.23	11.20	
7744	10.25	67.88	9.19	
7746	23.58	126.77	11.75	
7747	24.25	95.47	17.03	
7748	27.00	146.74	14.83	
7752	39.60	327.27	16.26	
7753	27.34	128.36	19.43	
8026	24.50	196.00	19.92	Normal diet, day 1.
8027	22.62	201.96	18.97	Low phenylalanine-tyrosine diet*, day 2.
8028	18.14	153.73	16.09	Low phenylalanine-tyrosine diet, day 3.
8033	24.13	138.68	13.31	Low phenylalanine-tyrosine diet, day 4.
8030	27.88	268.08	15.45	Low phenylalanine-tyrosine diet, day 5.
8031	23.04	160.00	15.86	Low phenylalanine-tyrosine diet, day 6.
8032	21.22	117.89	25.88	Normal diet, day 7.
8034	21.47	150,14	24.35	Normal diet, day 8.
8038	19.60	104.26	5.51	Received Aldomet, day 1.
8039	24.00	133.33	7.56	Received Aldomet, day 2.
8041	24.86	114.04	11.30	Received Aldomet, day 3.
8042	30.92	128.30	11.11	Received Aldomet, day 4.
8043	31.01	130.29	12.40	No Aldomet, day 5.
8044	27.20	98.55	12.59	No Aldomet, day 6.

* Lofenalac® (Mead Johnson) with tyrosine omitted.

+ The dopamine values are included in this table for comparative purposes.

c. Ganglioneuroma

Two cases of ganglioneuroma were studied. HVA was determined in both pre-operative and post-operative urine specimens (See Table XXIII).

Case A was an 11.5-year old girl with a ganglioneuroma. Urines 6460 and 6467 were pre-operative samples while sample 6491 was collected after removal of much of the thoracic tumor. The HVA values dropped substantially after the removal of the tumor. Although urine 7730 showed high HVA levels, no recurrence of the tumor was noted when the patient returned to the hospital for a spine fusion operation necessitated by the tumor. Despite the high HVA value, the girl was free radiologically and clinically of the ganglioneuroma symptoms. The father of the patient had had a pheochromocytoma removed two years previously. Because of this, it was decided to investigate the mother and a urine sample was obtained. The HVA value was within the upper limits of the normal range.

Patient B was a five-year old boy with lung lesions. Urine 7618 was collected prior to surgery. Samples 7622 and 7624 were collected after removal of a mediastinal mass. The HVA levels were substantially lower after surgery.

Patient C was a 17.5-year old male with a presumed ganglioneuroma. This patient suffered from cerebral palsy and mental retardation. Because of radiological and clinical symptoms, he was recommended for biopsy to determine whether he had a ganglioneuroma. This was refused, but because of elevated HVA (and other catechol values), it is highly probable that he does have a catechol-producing tumor.

TABLE XXIII

Excretion of Homovanillic Acid in Urines of Patients with Ganglioneuroma

Patient	Number	mg.	mg./gr. creatinine	Remarks
A	6460	17.60	16.92	Pre-operative
A	6467	14.60	16.27	Pre-operative
A	6491	6.89	14.60	Post-operative
A*	7730	16.25	30.20	
B	7618	25.80	57.08	Pre-operative
B	7622	6.85	16.04	Post-operative
B	7624	4.30	10.00	Post-operative
C	7642	28.50	33.61	

* Mother of patient A showed at this time (urine number 7731) 11.61 mg. HVA/ 24 hours, or 13.24 mg./gm. creatinine.

d. Malignant melanoma

In the single case of malignant melanoma available for study, the patient was suffering from multiple metastases. The patient's HVA values fall within the normal range, except for the samples collected after triethylenethiophosphoramide (Thio-TEPA) therapy. This agent may have disrupted tissue which released extra amounts of dopamine which was eventually metabolized to HVA.

TABLE XXIV

Excretion of Homovanillic Acid in Urine of Patient with Malignant Melanoma

Number	HVA mg.	Dopamine (mg.)+	Remarks
3807	6.66	-	
3808	8.84	3.90	12 hours before thio-TEPA
3809	15.47	7.41	after thio-TEPA therapy
3810	10.80	7.31	after thio-TEPA therapy

\+ The dopamine results are included in this table for comparative purposes.

3. Other disease states

a. Hyperkinetic syndrome

Urines from patients with hyperkinetic syndrome were collected at the Montreal Children's Hospital and made available to us through the courtesy of Dr. J. Werry. Analysis of several urinary amines and amine metabolites were carried out to determine whether a metabolic disturbance in those areas could be detected in this syndrome. Only the HVA values are reported here. The results show no difference in HVA excretion between normal children and those with hyperkinetic syndrome.

TABLE XXV

Excretion of Homovanillic Acid in Urines of Patients with Hyperkinetic Syndrome

Patient	Number	Sex	Age	mg.	mg./gr. creatinine
A	7670	M	9 7/12	7.00	11.63
B	7672	M	10 6/12	7.58	6.96
C	7688	M	8 7/12	5.28	9.76
D	7711	M	7 6/12	4.69	8.03
E	7750	F		9.00	18.14
F	8045	M	9	8.06	14.87
G	8057	M	13	7.91	9.12
H	8058	M	9	9.24	19.66
I	8059	M		6.03	10.98

IV. DISCUSSION

The discussion in this section will focus on three main topics. A discussion of the material described in Section III (Development of Methods) will concern 1) the preliminary methods tried and eventually rejected and 2) the advantages of TLC. Finally, a brief analysis of the results in Section III will be presented.

It should be emphasized that the main objective of this study was to develop a rapid method for the measurement of HVA which eventually could be set up as a routine procedure.

A. DISCUSSION OF PRELIMINARY METHODS

Many compounds can be extracted with ethyl acetate from acidified urine. Separation of HVA from some of these compounds was accomplished with column chromatography. When urine extracts were added to silica gel columns, HVA, popac, and an unknown compound (presumed to be phloretic acid and referred to as phloretic acid hereafter) were eluted with a mixture of 5% methanol in benzene or 5% methanol in methylene chloride. It was shown that HVA could not be isolated from the extracts solely by column chromatography, for, by using paper chromatography it was observed that the "HVA fraction" eluted from the column contained three compounds, HVA, popac, and phloretic acid. These compounds could also be separated when urinary extracts were applied directly to paper chromatograms. Thus the column chromatographic procedure was abandoned, and the isolation of HVA was attempted using paper chromatography.

A solvent system was needed that would give a clearer separation of HVA from neighbouring compounds and develop the chromatograms quickly.

Solvent systems giving good separations , but requiring lengthy developing times (16-24 hours) were not suitable. Useful separations were achieved with benzene:propionic acid:water (2:2:1) and benzene: acetic acid:water (6:7:3). The developing times were relatively rapid - six to eight hours for the former mixture and five hours for the latter. However, difficulties arose with urine samples containing large amounts of compounds.

In these instances the spots spread horizontally and vertically, often overlapping other spots from the same extract and adjacent extracts. We were unaware of the amount of HVA in a urine sample before the extraction. If the concentration of HVA was very high, the spots representing different compounds overlapped and we were unable to isolate the entire spot belonging to a single compound. Thus we had to repeat the procedure with more space between the applied spots and/or with a greater dilution of the sample applied to the chromatogram.

We were able to obtain HVA, popac, and phloretic acid in the same column chromatography eluate. These compounds could be separated on paper. Urine extracts might also be resolved into several compounds on paper, but the resolution was not as clear as that obtained with the column chromatography eluates. This process limited severely the number of samples that could be determined, and was time-consuming. We also had difficulties eluting the compounds from the paper chromatograms. Although the method appeared to be suitable qualitatively, it was not at all efficient quantitatively. At this point, we commenced work with TLC, and the above experiments were not extended.

B. DISCUSSION OF THIN-LAYER CHROMATOGRAPHY

The use of TLC has two advantages over other techniques for determining HVA: (1) it requires no specialized apparatus, except the gel-dispenser, as compared with gas chromatography and high voltage electrophoresis; and (2) it is relatively rapid, by comparison with paper chromatography. The entire procedure can be completed in ten hours, and may be interrupted at certain obvious stages. Extraction of new samples can be carried out while previous ones are being chromatographed.

Excellent quantitative separations were obtained with TLC in about 90 minutes. The spots were more distinct and smaller concentrations of HVA could be detected than on paper chromatography. There was no cause to worry about overloading spots because they did not spread laterally or vertically as was the case with paper chromatography.

1. Preparation of plates

The optimum thickness of silica gel on the plates was 0.25 mm. Seven plates were prepared at a time. Five plates were usually used for each experiment necessitating a new set of plates for each experiment.

The fact that it is almost impossible to duplicate conditions for preparing the plates leaves margin for error and contributes to uncontrollable experiment variation. Some of the factors causing discrepancies in sets of silica gel coated plates are described below.

1. Variability in consistency of slurry. Plates are prepared using 2 ml. water to 1 gm. silica gel. A too watery, or too thick slurry tends to produce plates that develop differently.

2. Drying. The length of drying time after the plates have been coated with silica gel and the atmospheric conditions may affect the mobilities of compounds.

3. Age. Fresh plates are best, although older plates may be used if they are placed in the oven to rid them of moisture ("activation" of the gel) before compounds are applied.

4. Dirty plates, slight variability in silica gel thickness in the same plate caused by lumps of gel in the applicator, or by uneven coating can also cause erratic results.

2. Spraying the plates and eluting homovanillic acid

The use of Folin's phenol reagent as a spray reagent indicated a homogeneous area for HVA that was isolated from nearby spots for other phenolic compounds normally occuring in urine. When the silica gel was extracted with water only a very pale blue colour appears in the eluate. It is possible that only a portion of the adsorbed HVA had reacted. Therefore a further Folin reaction using the same ingredients as those used for the spray must be carried out with the eluates. This allows the remainder of the unreacted HVA to react with the phenol and give a full colour development.

A comparison was made between spots sprayed with the Folin reagent for phenols, eluted, and then carried through the colour reaction with silica gel areas containing HVA and not sprayed with the colour reagent, but eluted in the same way and carried through the same colour reaction. No difference was noted in the results. Therefore all of the HVA must have reacted with the phenol reagent in both cases.

If the plates were sprayed with diazotized sulphanilic acid, the silica gel eluted, and then diazotized sulphanilic acid or phenol reagent added to the eluate, the ensuing results were erratic. Thus the final reaction was evolved using a phenol reagent spray followed by a phenol reagent colour reaction.

For qualitative reactions, diazotized sulphanilic acid was the best spray, giving a variety of colours to compounds on silica gel. Although the colours tended to fade after a while, they were regenerated by placing the plate in a jar of ammonia vapours. Colours for all compounds with the phenol spray were shades of blue, the intensity of the colour depending on the concentrations of the compounds.

3. Removal of silica gel from plates

A few problems were encountered with quantitative measurements. The silica gel containing HVA must be scraped from the plates without including other compounds. As previously described in this thesis, the solvent front did not ascend the plate evenly. It was more mobile at the edges than at the center. Several attempts to compensate for this by adding more solvent, lining the sides of the chambers with filter paper, or varying the number of plates in a chamber were unsuccessful. A concave dip was formed in the solvent front as a result of this uneven mobility. The low spot of the dip was usually at the center of the plate, but sometimes was as far removed as six cms. from the center. The R_f values were quite unreliable, except for the compounds spotted in the one or two positions adjacent to the reference compounds. Temperature controls were not used and this may also have caused a daily variation in R_f values. R_f values were measured from approximately the center of the spot.

The original procedure to obtain the colour reaction was to spot HVA in pilot strips along the vertical edges of the TLC plates, cover the rest of the plate, and spray with diazotized sulphanilic acid. Spraying the pilot strips of HVA allowed us to visualize spots about one cm. in diameter. A horizontal line was drawn across the plate connecting positions about 0.25 cms. above these spots on each edge of the plate. Another horizontal line was drawn across the silica gel at a point three cms. below the previous point. The area between these lines, for each sample, was scraped off the plate, eluted, and the Folin reaction carried out with the eluate. In many cases other compounds were included in this area. Thus the values for HVA were often too high in these cases as we were not just eluting HVA, but other substances too. This was due to the solvent dip. Other possible interfering compounds were not included in the areas in close proximity to the reference compounds along the edges of the plates, and the values of HVA from these areas were comparable to the values obtained with the final procedure.

The main interference came from substances with a higher R_f than HVA (possibly vanillic and dihydroferulic acids). This was due to the decrease in mobility of HVA when the solvent front moved more slowly. Compounds with higher R_f values also had their mobilities decreased. Therefore they were often present in the area of silica gel scraped off the plate and eluted. Compounds with lesser mobilities than HVA (popac and phloretic acid) interfered to a lesser degree.

The problem of interfering compounds was ultimately solved by spraying the plates with phenol reagent to locate the "HVA area" prior to removing and eluting the silica gel.

4. Retarding effect of urine on homovanillic acid

HVA in urinary extracts had a lesser mobility on paper chromatography and TLC than the authentic compound. The reason for this retarding effect of urine is not known. It is possible that there is competition between HVA and other urinary components for positions on the chromatograms. Several other compounds in urinary extracts also had a lower R_f than did the authentic compound (See Table IX, page 53).

5. Possible modifications of the method

Despite the apparent validity of the method at this time, it is possible that several modifications and improvements can be carried out. We know that extraction of urine with methylene chloride removes fewer compounds from urine than does an ethyl acetate extract. If the urine were extracted with methylene chloride, several compounds would be left in the aqueous phase. If the aqueous phase was then extracted with ethyl acetate and the extract chromatographed, the chromatogram would have only the compounds that were not extracted with methylene chloride. It is quite feasible that the separation would be more distinct as there would be less spots on the chromatogram. For example, a compound is not extracted from urine with methylene chloride, but is with ethyl acetate. The methylene chloride may extract some of the compounds with R_f values in the neighbourhood of this compound . On chromatographing the ethyl acetate extract, the compound may now be in an area with fewer neighbouring compounds, and thus available for quantitative determinations.

TLC may also be tried without purchasing the specialized equipment needed for it. The gel dispenser is a very useful part of the equipment, but Lees and DeMuria (79) have used glass rods to spread silica gel over plates of any size. Adhesive tape layered along the sides of the glass determines the thickness of the silica gel coating.

Gritter and Albers (80) sprayed silica gel plates with water to determine the position of the spots. The spots showed as white opaque spots against a translucent background. This did not prove successful when we tried it. Perhaps the phenols are not as soluble in water and therefore do not respond well to this method.

An attempt to determine the amount of compound in a spot by measuring the area of the spot and then calculating the area:weight relationships is described by Purdy and Truter (81). Determining the area is a tedious task and not too accurate, especially when the spots do not spread to a great extent as was the case with our work.

The method as developed thus far is quite flexible. Other phenolic compounds can also be measured in the same way as HVA by appropriate adaptations in the present technique. Differences in solvent systems, temperatures, developing time, could well isolate other compounds for measurement. Other types of silica gel differing slightly from Silica Gel G may be used. Cellulose, or polyamide powder, might prove to be better for determinations of other metabolites of dopamine and dopa.

6. Validation of method

Proof that the compound we were measuring was HVA is contained in this section. The evidence comes from chromatography and colour reactions.

When urine extracts were added to silica gel columns, the urinary HVA was eluted in a fraction similar to that which eluted authentic HVA from the columns. Paper and thin-layer chromatography of this material ("HVA fraction") established the presence of three phenolic spots, including HVA. This was true when both methanol:benzene and methanol: methylene chloride respectively were used as eluants. The R_f values of the authentic HVA thus eluted were similar to the values of urinary HVA on both paper and thin-layer chromatograms. The R_f values for the urinary HVA extracted with both ethyl acetate and methylene chloride were also similar when compared with those for authentic HVA.

The separations of HVA from other urinary components when urine extracts were applied to chromatograms were similar to the separation of authentic HVA from a mixture of pure compounds that may be found in urine. The evidence from chromatographic mobility studies is summarized in Table XXVI.

TABLE XXVI

Comparison of R_f Values of Authentic and Urinary Homovanillic Acid

Solvent system	R_f authentic HVA	R_f urinary HVA
Paper chromatography		
Benzene:propionic acid:water (2:2:1)	0.68	0.65
Two-dimensional paper chromatography		
Benzene:acetic acid:water (6:7:3)	0.19	0.20
2% Formic acid	0.26	0.26
Thin-layer chromatography		
Benzene:acetic acid:water (2:3:1)	0.60;0.64	0.57;0.59
Benzene:propionic acid:water (2:3:1)	0.83	0.78*
Chloroform:acetic acid:water (2:2:1)	0.79	0.77

* Very low due to solvent dip, colours used to identify similar compounds.

From the above table we can see that with the solvent systems tried, in each case the R_f of urinary and authentic HVA were similar.

Colour sprays and reactions in test tubes produced colours with urinary HVA that were similar to the colours formed by authentic HVA, i.e. the compound moving with the same R_f as the authentic HVA produced a colour identical to that of the authentic HVA (purple with stable diazo salts and diazotized sulphanilic acid).

The comparison of urine extracts, extracts of urine with added HVA, and extracts of aqueous solutions of HVA, when chromatographed, showed similar mobilities for the spots corresponding to HVA.

C. DISCUSSION OF RESULTS

1. Homovanillic acid excretion in healthy persons

There was very little difference in the normal daily values of HVA whether they were determined after an ethyl acetate or methylene chloride extraction. There was no detectable difference between the values for males and females, nor was there any correlation in values with respect to age, even when the children's urines were included. However, only one of the children was less than six years old and it would be valuable to extend the age range downwards. Values obtained with the diazotized sulphanilic acid reaction were usually higher, and well above the normal range later established with Folin's reagent or reported by others (See pages 89 and 90). Our values compared quite favourably with those obtained by other authors (See Table III, page 26).

Variability of the determination was assessed by measuring HVA in given urine samples, on different days. A summary of the mean values and ranges of HVA is set out below.

Number	Number of determinations	Range. mg. of HVA/24 hours	Mean. mg. of HVA/24 hours
7669	3	11.56 - 14.45	12.71
7678	3	8.40 - 10.08	9.18
7680	5	7.22 - 8.60	8.01
7683	5	5.52 - 6.44	5.85
7684	3	8.96 - 9.80	9.24
7687	3	8.50	8.50
7689	4	5.16-6.17	5.38

2. Excretion of homovanillic acid in diseases of the basal ganglia

The previous work in this laboratory concerning measurement of certain compounds in diseases of the basal ganglia has centered on the measurement of catecholamines (82). Measurement of HVA has only been used in the last three years in an attempt to substantiate some of the results. Urines from patients with Parkinson's disease, Wilson's disease, and Huntington's chorea were available for the studies reported in this thesis.

a. Parkinson's disease

Parkinson's disease involves a central deficiency of dopamine and other amines (56, 83), subnormal excretion of dopamine in the urine (57, 84, 85), and degenerative changes in some of the basal ganglia (86, 87). There is a reduced ability to convert L-dopa to urinary dopamine and dopac (84). Consequently one might expect a reduced amount of HVA in the urine, too. Inconclusive results were obtained in the two cases in which urinary HVA was measured during this study. Both cases were at the lower extremity of the range for normal values of HVA in the urine. Greer and Williams (88) have also shown recently that HVA was not significantly different in patients with Parkinson's disease as compared with normal subjects in a similar age group.

b. Wilson's disease

Wilson's disease (hepatolenticular degeneration) is characterized biochemically by defective copper metabolism, as well as excessive excretion of various metabolites in the urine. These include amino acids, peptides, phosphate, and uric acid among others; their excretion is believed to arise from a defect in tubular reabsorption.

In the six cases cited in this thesis, certain of the cases excreted abnormally large amounts of one or more of the following compounds: dopamine, dopac, HVA, 5-HIAA (89). However, it is not known if this is due to renal tubular effect.

In patient A, HVA values were significantly higher than normal. The mean dopamine output in this patient was also greater than normal, but not significantly so.

Patient B had been on penicillamine therapy for some time. The excretion of urinary HVA was within the normal range. However, the dopamine values were greater than normal, but not significantly so. A relationship between penicillamine treatment and urinary or brain catecholamines is not known.

HVA values in patient C were elevated as were the dopac values. The excretion of dopac was also higher than normal in patients D and E. HVA values were slightly elevated in patient E. Patients D, E, and F had high values for urinary 5-HIAA.

c. Huntington's chorea

Huntington's chorea is a hereditary disease involving disorder of the basal ganglia. Normal values were obtained in these cases and this agrees with the findings of Williams et al. (11). The urinary dopamine is also not significantly different from normal in this disease (57). Dopac, noradrenaline, adrenaline, and 5-HIAA were also found to be within the normal range (Sourkes, unpublished results). Ehringer and Hornykiewicz (56) found normal values for cerebral dopamine in this disease. Thus, just as in Parkinsonism, cerebral and peripheral results of dopamine analysis parallel one another in Huntington's chorea.

This may also be true for HVA as elevated values have not been observed to date in our laboratory. No change was noted in the urinary HVA levels for patients treated with Aldomet. Perhaps the dose of Aldomet was too low to effect a result.

D- and L-dopa load tests were performed on advanced Huntington's chorea patients. Some results were unreliable due to difficulties of collecting urine from the patients. Patients given 200 mg. of D-dopa orally showed slight, if any, increase in urinary HVA over a six hour period. Those given a similar dose of L-dopa showed marked increases in the amount of HVA excreted over a similar period. The amounts of HVA excreted after L-dopa were slightly lower in the patients with Huntington's chorea than in the control subjects. When mg./gm. of creatinine were calculated, the values of HVA for Huntington's chorea were within the range of the controls (See pages 74 and 75). This shows that L-dopa must be responsible for most of the urinary HVA in humans. D-dopa is not metabolized as quickly in humans. Due to experimental difficulties and the short period of urine collection, an accurate estimate on the amount of dopa excreted as HVA is rather difficult to assess.

3. Excretion of homovanillic acid in diseases of catecholamine producing tumors

These diseases feature a change in the amount of functioning tissue, usually a tumor type growth. For the most part, HVA values were found to be elevated in these diseases. The diseases include pheochromocytoma, neuroblastoma, ganglioneuroma, and malignant melanoma.

a. Pheochromocytoma

Pheochromocytoma is a tumor of chromaffin tissue in the adrenals. Catecholamine production, and especially that of adrenaline, is usually high in this disease. HVA was found to be either normal or higher than normal in the cases studied for this thesis. Robinson and Smith found normal HVA values (13).

Patient A, a Mexican, showed normal HVA values. His dopac and dopamine values were also within the normal range.

Patient B had a recurrence of the tumor with metastases. Urines collected after the metastases gave higher values for HVA. Dopamine and dopac were also elevated in these urines (Sourkes, unpublished results). It is worth noting that the urinary dopamine, like HVA, is not always elevated in pheochromocytoma (13). Of five cases studied in Dr. Sourkes' laboratory, two values were normal, one was lower than normal, and two were higher than normal.

Values for patient C were within the upper limits of the normal range.

b. Neuroblastoma and ganglioneuroma

In the cases of neuroblastoma and ganglioneuroma studied for this thesis, HVA values were well above normal and exceedingly high in some cases. In both of these diseases, abnormally large amounts of several catechols have been detected in the urine (4). The urinary excretion of dopac and dopamine have now been demonstrated to reflect frequently the clinical state of the patient followed through the course of the disease (77, 82). The data for these conditions provided in the results establish in a similar fashion the usefulness of HVA determinations.

Several determinations were made on daily urines from patient F. Her values were high. When the patient was placed on a low phenylalanine diet, her HVA values fell slightly after the first day, although they were still above the normal value, but they then rose again. The same results were observed when the patient was given Aldomet. Her HVA values decreased slightly the first day, but then rose again the following day. The daily variation in HVA values coincided with the daily variation in dopac and 5-HIAA values.

Both children with ganglioneuroma had high HVA values in pre-operative urine specimens. Removal of tumor tissue resulted in a sharp fall in the urinary HVA values.

Williams and Greer (90) have shown elevated HVA values in six of seven neuroblastoma cases ranging in ages from one month to four years. They reported values of 25 - 830 mg./gm. of creatinine. Normal values for 24 children - one month to 12 years - were 6.5 mg./gm. of creatinine (mean) with a range of 1.2 to 19.0 mg./gm. of creatinine. These values are lower than those reported by von Studnitz (4) (See Table III, page 26).

c. Malignant melanoma

The patient studied in this case excreted normal amounts of HVA. However, dopamine values were higher than normal, ranging from 3.90 to 7.41 mg./24 hours. Duchon and Gregora (8) reported levels of HVA two to four times greater than normal in cases of generalized melanoma.

4.Other disease states

The urines from several children with hyperkinetic syndrome were available for us to analyse. HVA values were normal in all cases.

V. SUMMARY

Homovanillic acid, one of the phenolic acids found as a normal constituent in human urine, is derived in metabolism from dopa and dopamine. A rapid method using thin-layer chromatography has been developed to measure urinary homovanillic acid and is described in this thesis. The method consists of extracting urine with ethyl acetate (methylene chloride is also suitable), and chromatographing the ethyl acetate extracts on glass plates coated with silica gel. The solvent used to develop the plates is the organic phase of a benzene:acetic acid:water (2:3:1) mixture. The eluted homovanillic acid is treated with Folin's phenol reagent and the absorbance is measured at 750 mμ in a spectrophotometer. Normal amounts of homovanillic acid in human urine are 8.23 ± 2.96 (mean ± Standard Deviation) mg./24 hours or 6.42 ± 2.28 mg./gm. creatinine. Higher values were found in some cases of neuroblastoma, ganglioneuroma, hepatolenticular degeneration (Wilson's disease), and pheochromocytoma. In other conditions studied, Huntington's chorea (six patients), Parkinson's disease (two patients), hyperkinetic syndrome (nine patients), and malignant melanoma (one patient), no abnormality of homovanillic acid was observed. The effect of a decarboxylase inhibitor, α-methyldopa, was studied in two cases. The effect of a diet deficient in precursors of homovanillic acid was tested in a case of neuroblastoma.

VI. BIBLIOGRAPHY

1. I.M. Heilbron, H.M. Bunbury, and W.E. Jones, Dictionary of Organic Compounds, Eyre and Spottiswoode, London, 1936.

2. F. Tiemann and N. Nagai, Ber. deut. chem. Ges., 10, 202,204 (1877).

3. W. von Studnitz, Scand. J. Clin. Lab. Invest., 12, Suppl. 48 (1960).

4. W. von Studnitz, Klin. Wochschr., 40, 163 (1962).

5. L. Mitchell, T.H. Evans, and H. Hibbert, J. Am. Chem. Soc., 66, 604 (1944).

6. H.E. Fisher and H. Hibbert, J. Am. Chem. Soc., 69, 1208 (1947).

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

8. J. Duchon and V. Gregora, Clin. Chim. Acta, 7, 443 (1962).

9. C.R.J. Ruthven and M. Sandler, Biochem. J., 83 , 30P (1962).

10. R.E. Greenberg and L.I. Gardner, J. Clin. Invest., 39, 1729 (1960).

11. C.M. Williams, S. Maury, and N.F. Kibler, J. Neurochem., 6, 254 (1960).

12. J.M. Smellie and M. Sandler, Proc. Roy. Soc. Med., 54, 327 (1961).

13. R. Robinson and P. Smith, Clin. Chim. Acta, 7, 29 (1962).

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

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

16. D.E. Duggan, R.L. Bowman, B.B. Brodie, and S. Udenfriend, Arch. Biochem. and Biophys., 68, 1 (1957).

17. R.T. Williams, J. Roy. Inst. Chem., p. 611, Nov. 1959.

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

19. D.M. Smith, R.M. Paul, E.G. McGeer, and P.L. McGeer, Can. J. Biochem. and Physiol., 37, 1493 (1959).

20. E.G. McGeer, M.C. Robertson, and P.L. McGeer, Can. J. Biochem. and Physiol., 39, 605 (1961).

21. F. Mauthner, Justus Liebigs Ann. Chem., 370, 373 (1909).

22. R. Pschorr, Justus Liebigs Ann. Chem., 391 , 23 (1912).

23. A. Klemenc, Monatsh. Chem., 33, 375 (1912).

24. Z. Kitasato, Acta Phytochim., 3, 175 (1927).

25. J. M. Gulland, J. Chem. Soc., p. 2872 (1931).

26. R.L. Douglas and J.M. Gulland, J. Chem. Soc., p.2893 (1931).

27. G. Hahn and O. Schales, Ber. deut. chem. Ges., 67B, 1486 (1934).

28. A.A.L. Challis and G.R. Clemo, J. Chem. Soc., p. 1692 (1947).

29. A. Ya Berlin, S.M. Sherlin, and T.A. Serebrennikova, Zhur. Obshchei Khim., 19, 759 (1949).

30. I.A. Pearl and D. L. Beyer, J. Am. Chem. Soc., 77, 3660 (1955).

31. K.N.F. Shaw, A. McMillan, and M.D. Armstrong, J. Org. Chem., 23, 27 (1958).

32. C.C. Sweeley and C.M. Williams, Anal. Biochem., 2, 83 (1961).

33. C.M. Williams and C.C Sweeley, J. Clin. Endocrinology and Met., 21, 1500 (1961).

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

35. F. DeEds, A.N. Booth, and F.T. Jones, Fed. Proc., 14, 332 (1955).

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

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

38. C.W. Murray, A.N. Booth, F. DeEds, and F. T. Jones, J. Am. Pharm. Assn., Scient. Ed., 43, 361 (1954).

39. P. Holtz, R. Heise, and K. Ludtke, Arch. exp. Path. u. Pharmakol., 191, 87 (1939).

40. P. Holtz, K. Gredner, and W. Koepp, Arch. exp. Path. u. Pharmakol., 200, 356 (1942).

41. M.S. Masri, A.N. Booth, and F. DeEds, Arch. Biochem. and Biophys., 85, 284 (1959).

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

43. J. Pellerin and A. D'Iorio, Can. J. Biochem. and Physiol., 33 1055 (1955).

44. A.N. Booth and F. DeEds, J. Am. Pharm. Assn., 47, 183 (1958).

45. C.M. Williams and A.A. Babuscio, Biochim. et Biophys. Acta, 47, 393 (1961).

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

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

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

49. M. Goldstein, A.J. Friedhoff, and C. Simmons, Biochim. et Biophys. Acta, 33, 572 (1959).

50. M. Goldstein, A.J. Friedhoff, S. Pomerantz, and C. Simmons, Biochim. et Biophys. Acta, 39, 189 (1960).

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

52. C.M. Williams, Anal. Biochem., 4, 423 (1962).

53. R. Robinson and P. Smith, Nature, 186, 240 (1960).

54. S.L. Tompsett, J. Pharm. and Pharmacol., 13, 747 (1961).

55. H. Zeisel, Z. Kinderheilk., 86, 89 (1961).

56. H. Ehringer and O. Hornykiewicz, Klin. Wochschr., 38, 1236 (1960).

57. A. Barbeau, G.F. Murphy, and T.L. Sourkes, Science, 133, 1706 (1961).

58. A. Carlsson and N.A.H. Hillarp, Acta Physiol. Scand., 55, 95 (1962).

59. C.M. Williams and M. Greer, Clin. Chim. Acta, 7, 880 (1962).

60. I. Sankoff and T. L. Sourkes, Can. J. Biochem. and Physiol., in press.

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

62. O. Folin and V. Ciocalteu, J. Biol. Chem., 73, 627 (1927).

63. P.B. Hawk, B.C. Oser, and W.H. Summerson, Practical Physiological Chemistry, 12th ed., Blakiston, Philadelphia and Toronto, 1947, p. 879.

64. I.A. Pearl and P.F. McCoy, Anal. Chem., 32, 1407 (1960).

65. H.K. Berry, H.E. Sutton, L. Cain, and J.S. Berry, Univ. Texas Publ. No. 5109, 22 (1951).

66. F.W. Sunderman, Jr., P.D. Cleveland, N.C. Law, and F.W. Sunderman, Am. J. Clin. Pathol., 34, 293 (1960).

67. P. Colombo, D. Corbetta, A. Pirotta, and G. Ruffini, J. Chromatog., 6, 467 (1961).

68. R.K. Ibrahim and G.H.N. Towers, Arch. Biochem. and Biophys., 87, 125 (1960); L.A. Griffiths, Nature, 180, 1373 (1957).

69. E. Stahl, Chem. Z., 82, 323 (1958).

70. D.M. Shepherd and G.B. West, J. Pharm. and Pharmacol., 4, 672 (1952).

71. T.L. Sourkes, Biochemistry of Mental Disease, Hoeber (Harper and Row), New York, 1962, p. 313.

72. S. Udenfriend, H. Weissbach, and B.B. Brodie, Methods Biochem. Anal., 6, 95 (1958).

73. I. Sano, K. Taniguchi, T. Gamo, M. Takesada, and Y. Kakimoto, Klin. Wochschr., 38, 5 (1960).

74. T.L. Sourkes, G. F. Murphy, B. Chavez, and M. Zielinska, J. Neurochem., 8, 109 (1961).

75. T.L. Sourkes, Arch. Biochem. and Biophys., 51, 444 (1954).

76. T.L. Sourkes, G. F. Murphy, and V.R. Woodford, Jr., J. Nutrition, 72, 145 (1960).

77. T.L. Sourkes, R.L. Denton, G.F. Murphy, B. Chavez, and S. Saint Cyr, Pediatrics, in press.

78. B.D. Drujan, T.L. Sourkes, D.S. Layne, and G.F. Murphy, Can. J. Biochem. and Physiol., 37 , 1153 (1959).

79. T.M. Lees and P.J. DeMuria, J. Chromatog., 8, 108 (1962).

80. R.J. Gritter and R.J. Albers, J. Chromatog., 9, 392 (1962).

81. S. J. Purdy and E.V. Truter, Analyst, 87, 802 (1962).

82. T.L. Sourkes in "Neurochemistry", edited by K.A.C. Elliott, I.H. Page, and J.H. Quastel, C.C. Thomas, Springfield, Ill., 1962.

83. O. Hornykiewicz, Deut. med. Wochschr., 87, 1807 (1962).

84. A. Barbeau, T.L. Sourkes, and G.F. Murphy in "Monoamines et Système Nerveux Central", edited by J. de Ajuriaguerra, Masson, Geneva, Georg, and Paris, 1962, p.247.

85. A. Barbeau and T. L. Sourkes, Rev. Can. Biol., 20, 197 (1962).

86. J.P. Martin, Lancet, i, 999 (1959).

87. D. Denny-Brown, Lancet, ii, 1099 and 1155 (1960).

88. M. Greer and C.M. Williams, Neurology, 13, 73 (1963).

89. T.L. Sourkes, G.F. Murphy, I. Sankoff, M.H. Wiseman-Distler, and S. Saint Cyr, in preparation.

90. C.M. Williams and M. Greer, J. Am. Med. Assoc., 183, 836 (1963).

VII. APPENDIX

A. INTRODUCTION

Pellerin and D'Iorio (i), and Shaw, McMillan, and Armstrong (ii) investigated the metabolism of dopa in the rat. Pellerin and D'Iorio injected DL-dopa-C^{14} into albino rats and recovered 80% of the radioactivity in the urine. HVA represented 16% of the radioactivity in the urine, or 20% of the injected dose.

Shaw's group injected L-dopa into rats and recovered 14.4% as free HVA in the urine. When dopac was injected into rats, 24.8% was recovered as free HVA.

B. METHODS

Albino rats were injected intraperitoneally with the compounds listed in Table A. Urines were collected for 24 hours and were then analysed for HVA as described in this thesis using the ethyl acetate extraction procedure.

C. RESULTS

The excretion of HVA and the percentage of the injected compound recovered as HVA is set out in Table A, page vii.

TABLE A

Formation of Homovanillic Acid from Compounds Injected into Rats

Compound injected (Expt. No.)	Number of rats	Total weight (gm.)	Dose injected (mg. per 100 gm. rat)	Amount given to all rats (mg.)	mg. HVA excreted in 24 hours	mg. excess HVA over normal excreted	mg. HVA excreted per 1,000 gm.	% recovered as HVA
Saline (1)	3	313	-	-	0.16	-	0.50	-
Saline (2)	4	495	-	-	0.27	-	0.55	-
Saline (3)	3	397	-	-	0.13	-	0.34	-
Saline (4)	4	426	-	-	0.27	-	0.62	-
Saline (5)	3	719	-	-	0.29	-	0.41	-
L-Dopa (1)	3	310	20 mg.	62.00	4.89	4.73	-	7.63
L-Dopa* (3)	3	407	10 mg.	40.70	3.47	3.34	-	8.19
DL-Dopa (2)	4	505	10 mg.	50.50	3.62	3.35	-	6.62
D-Dopa* (3)	3	405	10 mg.	40.50	1.37	1.23	-	3.04
Dopamine (4)	3	322	3 mg.	9.66	2.24	1.98	-	20.48
Dopamine (5)	3	718	3 mg.	21.54	4.44	4.15	-	19.25
3-Medopamine (3)	3	411	10 mg.	41.10	5.25	5.12	-	12.45
3-Medopamine (5)	3	723	3 mg.	21.69	5.76	5.47	-	25.21
Dopac (4)	3	320	3 mg.	9.60	1.45	1.18	-	12.34
Dopac (5)	3	724	3 mg.	21.72	2.64	2.35	-	10.80
Homovanillic acid (3)	3	321	3 mg.	9.63	6.08	5.82	-	60.42
Caffeic Acid* (2)	4	502	10 mg.	50.20	0.62	0.35	-	0.70
Ferulic acid* (2)	4	498	10 mg.	49.80	0.37	0.10	-	0.20

* Injected as suspensions.

All results are corrected for the amounts of homovanillic acid excreted by control rats in the individual experiments. The experiment numbers are denoted by the numbers in parenthesis in the column containing the compound injected.

D. DISCUSSION

Groups of normal rats excreted from 0.34 to 0.62 mg. of HVA/kg. of rat. An adult human weighing 50-70 kg. excretes an average of 8.23 mg./24 hours with a range of 4.56 - 13.61 mg./24 hours. This means that rats excrete from three to five times as much HVA as humans do. This agrees with Shaw et al. (ii) who found that rats excreted three times as much HVA as humans when calculated on a weight basis.

L-dopa was twice as effective as D-dopa as a precursor of HVA. Murphy and Sourkes (iii) have shown that D-dopa produces more dopamine in vivo than L-dopa. The L isomer is metabolized in most organs of the body while the D form is chiefly metabolized in the kidney. The D isomer forms dopamine which is quickly excreted by the kidney before either deamination or methylation to HVA can be accomplished.

If we assume that equal amounts of D- and L-dopa form the racemic mixture, we may conclude that 4% and 2% of the L- and D-dopa respectively is metabolized to HVA. This agrees with the HVA recoveries for the injections of D- and L-dopa.

Dopac and 3-Medopamine are believed to be the immediate precursors of HVA in man, with dopamine being the precursor of these two compounds. One would expect increasingly greater recoveries along the pathway to HVA, but this did not occur. We may imagine dopamine forming HVA by a third pathway, but it is difficult to conceive such a method.

We are thus left with the following possibilities: 1) there is a greater availability for dopamine at certain enzyme sites; 2) dopamine metabolism in the rat is more favored than that of dopac or 3-Medopamine; 3) there is more absorption of dopamine from the intraperitoneal cavity; 4) there is excretion of some of the dopac and 3-Medopamine in a conjugated form.

Only 60% of the injected dose of HVA was recovered as HVA. It is rather surprising that only this amount was recovered because it is believed that HVA is a terminal metabolite. It would be interesting to determine whether HVA can be demethylated to dopac, or to what extent HVA may be excreted as a conjugated product in the rat. The disposition of the injected HVA in the tissues may have been such that only two thirds of the HVA was recovered in the urine.

Ferulic and caffeic acids did not alter the output of HVA. If they are converted to HVA in the rat, this must be within the normal variation.

E. BIBLIOGRAPHY

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

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

iii. G.F. Murphy and T.L. Sourkes, Arch. Biochem. and Biophys., 93, 338 (1961).