GAS CHROMATOGRAPHIC STUDIES ON PHENOLIC AND INDOLIC ACID AND ALCOHOL PRODUCTION IN MAN AND RAT

by

Farouk Karoum

A Thesis submitted for the Degree of
Doctor of Philosophy
at the
University of London
Faculty of Science

APRIL 1970

ACKNOWLEDGEMENT

I would like to express my thanks to Dr. M. Sandler and Dr. C.R.J. Ruthven for their constructive criticisms, continued guidance and encouragement during the course of this work.

I am also most grateful to Mr. J. Arthur for instructions and cooperation in photographing and printing most of the figures shown in this thesis.

My thanks goes to those whose help made this thesis work possible:

To Dr. J. Chapman of the GEC-AEI (Electronics) Ltd. Manchester for carrying out mass-spectrometric studies on some phenolic compounds.

Mr. B. Newman, hospital statistician at the Middlesex Hospital, London, for assistance in the statistical analysis of results.

ABBREVIATIONS

The abbreviations shown for the compounds listed below have been used throughout the thesis.

A	adrenaline, β (3,4-dihydroxyphenyl) β-hydroxy-N-methyl ethylamine
COMT	catechol-O-methyltransferase
D	dopamine, β-(3,4-dihydroxyphenyl) ethylamine
DHMA	3,4-dihydroxymandelic acid
DHPE	3,4-dihydroxyphenylethanol
DHPG	3,4-dihydroxyphenylglycol
DHPPA	3,4-dihydroxyphenylpyruvic acid
DOPA	β (3,4-dihydroxyphenyl) alanine
DOPAC	3,4-dihydroxyphenylacetic acid
GLC	gas liquid chromatography
GC	gas chromatography
GC-MS	combined gas chromatography/mass spectrometry
HMBA	4-hydroxy-3-methoxybenzylalcohol
HMPE	4-hydroxy-3-methoxyphenylethanol
HMPG	4-hydroxy-3-methoxyphenylglycol
HVA	homovanillic acid, 4-hydroxy-3-methoxyphenylacetic acid
5HT	5-hydroxytryptamine
5HIAA	5-hydroxyindoleacetic acid

5HTOH	5-hydroxytryptophol
MAO	monoamine oxidase
ME/TE	methylester/trimethylsilylether
m-HPAA	m-hydroxyphenylacetic acid
MU	methylene unit
NA	noradrenaline, β-(3,4-dihydroxyphenyl) β-hydroxy-ethylamine
o-HPAA	o-hydroxyphenylacetic acid
PE-N-MT	phenylethanolamine-N-methyltransferase
p-HMA	p-hydroxymandelic acid
p-HPE	p-hydroxyphenylethanol
p-HPG	p-hydroxyphenylglycol
p-HPAA	p-hydroxyphenylacetic acid
p-HPLA	p-hydroxyphenyllactic acid
p-HPPA	p-hydroxyphenylpyruvic acid
P-N-MT	phenylalkylamine-N-methyltransferase
TE/E	trimethylsilyl ether/ester
TLC	thin layer chromatography
VMA	4-hydroxy-3-methocymandelic acid

ABSTRACT

The theory and practice of gas chromatography have been described in detail. Such knowledge provides an essential background to an understanding of the various parameters and modifications of earlier procedures adopted in the gas chromatographic procedure finally devised. With this procedure it has been possible to measure accurately large numbers of aromatic acids and alcohols in urine.

The normal excretion range for a number of such compounds of importance in clinical practice was established. In general, values were in agreement with those obtained by other procedures both in man and rat. Satisfactory quantitative assay procedures had not previously been available for some of these compounds such as p-HPLA put out in excess in tyrosyluria, statistical analysis revealed a positive correlation between jaundice during the first week of life and tyrosyluria, which was more strongly associated with low gestational age than with low birth weight. A positive correlation between urine volume and the total excretion of HVA and VMA was also observed in this period of life.

Urine samples from a number of patients with carcinoid syndome were analysed for indolic metabolites, mainly for 5HIAA and 5HTOH. In all, an increased 5HIAA output was

observed accompanied by an elevated 5HTOH excretion. 5HTOH constitutes about 1% of the total 5HIAA excreted.

In urine samples from patients with catecholamine secreting tumours, a wide spectrum of phenolic metabolites was studied. As might have been expected, the metabolites most useful for the diagnosis of phaechromocytoma were VMA and perhaps HMPG. These together with HVA, DOPAC and HMPE were diagnostically helpful in the assessment of neuroblastoma. The occurrence of other metabolites such as p-HPLA, VLA, DHMA and DHPG was critically assessed.

Intravenous administration of reserpine caused a shift in the further metabolism of aldehyde intermediates of catecholamines and some other monoamines from a predominently oxidative pathway to a reductive one, resulting in an increased excretion of alcoholic metabolites at the expense of acids during the first nine hours after dosage.

In a group of post encephalic and idiopathic Parkinsonian patients, an extensive study of the excretion of phenolic acids and alcohols was carried out before, during and after oral L-DOPA therapy. No abnormality in catecholamine metabolite output could be detected in any of the pre-treatment patients. During treatment, a dramatic increase in the excretion of HVA and DOPAC were observed. The ratio of DOPAC to HVA was much higher than was found in neuroblastoma. A slight increase

only of β -hydroxylated catecholamine metabolites was observed, indication that DOPA was unlikely to be acting by virtue of its conversion to noradrenaline. Other metabolites which were elevated after L-DOPA include VLA, p-HPLA and m-HPAA. Experimental evidence has been provided to show that the latter owes its origin to p-dehydroxylation either of L-DOPA or one of its dihydroxy-metabolites by gut flora.

INDEX

Page

INTRODUCTION

Animals are well equipped with a versatile range of enzymes with which to convert precursor aromatic materials to the wide variety of compounds found in and excreted by the body (Williams, 1959). While most urinary aromatic metabolites in man (Armstrong, Shaw and Wall, 1956; Armstrong Shaw, Gortatowski and Singer, 1958; Mathiell and Revol, 1968) are probably derived from ingested substances foreign to the body economy (Williams, 1959) including drugs, flavourings, pesticides and beauty preparations, a proportion are derived from endogenous sources. Because the body is not easily able to split the benzene ring, this moiety forms a useful marker whose characteristic properties can be employed as the basis of a variety of physico-chemical approaches to assay. Such assay of the phenolic or indolic acids and alcohols, which are the end-results of a number of important metabolic pathways, has helped to shed considerable light on a variety of physiological and pathological states. Many different measuring principles have been utilised for the assay of these compounds. Visible and ultra-violet spectrophotometry (Sandler and Ruthven, 1959 a,b; Sunderman, Cleveland, Law and Sunderman, 1960; Sandler and Ruthven, 1961; Fellman, Severson, Robinson and Fujita, 1962; Pisano, Crout and Abraham, 1962; Ruthven and Sandler, 1962, 1964,

1965; Georges, 1964) and fluorimetry (Udenfriend, 1962) have perhaps been employed most widely for individual assays although many other approaches have been brought to bear on these problems including biological assay (Vane, 1964; Everett, 1964), infra-red spectrometry (Mandell, 1965; Warren Eisdorfer, Thompson and Zarembo, 1965), spectrophosphorimetry (St.John, Brook and Biggs, 1967), isotope dilution (Weise, McDonald and LaBrosse, 1961) and immunoassay (Filipp and Schneider, 1964; Ranadive and Sehon, 1967 a, b, c). A discussion of these aspects is beyond the scope of this thesis. Full reviews of assay procedures for indolic compounds are provided by Sandler (1963; 1968) and Hanson 1966. Information pertaining to the relatively large field of phenolic metabolite measurement is more widely scattered through the literature although useful reviews of some areas of the topic are provided by Ruthven (1963) and Sandler and Ruthven (1965).

Paper chromatography was one of the first techniques employed to study urinary phenolic (Armstrong et al, 1956) and indolic (Jepson, 1955; Armstrong et al, 1958) metabolites; it has subsequently been used widely (Jepson, 1969; Smith, 1969; Sandler and Ruthven, 1969) to unravel complex metabolic problems where semiquantitative information about a large number of different metabolites was required. Although many of these assays are now performed by thin layer chromatography (see Smith, 1969) which despite certain

drawbacks is quicker and more sensitive than paper chromatography, the approach is still at best semiquantitative unless tedious manoeuvres involving eluting material from the chromatogram (Sankoff and Sourkes, 1963) are employed. This difficulty is overcome by the introduction of gas chromatography which with its quality of speed, sensitivity, high resolution and quantitative interpretation (Dalgliesh, Horning, Horning, Knox and Yarger, 1966; Karoum, Ruthven and Sandler, 1968) has proved very useful in the study of a variety of diseases characterised by the urinary excretion of a wide spectrum of different metabolites (see later).

To carry out gas chromatographic investigations of phenolic and indolic metabolites, it is necessary to be familiar with many different metabolic pathways and their abnormalities. A sound understanding of the underlying principles of gas chromatography is also necessary for the planning and execution of suitable procedures. The first three chapters of this thesis are designed to go some way to providing this background.

CHAPTER 1

Biochemistry of aromatic amines

Metabolism of phenylalanine

This metabolic pathway has been particularly well studied because of the discovery of a number of inborn errors of metabolism involving its earlier enzymatic steps. One ramification of the pathway results in the production of catecholamines with their vast literature (see Second Symposium on Catecholamines, 1966; Glowinski and Baldessarini, 1966; Hornykiewicz, 1966; Iverson, 1967).

Phenylalanine-4-hydroxylase (E.C. 1.14.3.1).

The first major step in the metabolism of phenylalanine is its hydroxylation to tyrosine by the enzyme phenylalanine-4-hydroxylase (Kaufman, 1957) which is mainly localised in the liver (Udenfriend and Cooper, 1952) and is fairly specific towards phenylalanine. It is now well established that the enzyme is different from either tryptophan (Renson, Goodwin, Weissbach and Udenfriend, 1961; Renson, Weissbach and Udenfriend, 1962; Hagen and Leonard, 1966) or tyrosine hydroxylases (Sandler and Ruthven, 1968). While it has a weak action on tryptophan (Renson et al, 1961 and 1962), it has no effect on tyrosine (Ikeda, Levitt and Udenfriend, 1965). Hydroxylation of phenylalanine in the adrenal medullar, the brain and organs supplied by the sympathetic nervous system, is believed to be catalysed by tyrosine hydroxylase (Udenfriend,

1966; Ikeda, Levitt and Udenfriend, 1967).

Liver phenylalanine hydroxylase, like tryptophan hydroxylase (Renson et al, 1961), consists of two protein fractions or enzymes (Udenfriend, Weissbach and Clark, 1955; Mitoma, 1956; Kaufman, 1959; Kaufman and Lovenberg, 1959). One effects the hydroxylation while the other keeps the coenzyme in an active form (Kaufman, 1959). Hydroxylation of phenylalanine (Kaufman, 1958; Kaufman, 1963 a, b) as of tyrosine (Brenneman and Kaufman, 1964; Kaufman, 1964) and tryptophan (Grahame-Smith, 1967; Jequie, Robinson, Lovenberg and Sjoerdsma, 1969) requires tetrahydropteridine or closely related derivatives, $NADPH_2$, Fe^{++} and atmospheric oxygen (Kaufman, 1966).

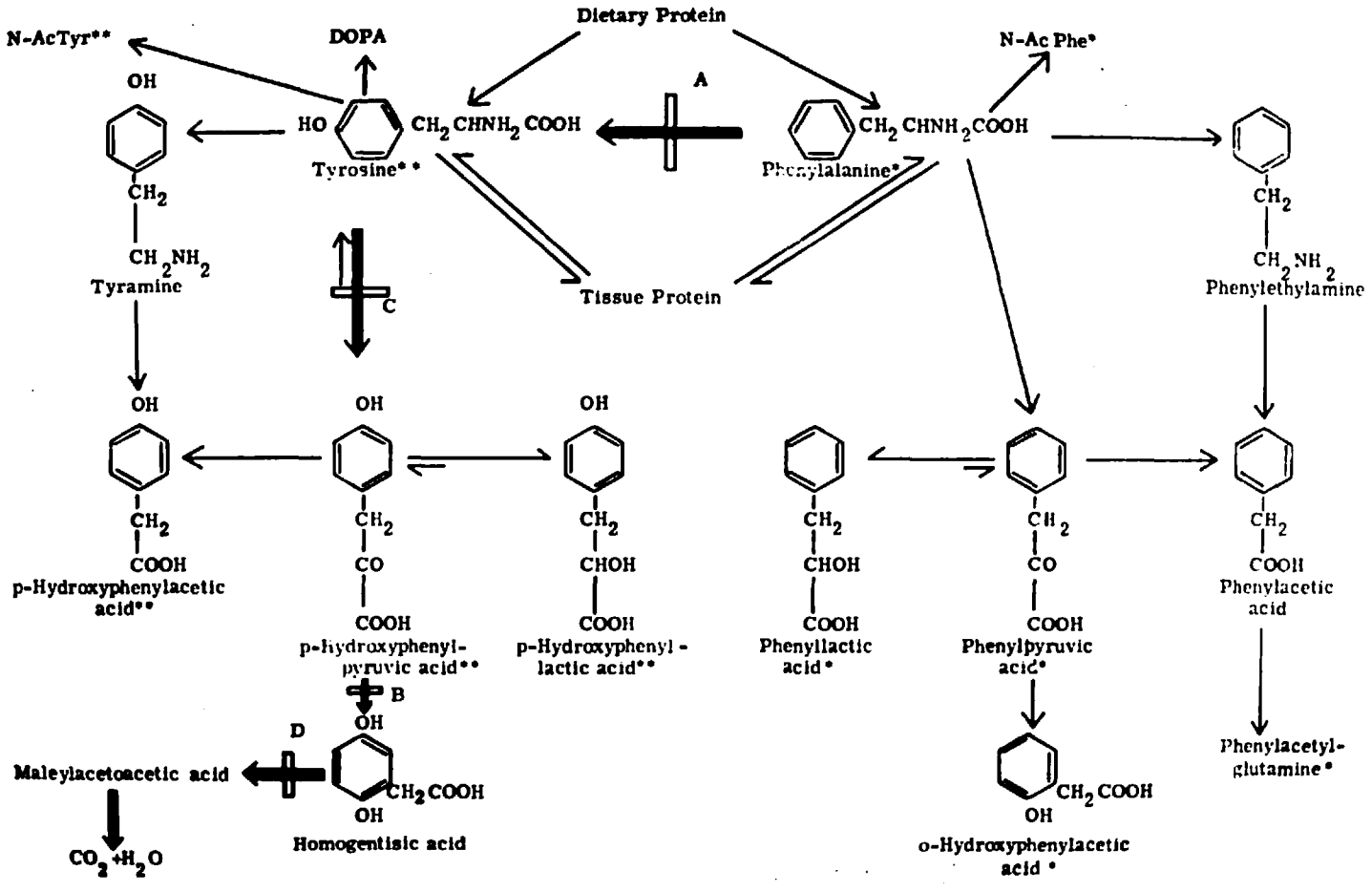
Tyrosine major pathway

Tyrosine is mainly metabolised through transamination by a liver transaminase (E.C. 2.6.1.5) (Canellakis and Cohen, 1956; Kenney, 1959; Jacoby and Ia Du, 1962; Haavaldsen and Horseth, 1965) to p-hydroxyphenylpyruvic acid (p-HPPA). There are apparently two forms of tyrosine transaminase, one soluble and the other mitochondrial. Very little of the soluble variety is found in the foetus and neonate compared with the mitochondrial form. After birth however, the activities of both rise steadily although that of the supernatant rises faster. As a result, the level of the soluble enzyme is higher than that of the mitochondria during the post-natal period and in adults (Koler, Vanbellinghen, Fellman, Jones

and Behrman, 1969). p-HPPA is oxidised by p-HPPA oxidase to homogentisic acid (Schefartz and Gurin, 1949; Taniguchi, Kappe and Armstrong, 1964), a reaction associated with what has now come to be called the "NIH shift" (Guroff, Daly, Jerina, Renson, Witkop and Udenfriend, 1967) coupled with decarboxylation. Further degradation of homogentisic acid involves oxidation by homogentisic acid oxidase, with splitting of the benzene ring to maleylacetoacetic acid which undergoes β-oxidation and enters the citric acid cycle (Fig. 1.1). For a more detailed account of the metabolism of tyrosine, see Dalgliesh (1955) and Knox, (1955).

Catecholamines biosynthesis

Dopamine, noradrenaline (NA) and adrenaline (A) are the main catecholamines found in mammals. Their synthesis is largely restricted to structures derived from the embryonic neural crest (Käser, 1966). They have thus been detected in many organs supplied by sympathetic nerves where the levels of catecholamines are directly related to the extent of sympathetic innervation. For example, in the vas deferens, the dense supply of sympathetic nerves (Sjostrand, 1965; Falck, Owman and Sjostrand, 1965; Owman and Sjostrand, 1965) is reflected by its high content of noradrenaline. It is now well established that NA is released from sympathetic nerve endings, acting as a transmitter (Euler, 1956).



The Metabolism of Phenylalaaine and Tyrosine.

Fig.1.1. **Key : A= metabolic block in phenylketonuria; B= metabolic block in tyrosinosis; (C) + B= metabolic block in neonatal hypertyrosinaemia; D= metabolic block in alkaptonuria. ▬ = Main degradative pathway. * Excreted in phenylketonuria. ** Excreted in "tyrosinoses".**

Dopamine (Holtz and Credner, 1942) which is present in high concentration in the basal ganglia of the brain may have a similar function (Hornykiewicz, 1966). Adrenaline might also play a role as a neuro-transmitter, particularly in the frog (Loewi, 1921; Axelrod, 1966) and certain birds (Axelrod, 1966).

Ever since the discovery of A by Oliver and Schäfer in 1894, the biosynthetic pathway of the catecholamines has been the subject of much speculation. The metabolic route suggested by Blaschko (1939) was finally established by radio-isotopic studies as the main pathway. During these studies, phenylalanine (Gurin and Delluva, 1947), tyrosine (Udenfriend and Wyngaarden, 1956; Goodall and Kirshner, 1957), DOPA and dopamine (Leeper and Udenfriend, 1956) were conclusively shown to be precursors of NA.

Tyrosine hydroxylase (E.C. 1.14.3.?)

The first step in the synthesis of the catecholamines was the last to be characterised. It involves the conversion of tyrosine to DOPA by tyrosine hydroxylase (Nagatsu, Levitt and Udenfriend, 1964). This enzyme has now been demonstrated in adrenal medulla, brain, heart and other sympathetically innervated tissues of a variety of animal species (Udenfriend, 1966). It has recently been shown to be located exclusively within the neurones in the adrenal glands (Nagatsu, Lyman, Rust and DeQuattro, 1969). The putative role of tyrosinase (E.C. 1.10.3.1.) in the hydroxylation of tyrosine (Udenfriend

1966), has now been completely dismissed. The subcellular distribution of the enzyme is debatable. While some authors have claimed that it is associated with subcellular particles in the adrenal medulla (Nagatsu et al, 1964; Petrack, Sheppy and Fetzer, 1968), brain (Nagatsu et al, 1964; Bagchi and McGeer, 1964) and sympathetically innervated tissues (Nagatsu et al, 1964), others have shown it to be essentially a cytoplasmic enzyme in the beef adrenal (Musacchio, 1968; Laduron and Belpaire, 1968; Musacchio and Wurzburger, 1969).

The cofactor requirements of the enzyme are the same as those of phenylalanine hydroxylase (Udenfriend et al, 1966; Petrack et al, 1968). The purified enzyme is specific for tyrosine and phenylalanine, in contrast to phenylalanine hydroxylase which could not hydroxylate tyrosine (Ikeda et al, 1965). However it does not hydroxylate D-tyrosine, tyramine or tryptophan (Udenfriend, 1966). This finding implies that the liver is not the only site of tyrosine production and that in nervous tissues and related chromaffin structures the conversion of phenylalanine to tyrosine and tyrosine to DOPA may be carried out by the same enzyme (Udenfriend, 1966; Sandler and Ruthven, 1969). This last view is supported by evidence for an extrahepatic source of tyrosine in phenylketonuria (Udenfriend and Bessman, 1953).

There is considerable evidence to suggest that hydroxylation of tyrosine is the rate-limiting step in the

biosynthesis of NA. Thus when tyrosine, DOPA and dopamine were in turn perfused through the guinea-pig heart the maximum rate of conversion to NA was only achieved with tyrosine, with a Michaelis constant (K_m) value approximately similar to that of tyrosine hydroxylase (Levitt, Spector, Sjoerdsma and Udenfriend, 1965). Further support was furnished from *in vivo* studies with inhibitors of tyrosine hydroxylase, from which the extent of inhibition of NA synthesis was found to be exactly the same as that of tyrosine hydroxylase (Udenfriend *et al*, 1966; Udenfriend, Zaltzman-Nirenberg, Gordon and Spector, 1966a). The rate of tyrosine hydroxylation in the bovine adrenal medulla (Petrack *et al*, 1968) was found to correspond well with the activities of DOPA decarboxylase and dopamine β-hydroxylase reported by Nagatsu *et al* (1964). This good correlation formed the basis of a suggestion put forward by Petrack *et al* (1968) that the rate of tyrosine hydroxylation *in vivo* may be limited by availability of substrate and cofactor rather than by catalytic activity of the enzyme. On the other hand, the possibility exists that a negative feed-back mechanism involving inhibition of tyrosine hydroxylase by A and NA, acts as the rate limiting factor in the biosynthesis of catecholamines (Nagatsu *et al*, 1964; Neff and Costa, 1966) although this view has been disputed (Petrack *et al*, 1968).

DOPA decarboxylase (5HTP decarboxylase)

This was the first enzyme involved in catecholamine biosynthesis to be characterised (Holtz, Heise and Ludtke, 1938; Holtz, 1939). It is widely distributed in mammalian tissues, including the central nervous system (Goodall and Kirshner, 1958). Its intracellular localisation in the cytoplasm (Laduron et al., 1968) indicates that decarboxylation of DOPA takes place outside the granule were tyrosine is hydroxylated - provided that tyrosine hydroxylase is accepted as a particulate enzyme (Udenfriend, 1966) but if tyrosine hydroxylase is a cytoplasmic enzyme, as is likely to be the case with the bovine adrenal medulla (Musacchio, 1968; Musacchio et al., 1969) then the reactions converting tyrosine to dopamine take place in the cytoplasm (Fig. 1.2).

DOPA decarboxylase has been claimed to be relatively non-specific for a number of L-aromatic amino acids (Lovenberg, Weissbach and Udenfriend, 1962). Certainly the likelihood of its identity with 5 HTP decarboxylase is high as is evident from their many similarities in tissue distribution (Westerman, Balzer and Knell, 1958), affinity towards both DOPA and 5HT (Westerman et al., 1958; Fellman, 1959; Worle and Aures, 1959; Yuwiler, Geller and Eiduson, 1959; Rosengren, 1960; Udenfriend, Lovenberg and Weissbach, 1960; Kuntzman, Shore, Bogdanski and Brodie, 1961; Hagen,

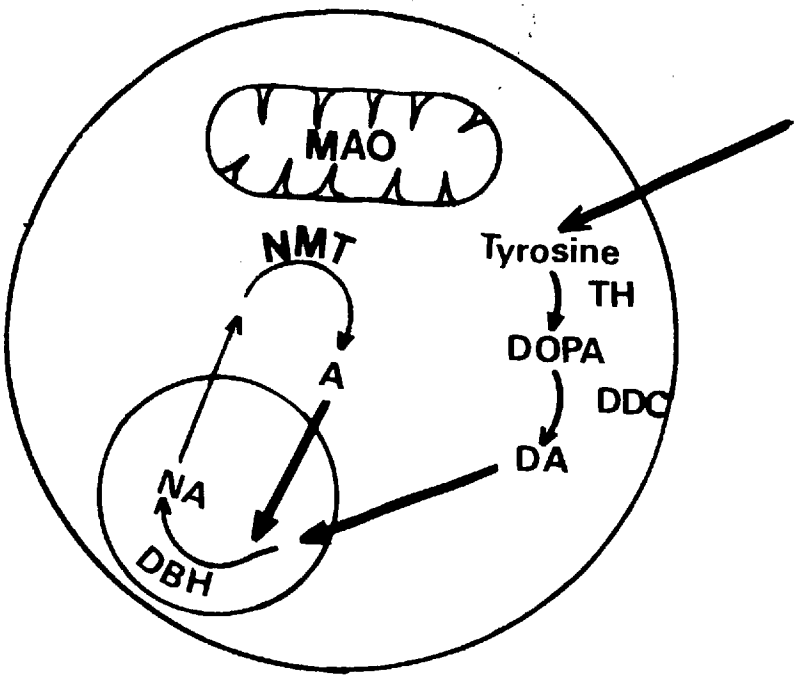


Fig. 1.2. Model for the intracellular biosynthesis of catecholamines in the adrenal medulla. Abbreviations used: TH = Tyrosine hydroxylase, DDC = dopa decarboxylase, DA = dopamine, DBH = dopamine- -hydroxylase, NA = noradrenaline, NMT = N-methyltransferase, A = adrenaline. After Laduron and Belpaire, (1968).

1962; Lovenberg et al., 1962), from competition experiments in which DOPA and 5HTP were incubated together or separately with preparations of the enzyme (Bertler and Rosengren, 1959; Fellman, 1959; Werle et al., 1959; Yuwiler et al., 1959; Hagen, 1962) and from inhibition studies (Westermann et al., 1958). It will decarboxylate to some extent o- and m-tyrosine (Blaschko and Sloane-Stanley, 1948; Blaschko and Holton, 1950) and other amino acids including tryptophan, phenylalanine and p-tyrosine (Lovenberg et al., 1962; Lovenberg, Barchas, Weissbach and Udenfriend, 1963) although to an extent which probably reflects an insignificant in vivo role (Awapara, Sandman, and Hanly, 1962; Awapara, Perry, Hanly and Peck, 1964). The enzyme is specific for the L-isomer of DOPA (Holtz et al., 1938; Holtz, 1939) and 5HTP (Clark, Weissbach and Udenfriend, 1954). It can also decarboxylate 4-hydroxytryptophan (Erspamer and Glasser, 1960; Erspamer, Glasser, Pasini and Stoppani, 1961), but cannot function when the hydroxyl group is on the 7 (Udenfriend, Clark and Titus 1953; Erspamer et al., 1961) 6 and 2 (Erspamer et al., 1961) positions.

Pyridoxal phosphate is needed as a cofactor (Garattini and Valzelli, 1965) but the manner in which enzyme and coenzyme interact is complicated, depending on source of enzyme, type of substrate, pH and the way the enzyme is stored (Awapara

et al., 1962; Sourkes, 1966).

DOPA decarboxylase is widely distributed in mammalian tissue (Gaddum and Giarman, 1956; Holtz, 1959) with the highest activities being found in the kidney, liver, adrenal medulla, sympathetic ganglia and intestine. High activities has also been found in certain neoplastic tissues e.g. in phaeochromocytoma (Burger and Langemann, 1956; Hagen, 1962; Langemann, Boner and Muller, 1962), carcinoid (Hagen, 1962; Langemann et al., 1962) and neoplastic mouse mast cell tissues (Lagunoff, Lam, Roeper and Benditt, 1957; Hagen and Lee, 1958; Hagen, Ono and Lee, 1960).

The pharmacological properties of compounds which act as DOPA decarboxylase inhibitors (Clark and Pegrund, 1961; Clark, 1963) have stimulated much investigatory activity in their mode of action and their therapeutic potentials, with particular emphasis on the treatment of hypertension (Martin, Brendel and Beiler, 1950; Hartman and Clark, 1955; Hartman, Akawie and Clark, 1955). A full discussion of this theme (Sandler and Ruthven, 1969) is beyond the scope of this thesis. It should be noted in passing however, that such compounds as α-methyldopa are unlikely to exert their anti-hypertensive effect by virtue of an inhibitory action on DOPA decarboxylase. Although there is some dispute about their precise mode of action, it is likely that decarboxylated

metabolites of the drug may compete with NA at its receptor site (Sourkes, 1965), acting as "false transmitters" (Kopin, Fischer, Musacchio, Horst and Weise, 1965).

Dopamine-β-hydroxylase (E.C.1.14.2.1.)

The next step in catecholamine biosynthesis is β-hydroxylation of dopamine to NA by dopamine-β-hydroxylase (Goldstein, Friedhoff, Simmons and Prochoroff, 1960). The enzyme was first characterised by Levin, Levenberg and Kaufman, 1959), although its action had previously been demonstrated both in vivo (Leeper and Udenfriend, 1956) and in vitro (Hagen, 1956; Neri, Hayano, Stone, Dorfman and Elmadjian, 1956; Goodall and Kirshner, 1957; Kirshner, 1959). The enzyme is located in the catecholamine storing granules (Kirshner, 1957; Musacchio, Kopin and Snyder, 1964; Oka, Kajikawa, Ohuchi, Yoshida and Imaisumi, 1957) probably bound to the inner surface of the vesicular membrane (Kaufman and Friedman, 1965). It is found in the adrenal medulla (Pisano, Creveling and Udenfriend, 1960), brain (Udenfriend and Creveling, 1959), heart (Chidsey, Kaiser and Braunwald, 1963), sympathetic nerve granules (Potter and Axelrod, 1963) and in phaeochromocytoma and neuroblastoma tumour tissue (Goldstein, Prochoroff and Sirlin, 1965a; Bohuon and Guerinot, 1966).

Following its purification from bovine adrenal particles (Levin Levenberg and Kaufman, 1960), studies have been carried

out on its composition, properties and kinetics (Goldstein, Lauber and McKereghan, 1965). It was shown to be a metallo-enzyme (Levin et al., 1960; Kirshner, 1962; Goldstein, 1962; Green, 1964) with copper as the prosthetic group (Friedman and Kaufman, 1965a; Goldstein, Lauber, Blumberg and Peisach, 1965; Friedman and Kaufman, 1965) and to have a molecular weight of 290,000 (Goldstein et al., 1965; Friedman et al, 1965, 1965a). The enzyme requires ascorbic and fumaric acids as cofactors (Levin et al., 1960). A catechol compound can substitute for ascorbic acid (Levin and Kaufman, 1961). Apart from these factors ATP (Levin et al., 1961; Goldstein and Contrera, 1962), a divalent metal such as Mn^{++}, molecular oxygen (Kaufman, Bridgers, Eisenberg and Friedman, 1962) and -SH groups (Goldstein, McKereghan and Lauber, 1963) are also needed.

The enzyme is nonspecific and accepts a variety of sympathomimetic amines including phenylethylamine, tyramine, epinine, 3-methoxydopamine and other phenylethylamine and phenylpropylamines (Levin et al., 1961; Goldstein and Contrera, 1961; Goldstein et al., 1962; Creveling, Daly, Witkop and Udenfriend, 1962; Bridges and Kaufman, 1962) as substrates. Some compounds structurally related to dopamine, which are not substrates for the enzyme, act as inhibitors (Goldstein, Musacchio, Kenin, Contrera and Rice, 1962; Creveling, van Der Schoot, and Udenfriend, 1962a; van Der Schoot, Creveling,

Nagatsu and Udenfriend, 1963). For further information on the inhibition of dopamine-β-hydroxylase, see Sandler et al., (1969).

Phenylethanolamine-N-methyltransferase (E.C.2.1.1.?)

In mammalian adrenal glands (Axelrod, 1966), the conversion of NA to its N-methylated analogue A is catalysed by phenylethanolamine-N-methyltransferase, PE-N-MT, (Axelrod, 1962) using S-adenosylmethionine as methyl donor (Harris, 1961; Axelrod, 1962). Other phenylethanolamines including normetadrenaline and octopamine, together with secondary amines such as A and metadrenaline, and drugs structurally related to phenylethanolamine will act as substrates (Axelrod, 1962; 1966). The enzyme is however, absolutely specific for phenylethanolamine derivatives and has no action on phenylethylamines (Axelrod, 1966).

PE-N-MT is present in the soluble supernatant fraction of the adrenal medulla of a variety of species (Axelrod, 1962) including man (Kitabchi and Williams, 1969). It is also present in relatively small amount in other organs e.g. brain (McGeer and McGeer, 1964; Pohorecky, Zigmond, Karten and Wurtman, 1969) and heart (Axelrod, 1962). The synthesis of A will therefore require NA to leave the chromaffin granules and enter the cytoplasm, where it is N-methylated. Adrenaline then returns to the storage granules to be stored until it is

liberated. There is now evidence however to suggest the intragranular synthesis of A in the bovine adrenal medulla (Goldstein, Gang and Joh, 1969). A wider distribution of the enzyme is found in some non-mammalian species, particularly amphibians such as the frog (Loewi, 1921; Falck, Haggendal and Owman, 1963; Axelrod, 1966) and some birds (Axelrod, 1966). It is thus possible that adrenaline acts as a neurotransmitter substance in these species (Axelrod, 1966).

Much recent information is available relevant to the production and control of A in the adrenal medulla. Adrenaline appear to be responsible for product inhibition of PE-N-MT (Fuller and Hunt, 1967). A very high local concentration of adrenal cortical corticosteroid is necessary for N-methylation (Wurtman and Axelrod, 1965; 1966; Wurtman, 1966). Thus a production in the adrenal medulla is probably controlled at two levels.

Phenylalkylamine-N-methyltransferase (E.C.2.1.1.?)

Phenylalkylamine-N-methyltransferase, P-N-MT, was first characterised by Axelrod and his colleagues (Axelrod, 1961; 1962a, b; Marki, Axelrod and Witkop, 1962). Unlike PE-N-MT, it is relatively non-specific and has been found to N-methylate phenylethanolamines, phenylethylamines and indolalkylamines. Like PE-N-MT, S-adenosylmethionine is required as methyl donor, but Mg^{++} or glutathione do not seem

to be necessary for the reaction.

P-N-MT has been detected in substantial amount in the parotid gland of a South American toad (Marki et al., 1962) and in human and rabbit lung (Axelrod, 1961; 1962b). It is present in relatively low activity in a number of mammalian organs including the adrenal medulla and the heart (Sandler et al., 1969). Its physiological role is unknown.

Minor biosynthetic pathways of catecholamines

Whether a compound is actually produced in the body depends not only on the existence there of an enzyme capable of its synthesis but on many other factors such as availability of substrate, subcellular localisation and enzyme control mechanisms. Nevertheless, it should be noted that most of the enzymes involved in catecholamine biosynthesis are not absolutely specific so that other products of their action are theoretically possible. Indeed many such minor metabolic pathways have been identified in various species. For a detailed review reference should be made to Sandler and Ruthven (1969). These pathways probably lead to the biosynthesis of certain biologically active amines such as tyramine and octopamine (Pisano et al., 1960; Musacchio and Goldstein, 1963) and synephrine (Axelrod, 1962). A schematic representation of some of them is shown in Fig. 1.3.

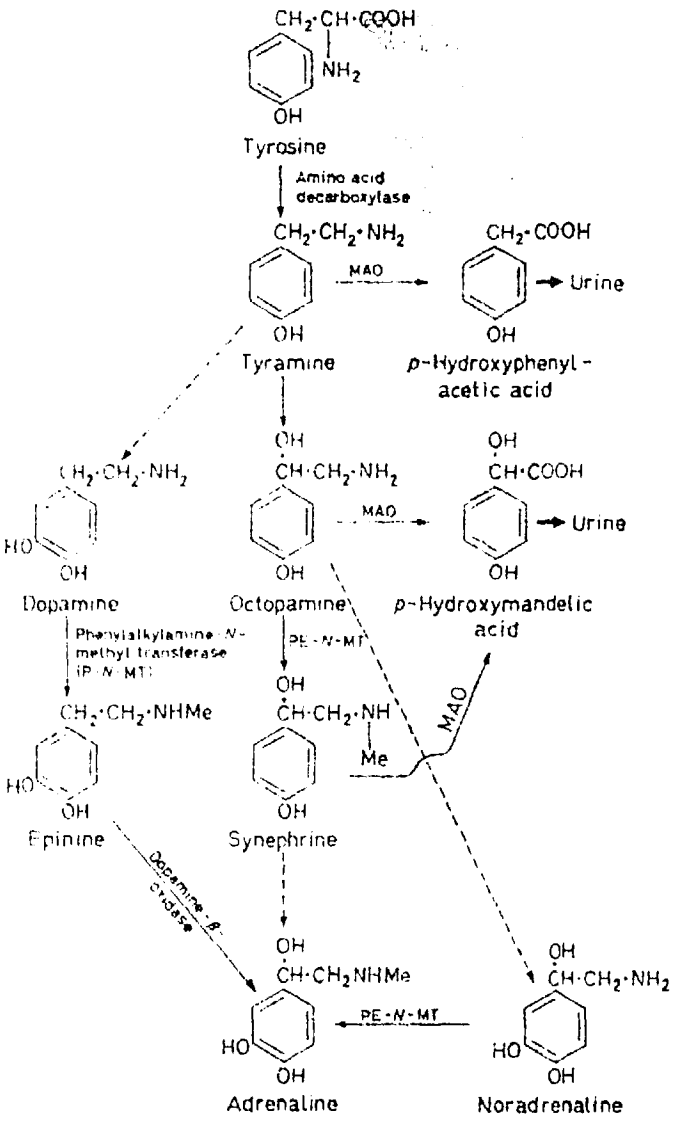


Fig. 1.3. Alternative pathways of catecholamine formation. Reproduced from Sandler and Ruthven (1969_a).

Fate of catecholamines in the organism

The inactivation of catecholamines and of other biologically active monoamines is achieved via two processes which together are responsible for the short duration of their effect. The first is associated with tissue uptake (Iversen, 1967 a, b; 1969) and the second with metabolic degradation.

The end-products of metabolism are excreted largely in the urine and play no further part in the body economy. The measurement of urinary metabolites of biologically active monoamines has been widely employed as a diagnostic index in both man and the experimental animal. The importance of this approach is reflected by the number and diversity of methodological approaches employed for such measurements. Gas chromatographic assay is but the latest addition to this methodological armamentarium but is likely to be the most fruitful.

Metabolism of catecholamines

As with those responsible for biosynthesis, the enzymes which bring about degradation of the catecholamines are relatively non-specific.

The major metabolic pathway of catecholamines is by oxidative deamination and O-methylation (Fig. 1.4). The

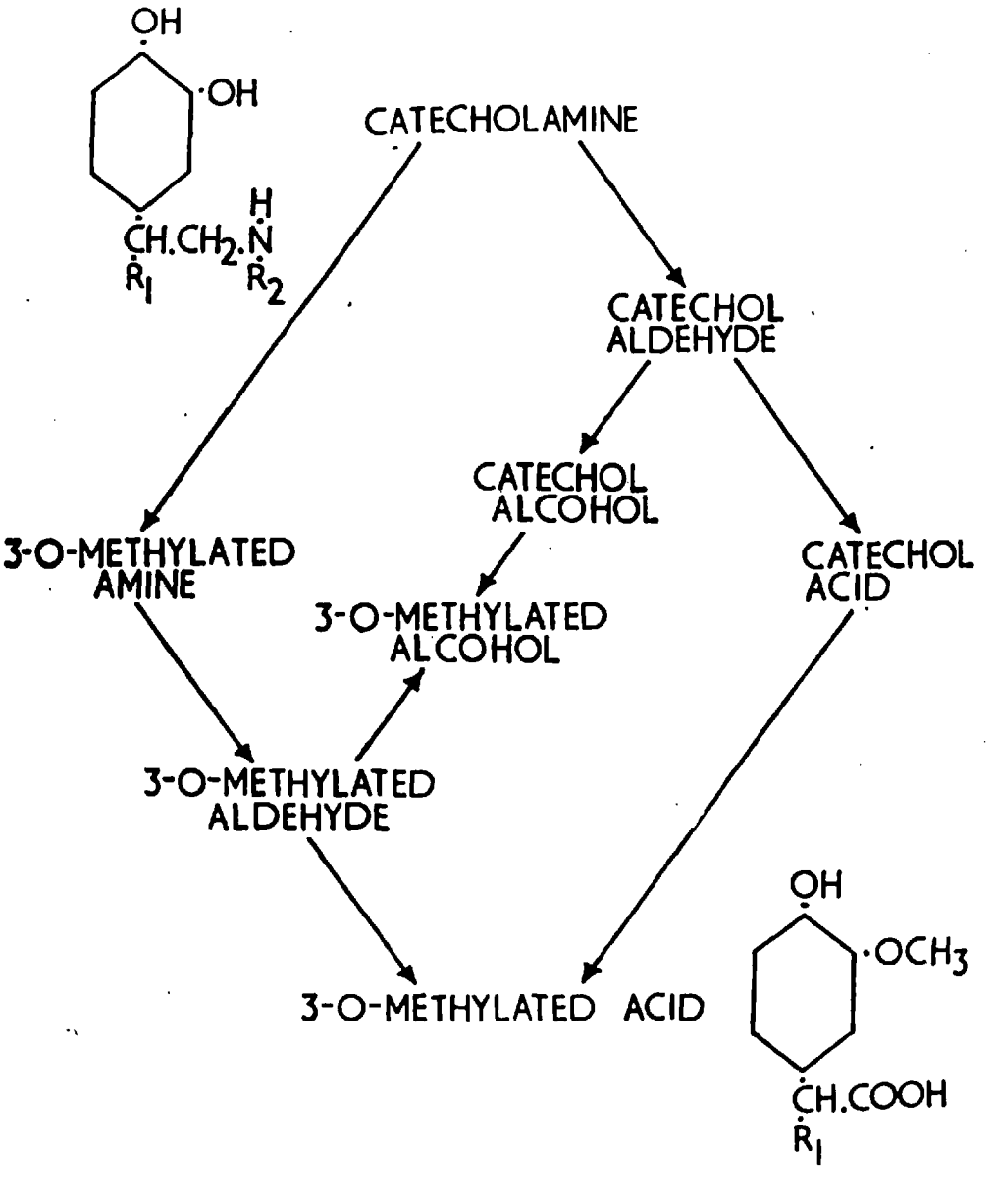


Fig. 1.4. General scheme of catecholamine metabolism; dopamine pathway - R_1 = H, R_2 = H, noradrenaline pathway - R_1 = OH, R_2 = H, adrenaline pathway - R_1 = OH, R_2 = Me

first is catalysed by monoamine oxidase (MAO) and the second by catechol-O-methyltransferase (COMT). Their relative importance appears to depend on a number of factors, not the least of which is anatomical site e.g. MAO may play a more important role in the brain and sympathetic nervous system (Glowinski et al., 1966) whilst COMT is more prominent extraneuronally (LaBrosse, Axelrod and Kopin, 1961; Axelrod, 1966) (Fig. 1.5). The agent responsible for amine liberation (Iversen, 1967; Kopin, 1968) also tends to produce a characteristic metabolic pattern. Thus nerve stimulation and tyramine appear to liberate NA from a small intracellular pool of rapid turnover where the primary inactivation mechanism is by COMT: conversely, drugs such as reserpine which release NA from a different, larger pool, tend to bring about inactivation primarily by MAO. For a full discussion of this problem see Kopin and Gordon (1962; 1963), Kopin (1965) and Sandler and Youdim (1968).

Monoamine oxidase (E.C. 1.4.3.4.)

Monoamine oxidase (MAO) catalyses the oxidative deamination of a number of aliphatic and aromatic monoamines to the corresponding aldehyde (Blaschko, 1952). It is relatively non-specific but its affinity towards its known substrates varies greatly with time and species source of the

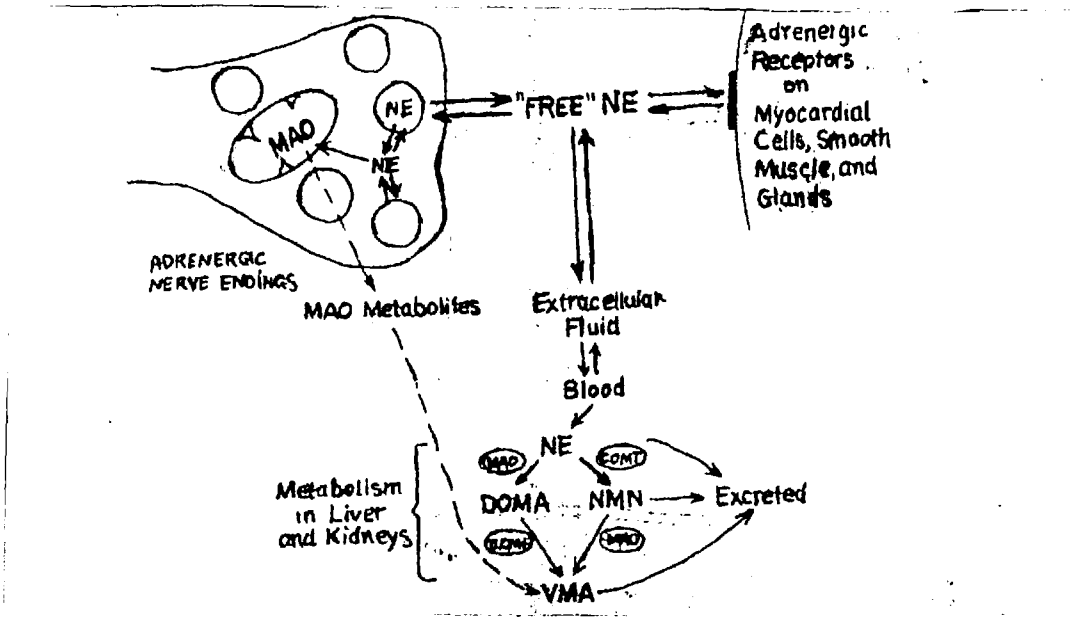


Fig. 1.5. Schematic representation of the metabolic fate of NA in adrenergic nerve endings, at receptor sites, and in the liver and kidneys. Note that urinary NA and NMN are derived from "free" NA while urinary VMA is derived from both "free" NA and intraneuronal NA. After Crout (1968).

enzyme. In general however, its activity is high against monophenolic and catecholic amines, moderate towards p-hydroxy substituted compounds and absent when the amine is attached to the ring (Blaschko, 1952; 1963). Dopamine, p-hydroxyphenylethylamine and tyramine are better substrates for MAO than A and NA (Blaschko, Richter and Schlossmann, 1937; Blaschko, 1952; 1963; Leeper, Weissbach and Udenfriend, 1958). The enzyme tends to exhibit some stereospecificity towards certain enantiamorphs, depending on the compound; (-)-noradrenaline and A are better substrates than the corresponding (+)-form, (Giachetti and Shore, 1966).

The distribution of the enzyme is very wide. It has been detected in many vertebrate organs and tissues where it is present in particulate organelles, mainly mitochondria (Oswald and Strittmater, 1963; Baudhin, Beaufay, Rahman, Sellinger, Wattiaux, Jacques and de Duve, 1964). A smaller proportion is probably present in the microsomal or supernatant fractions (de Champlain, Axelrod, Krakoff and Mueller, 1968). Within the mitochondria, MAO activity is probably localised on the outer membrane (Schnaitman, Erwin and Greenawalt, 1967; Boadle and Bloom, 1969).

Latterly, MAO has been extensively studied in a solubilised, purified and stable form (Barbato and Abood, 1963; Gorkin, 1963; Coq and Baron 1964; Guha and Kirshna Murti,

1965; Nara and Yasunobu, 1966; Youdim and Sourkes, 1966). It has a molecular weight of about 290,000 and its absorption spectrum shows two extinction peaks at 280 nm and 410 nm (oxidised form) or 460 nm (reduced form) (Youdim et al., 1966). The enzyme is considered to be flavoprotein (Wiseman-Distler and Sourkes, 1963) with 8 - SH groups associated with each molecule (Erwin and Hellerman, 1967). Inhibition of the enzyme by chelating agents (Gorkin and Romanova, 1959) points to the involvement of a covalent metal, but attempts to identify it have not been successful (Youdim et al., 1966; Erwin et al., 1967).

The inhomogeneity of the enzyme towards inhibitors (Pletscher, 1966) together with kinetic studies, provide indirect evidence that MAO is not a single enzyme (Gorkin et al., 1959; Gorkin, 1963; Wiseman-Distler et al., 1963; Nara et al., 1966; Gorkin, 1966; Pletscher, 1966). Recently, direct evidence (Youdim and Sandler, 1967; Collins, Youdim and Sandler, 1968; Youdim, Collins and Sandler, 1968; Youdim, Collins and Sandler, 1969), employing disc electrophoresis, has established the existence of multiple forms of MAO which migrate at different rates through polyacrylamide and possess differing physico-chemical characteristics. The possibility of this finding being an artifact cannot be completely excluded, however, although the constant and reproducible

electrophoretic behaviour of these bands in relation to the tissue and species from which they derive makes this unlikely.

Catechol-O-methyltransferase

Methylation of substituted cyclic compounds constitutes an important detoxication or inactivation mechanism in all living organisms. Of the many methylating enzymes, COMT is among the most important because of its role in the inactivation of circulating catecholamines (Axelrod, 1959). Together with MAO, it is largely responsible for the metabolic degradation of catecholamines.

The enzyme is widely distributed; high concentrations are present in the nervous system although the highest levels have been detected in the liver and kidneys (Axelrod, Albers, and Clemente, 1959a). It has been detected in neuroblastoma tissue (La Brosse and Karon, 1962). Its activity doubles in the rat uterus during pregnancy (Wurtman, Axelrod and Potter, 1964), and appreciable amounts have been detected in the human placenta (Iisalo and Castron, 1967). Within the cell, COMT is mainly confined to the soluble supernatant fraction (Axelrod and Tomchick, 1958). An atypical form of COMT appears to be present in the microsomal fraction of liver homogenates. This variant differs from the soluble

form with respect to pH optimum and response to cold stress and benzpyrene (Inscoe, Daly and Axelrod, 1965). Polyacrylamide gel electrophoresis has revealed multiple bands of activity suggesting the existence of isoenzymes (Anderson and D'Iorio, 1968).

Studies on the purified enzyme (Axelrod et al., 1958; Anderson, Ziegler and Doeden, 1968) using both isotopic and non-isotopic procedures (Axelrod, 1966) and Sephadex column chromatography (Anderson et al., 1968), have shown it to exhibit no stereo-specificity but specific for catechols as a class. It appears to have a molecular weight of 29,000 and employs S-adenosylmethionine as methyl donor. For the reaction to take place Mg^{++}, or another divalent cation such as Mn^{++}, Co^{++}, Ca^{++}, Zn^{++}, Fe^{++} or Ni^{++} in descending order, is necessary as cofactor.

O-Methylation tends to occur predominently at the 3-position although, depending on the nature of the side chain, the 4-hydroxyl group can be affected (Senoh, Daly, Axelrod and Witkop, 1959; Daly, Axelrod and Witkop, 1960). All naturally occurring catechols are O-methylated in the 3-position and it has been difficult to find evidence for 4-methylation of these compounds in vivo (von Studnitz, 1967).

Aldehyde oxidation and reduction

The aldehyde resulting from the oxidative deamination

of a monoamine by MAO may be further metabolised by two alternative reactions involving its transformation by aldehyde dehydrogenase to the corresponding acid or by aldehyde reductase to the alcohol. The proportion of intermediate aldehyde further metabolised by an oxidative or a reductive pathway depends on the nature of the side chain and, in particular, on whether a β-hydroxyl group is present (Breese, Chase and Kopin, 1968; 1969). Thus phenylethylamines are predominantly degraded to their corresponding acid, whereas reduction to the alcohol assumes greater importance in the further metabolism of phenylethanolamines; the proportion depends to a large extent on the species. Reduction seems to predominate in some tissues, notably the brain (Mannarino et al., 1963; Glowinski et al., 1965; 1966; Rutledge and Jonsson, 1967) where NA is mainly metabolised to HMPG. Even in the brain however, phenylethylamines e.g. dopamine (von Euler, 1958; Rosengren, 1960; Sharman, 1963) and tyramine (Breese et al., 1968) are mainly metabolised to their corresponding acid. In man, a greater proportion of the β-hydroxylated catecholamines, A and NA, is converted to VMA than in species such as the rat where HMPG is the major metabolite (Kopin, Axelrod and Gordon, 1961; Kopin and Gordon, 1962). Dopamine and other phenylethylamines are almost wholly metabolised by the

oxidative route, independently of species, and only very small amounts of alcohols such as HMPE (Goldstein, Friedhoff, Pomerants and Contrera, 1961) and dihydroxyphenylethanol (Goldstein, Friedhoff, Pomerants and Simmonds, 1960; Goldstein et al., 1960$_a$) and p-hydroxyphenylethanol can normally be detected (see context of this thesis).

A number of different agents, including ethanol (Smith, Gitlow, Gall, Wortis and Mandlowitz, 1960; Davis, Brown, Huff and Cashaw, 1967) and reserpine (Sandler and Youdim, 1968), produce a shift in the normal metabolism of the catechol aldehyde from an oxidative to a reductive pathway. The underlying mechanism of this shift will be discussed in some detail in chapter 9.

Other metabolic routes

Apart from the metabolic route of degradation already described above, there exist other pathways for the metabolism of catecholamines and their precursor, L-DOPA. Although quantitatively these pathways are of minor importance, in certain pathological conditions associated with increased production of catecholamines and their precursors they may become exaggerated.

Of particular interest is that involving the transamination of DOPA or its O-methylated derivative,

vanilalanine, to their corresponding pyruvic acids, dihydroxyphenylpyruvic (DHPPA) and vanilpyruvic (VPA) acids. These keto acids might further be reduced to their lactic forms giving dihydroxyphenyllactic (DHPLA) and vanillactic (VLA) acids respectively (Fig. 6). Thus in melanoma (Coward, Smith and Middleton, 1967; Duchon, Matous and Prochazkova, 1967), neuroblastoma (Gjessing, 1963$_a$; 1965) malignant phaeochromocytoma and a number of other disorders such as cancer and rheumatoid arthritis (Smith, 1965), an increased urinary output of these metabolites has been reported. In this connection there is evidence of an alternative pathway (Gey, 1968) whereby p-HPPA gives rise to DOPA both <u>in vivo</u> and <u>in vitro</u>, presumably through m-hydroxylation followed by transamination.

Other metabolic routes which appear to be less important than transamination include <u>in vivo</u> O-demethylation of normetadrenaline, metadrenaline and other methylated catechols (Axelrod and Saara, 1958$_a$; Daly <u>et al</u>., 1960)and <u>in vivo</u> N-demethylation of metadrenaline in the rabbit (de Potter, Bacq, Oriel, De Schaepdryver and Renson, 1963) and the cat (Verley, Koch and Hunebelle, 1962); <u>in vitro</u>, metadrenaline has been connected to normetadrenaline by a rabbit liver microsomal preparation (Axelrod, 1960). Other minor metabolic routes include ring dehydroxylation (DeEds, Booth and Jones,

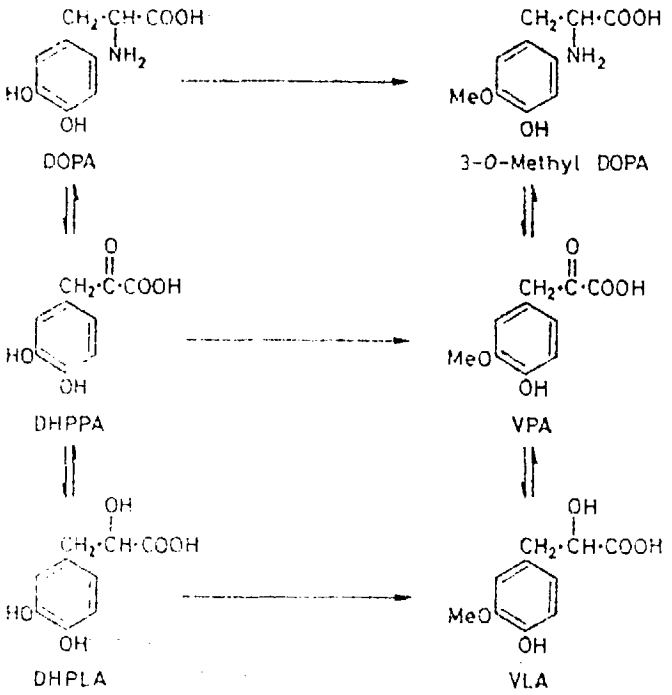


Fig. 1.6. Alternative pathways of DOPA metabolism. Reproduced from Sandler and Ruthven (1969_a).

1957; Booth, Emerson, Jones and DeEds, 1957; Scheline, Williams and Wit, 1960; Shaw, Gutenstein and Jepson, 1961; Dayman and Jepson, 1969), β -dehydroxylation (Sandler, Ruthven, and Contractor, 1963; Sandler, Ruthven and Wood, 1964$_a$; Goodall and Alton, 1965) and the production of vanillic acid from A and NA (Imaizumi, Yashida and Kita, 1958) and DOPAC (Alton and Goodall, 1969). For a more detailed account of these minor pathways, see review by Sandler and Ruthven (1969$_a$).

Tryptophan metabolism with intact indole ring

Tryptophan plays a versatile role in mammals (Meister, 1965) as an indispensible amino acid serving in the synthesis of protein and as the precursor of the important biologically active compounds tryptamine, 5-hydroxytryptamine (Serotonin, 5HT), melatonin and nicotinic acid. Although it has been studied since the beginning of this century (Hopkins and Cole, 1902), little was known about metabolic pathways other than that involving ring-splitting (Dalgliesh, 1955$_a$; Meister, 1965) until the early nineteen fifties when the potent pharmacologically active agent 5HT began to be intensively investigated (Erspamer, 1954; Page, 1954;, 1958). Since that time, a vast literature has arisen on this subject which has been well summarised in recent reviews (Garattini and Valsalli, 1965; Erspamer, 1966) and symposia (Garattini, Shore,

Costa and Sandler, 1968; Symposium on 5-hydroxytryptamine, 1968). The 5-hydroxyindole pathway of tryptophan metabolism has received attention out of all proportion to its quantitative importance. It has been estimated (Udenfriend, Weissbach and Sjoerdsma 1956) that no more than 1% of body tryptophan is metabolised in this way. Most of the impetus has been clinical however, derived from two main areas of investigation; carcinoid tumours which give rise to large amounts of indoles (Kahler and Heilmeyer, 1961) and studies in the aetiology of mental disease in which 5-hydroxyindoles may be implicated (Stoll, 1947; Gaddum, 1953a, b; Woolley and Shaw, 1954; Woolley et al., 1954).

Another pathway of tryptophan metabolism in which the indole ring remains intact has indoleacetic acid as its end-product. This compound possesses great significance in the plant kingdom as one of the most important growth substances (Kogl, Haagen-Smit and Erxleben, 1933). Its significance in mammals however is obscure.

5-Hydroxyindole pathway of tryptophan metabolism

Since the discovery of 5-hydroxytryptophan decarboxylas (Udenfriend et al., 1953; Clark et al., 1954) in mammalian kidney, 5-hydroxylation of tryptophan was strongly suspected as the initial step in the pathway leading to the production of 5HT (Udenfriend, 1958). Both 5HTP (Udenfriend, Weissbach

and Boganski, 1957; Udenfriend and Weissbach, 1958a) and tryptophan (Donaldson, Gray and Letsou, 1959) were shown to be suitable precursors of 5HT. After what was likely to have been a false start (Cooper and Melier, 1961), direct *in vitro* evidence for one type of hydroxylation was provided in 1961 when tryptophan was shown to be converted to 5HTP by a soluble rat liver supernatant (Friedland, Wadzinski and Waisman, 1961). This observation was soon confirmed by Renson, Goodwin, Weissbach and Udenfriend (1961). After some early confusion as to the identity of the enzyme responsible - the hepatic enzyme is now thought to be phenylalanine hydroxylase (Hagen and Cohen, 1966) - the tryptophan hydroxylating enzyme was at length shown to be quite distinct (Renson *et al.*, 1961), see section on phenylalanine hydroxylase.

The enzyme tryptophan hydroxylase was first identified by Grahame-Smith (1964c) in carcinoid tumour and has since been found in a variety of tissues where 5HT is known to be present, including intestinal mucosa (Lovenberg, Jequier and Sjoerdsma, 1968), brain (Grahame-Smith, 1964 b, c; Grahame-Smith and Moloney, 1965; Green and Sawyer, 1965; 1966; Nakamura, Ichiyama and Hayaishi, 1965; Gal, 1965; Gal, Armstrong and Ginsberg, 1966; Lovenberg, Jequier and Sjoerdsma, 1967), and platelets (Lovenberg *et al.*, 1968). It is mainly a soluble or perhaps an easily solubilised

mitochondrial enzyme (Ichiyama, Nakamura, Nishizuka and Hayaishi, 1968; Lovenberg et al., 1968) and exhibits the characteristics of a typical mixed function aromatic ring hydroxylase (Lovenberg et al., 1968). As with tyrosine hydroxylase (Nagatsu et al., 1964), the use of mercapto-ethanol was found to be essential to obtain in vitro enzyme activity in mammalian tissues (Hosoda and Click, 1966; Sato, Jequier, Lovenberg and Sjoerdsma, 1967; Lovenberg et al., 1968). For this in vitro reaction however, there is an apparent requirement for ferrous iron (Lovenberg et al, 1968). There is now sufficient evidence available to establish the rate limiting function of the enzyme in the synthesis of 5HT (Ichiyama et al., 1968; Green, 1968; Lovenberg et al., 1968). The enzyme is specific for the L-form of tryptophan on which hydroxylation occurs only on the 5-position (Udenfriend, Creveling, Posner, Redfield, Daly and Witkop, 1959) in contrast to the tryptophan hydroxylase of the microorganism Chromobacterium Violaceum (Mitoma, Weissbach and Udenfriend, 1955, 1956) which hydroxylates tryptophan in either the 5- or the 6- position (Contractor, Sandler and Wragg, 1964). D-tryptophan may be metabolised (Meister, 1957) but has first to be converted to the L-form via D-amino acid oxidation to indolepyruvic acid followed by transamination (Greenstein and Winitz, 1961).

The further metabolism of 5HTP possesses many points of similarity to that of DOPA where the basic degradation sequences and mechanisms of inactivation are closely analogous.

Further metabolism of 5HTP is through decarboxylation to 5HT which may then be stored within special intracellular granules whose ultrastructure depends on the anatomical site. 5HT storage is found in the enterochromaffin cells of the alimentary mucous membrane (Erspamer and Asero, 1952), blood platelets (Rand and Reid, 1951; Maynert and Isaac, 1968) spleen (Erspamer, 1965) and other sites. A small proportion of 5HTP may be metabolised via transamination to 5-hydroxy-indolepyruvic acid (5HIPyA), a minor pathway which has been demonstrated <u>in vitro</u> by Sandler, Spector, Ruthven and Davison (1960) and Spencer and Zamchek (1960). 5HIPyA may be converted non-enzymatically to 5-hydroxyindoleacetaldehyde (5-HIAAld) (Udenfriend, 1957) and then oxidised to 5HIAA by aldehyde dehydrogenase (Weissbach, Udenfriend and Redfield, 1957). Alternatively oxidative decarboxylation or non-enzymic oxidation of 5HIPyA may give rise directly to 5HIAA in a similar manner to the degradation pathway postulated for IPyA by Weissbach, King, Sjoerdsma and Udenfriend (1959) (see Fig. 1.7).

The enzyme responsible for the decarboxylation of 5HTP, 5HTP decarboxylase, is probably identical with DOPA decarboxylase; its properties, distribution and general

5-HYDROXYLATION PATHWAY OF TRYPTOPHAN METABOLISM

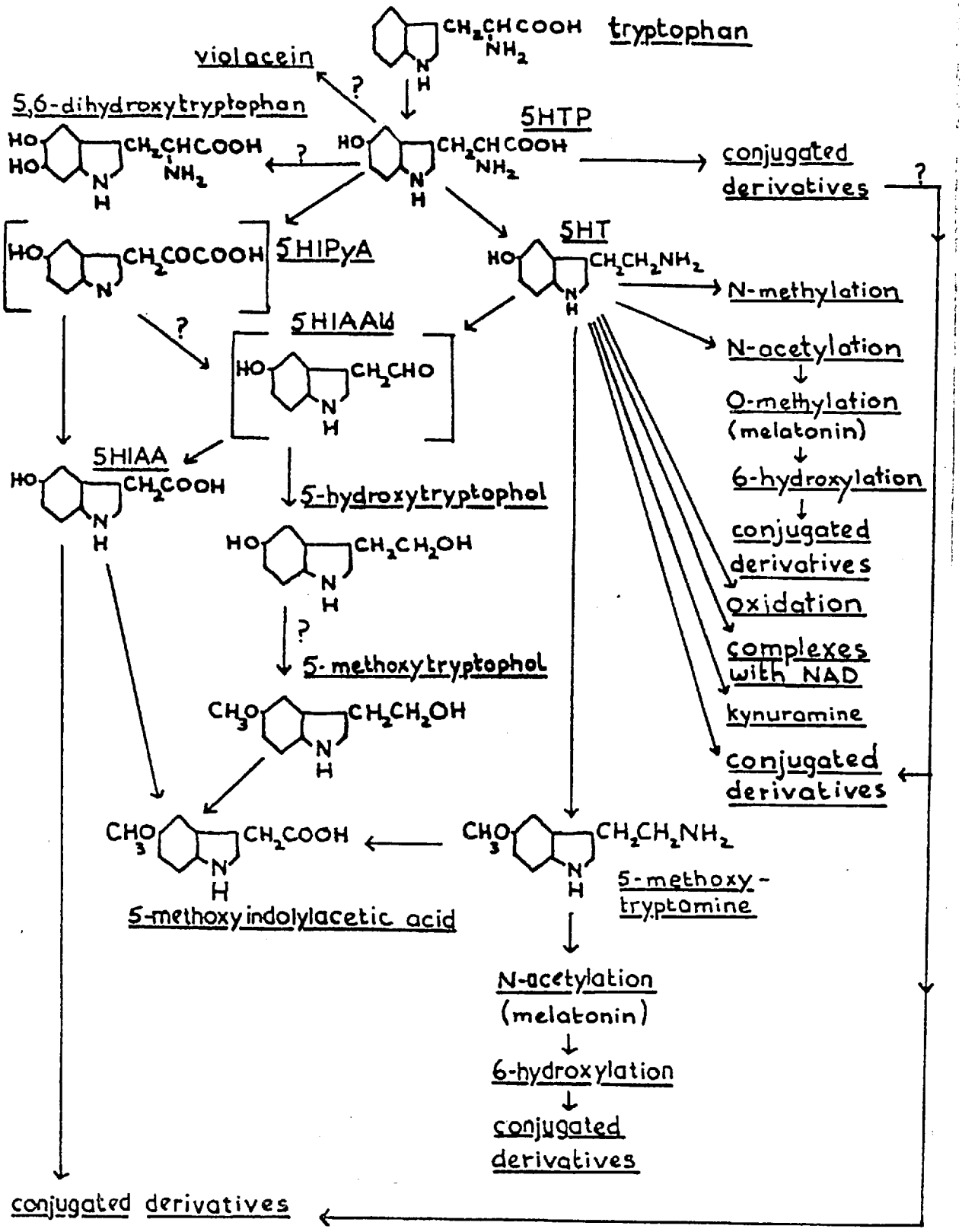


Fig. 1.7.

characteristics are fully described in the earlier section on DOPA decarboxylase.

The metabolic degradation of 5HT may proceed by a variety of reactions, as shown on Fig. 1.7. The most prominent involves oxidative deamination by MAO to 5-hydroxyindoleacetaldehyde (Udenfriend, Titus, Weissbach and Peterson, 1956b). The enzyme has been discussed more fully in an earlier section. The aldehyde is metabolised largely by aldehyde dehydrogenase to 5HIAA (Freyburger, Graham, Rapport, Seay, Govier, Swoop and Van Der Brook, 1952; Sjoerdsma, Smith, Stevenson and Udenfriend, 1955; Udenfriend et al., 1956b; Erspamer, 1956) which is the major metabolite of both endogenous and exogenous 5HT (Erspamer, 1961; Erspamer and Bertaccini, 1962; Garattini and Valzelli, 1965). A small proportion however, may be reduced by aldehyde reductase to 5HTOH (Kveder, Iskric and Keglevic, 1962). An increased output of this alcohol has been noted in the urine of patients with carcinoid disease (Davis, Cashaw, Huff and Brown, 1961). The conversion of aldehyde to acid or alcohol is apparently dependent on the $NAD/_{NADH_2}$ ratio, since the addition of $NADH_2$ to rat liver homogenate incubated with labeled 5HT increased the formation of 5HTOH at the expense of 5HIAA (Feldstein and Wong, 1965a, b). *In vitro* brain homogenates have been found to convert 5HT to 5HTOH

(Eccleston, Moir, Reading and Ritchie, 1966; Feldstein and Williams, 1968), but here $NADPH_2$ was found to promote the reaction more efficiently than $NADH_2$.

After the discovery of high concentrations of 5HT in carcinoid tumour tissue (Lembeck, 1953) its major metabolite 5HIAA was shown to be excreted in greatly increased amount in the urine of affected subjects (Page, Corcoran, Udenfriend, Sjoerdsma and Weissbach, 1955; Sjoerdsma, Weissbach and Udenfriend, 1955_a; Udenfriend et al, 1956_a). Urinary 5HIAA assay has since been widely used as in vivo index of 5HT turnover, although careful dietary control must be instigated because of the presence of high concentrations of 5HT in certain fruits such as banana (Anderson, Ziegler and Doeden, 1958; Udenfriend, Lovenberg and Sjoerdsma, 1959_a) and walnut (Kirberger, 1962). The normal urinary output is rather less than 10 mg/24 hr., depending on the method of assay (Sandler, 1963, 1968).

5HIAA may, at least theoretically, arise from a variety of hydroxyindolealkylamines such as bufotenidine (Erspamer, 1955), bufotenine (Erspamer, 1955; Gessner, Khairallah, McIsaac and Page, 1960) and possibly from their derivatives, 5-hydroxy-N-acetyltryptophan and 5-acetoxy-N-acetyltryptophan both of which were found to be excreted as 5HT when administered in large quantities (Erspamer, Glässer and Nobili, 1961). Their occurrence in man is perhaps doubtful. Although

N-methyl-5-hydroxytryptophan and bufotenine were claimed to be present in human urine (Bumpus and Page, 1955), this finding could not be confirmed after careful search (Rodnight, 1956).

Apart from the major metabolic route of 5HT described above, there exist minor pathways which may not involve oxidative deamination. Other pathways include transamination (Sandler et al., 1960; Spencer et al., 1960), conjugation as sulphate (Snow, Lennard-Jones, Curzon and Stacey, 1955; Curzon, 1957; Chadwick and Wilkinson, 1960) or glucuronide (Weissbach, Redfield and Udenfriend, 1958; McIsaac and Page, 1958; Airaksinen, 1961), N-methylation by P-N-MT (Axelrod, 1961, 1952_a), N-acetylation by a non-specific cytoplasmic enzyme (Weissbach, Redfield and Axelrod 1960_a) and O-methylation (Axelrod and Weissbach, 1960, 1961). The last two reactions are associated with the formation of melatonin in the pineal gland (Quay, 1968; Wurtman, Axelrod and Douglas, (1968). It should perhaps be mentioned that the authenticity of melatonin has been questioned by Feldstein and Williams (1968) who suggest that it might be an artifact formed by a reaction between 5-methoxytryptamine and the organic solvent used for the extraction; alternatively it might be a different indole, perhaps 5-methoxytryptophol which has many properties similar to those of melatonin. This, however, is very much a minority view (Axelrod, 1968). A further pathway of 5-hydroxyindoleacetaldehyde recently described involves the

loss of one carbon atom from the side chain, giving 5-hydroxyindollacetic acid (Fig. 1.8) (Kvender and Iskric, 1965; Keglevic, Kvender and Iskric, 1968).

Indoleacetic acid pathway of tryptophan metabolism

It is possible that a proportion of human urinary indoleacetic acid is derived from the action of gut flora on tryptophan. This view however is extrapolated entirely from observations on certain pathological conditions where tryptophan absorption is defective such as malabsorption syndrome (Blaschko and Levine, 1966) and Hartnup disease (Baron, Dent, Harris, Hart and Jepson, 1956; Jepson, 1966) and where a considerably increased urinary excretion of the acid is found. This elevation is dramatically abolished and in some cases output lowered to less than normal (5 to 8mg/24 hr), (Weissbach, King, Sjoerdsma and Udenfriend, 1959) by gut sterilization as shown in Hartnup disease by several groups (Shaw, Redlich, Wright and Jepson, 1960; Halvorsen, 1963; De Lacy, Hooft, Timmermans and Snoeck, 1964) and in non-tropical sprue (Weissbach et al, 1959). On balance however and excluding vegetable sources, it seems likely that by far the greater proportion of IAA normally present in the urine is produced endogenously from tryptophan, either from decarboxylation to tryptamine (Weissbach et al., 1959; Hess, Redfield and Udenfriend, 1959) and subsequent oxidative

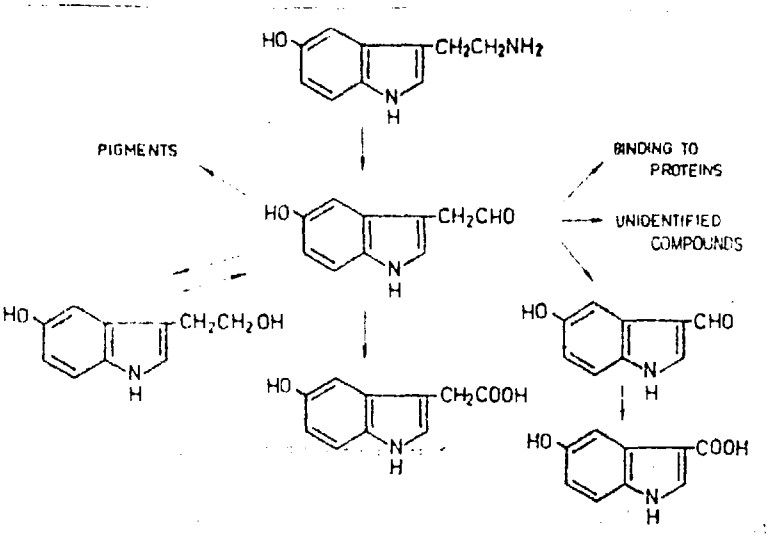


Fig. 1.8. Different pathways of 5-hydroxyindole-acetaldehyde metabolism. Reproduced from Keglevic et al.,(1968).

deamination, or via transamination to indolepyruvic acid (Fonnum, Haavaldsen and Tangen, 1964; Haavaldsen, 1964; Tangen, Fonnum and Haavaldsen, 1965) followed by decarboxylation (Weissbach et al., 1959) (Fig. 1.8).

Other minor metabolic route connected with the IAA pathway are N-methylation of tryptamine (Axelrod, 1961; 1962) and 6-hydroxylation of tryptamine (Jepson, Udenfriend and Zaltzman, 1959; Jepson, Zaltzman and Udenfriend, 1962).

Indole pathway of tryptophan

The production of indole from tryptophan by tryptophanase from mammalian gut flora is well recognised (Happold, 1950). Further metabolism of indole takes place partly by the further action of gut flora and partly within the mammalian tissues (Fig. 1.8). The pathway starting with the conversion of indole to isatin has been assigned to bacterial action by Sakamoto, Uchida and Ichihara (1953). Methylation of indole to 3-mthyl indole (skatole) occurs by a similar mechanism as is evident by its reduced excretion after gut sterilization (Rodnight, 1961). Indole is a suitable substrate for hydroxylation by the liver microsomal enzyme which preferentially hydroxylates at the 3 position (Jepson, 1962) forming indoxyl from indole (Posner, Mitoma and Udenfriend, 1961). Hydroxylation also takes place at the 6-position giving rise to 6-hydroxy-indole (Jepson,

IAA PATHWAY OF TRYPTOPHAN METABOLISM

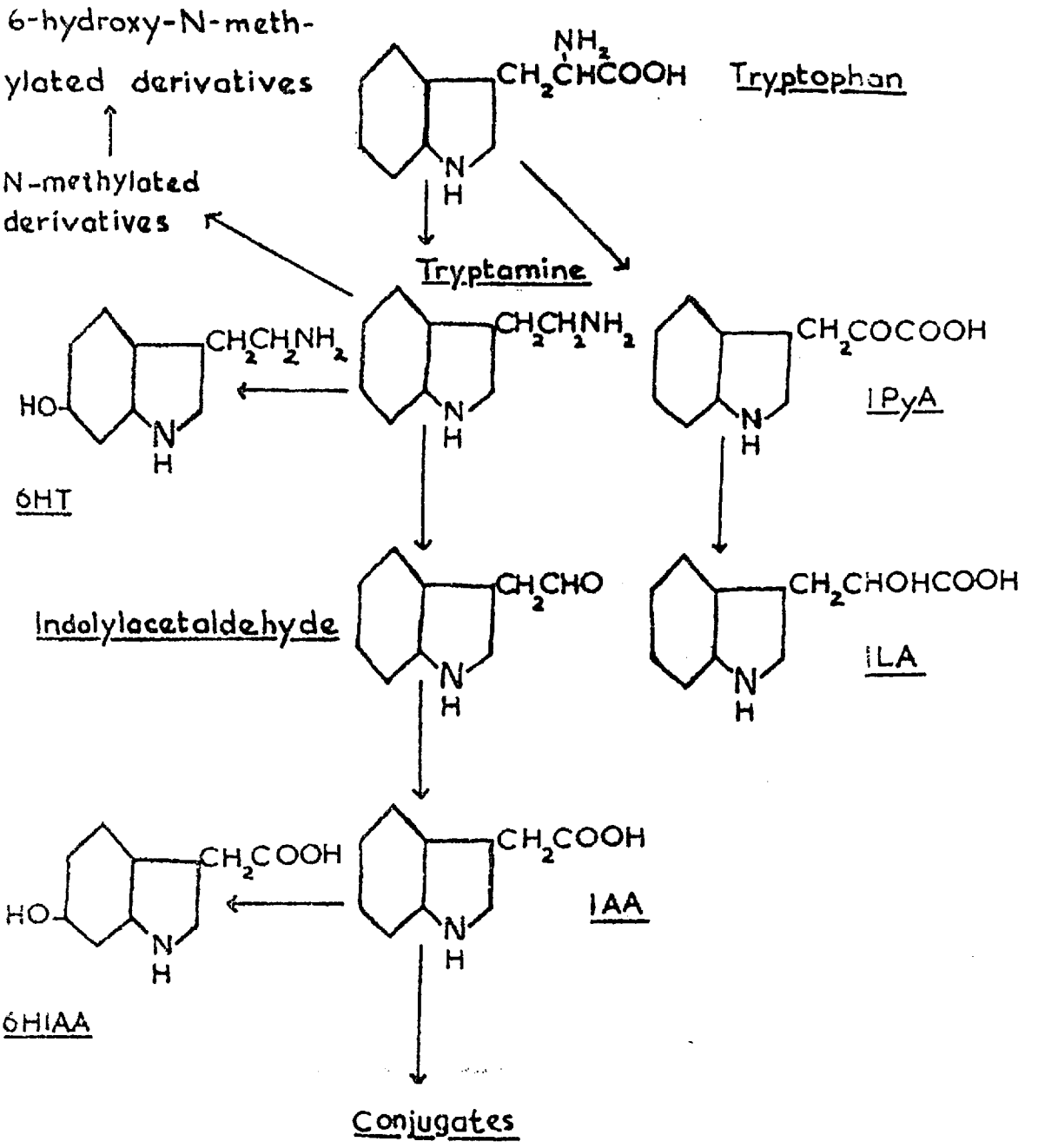


Fig. 1.9.

FORMATION AND METABOLISM OF INDOLE

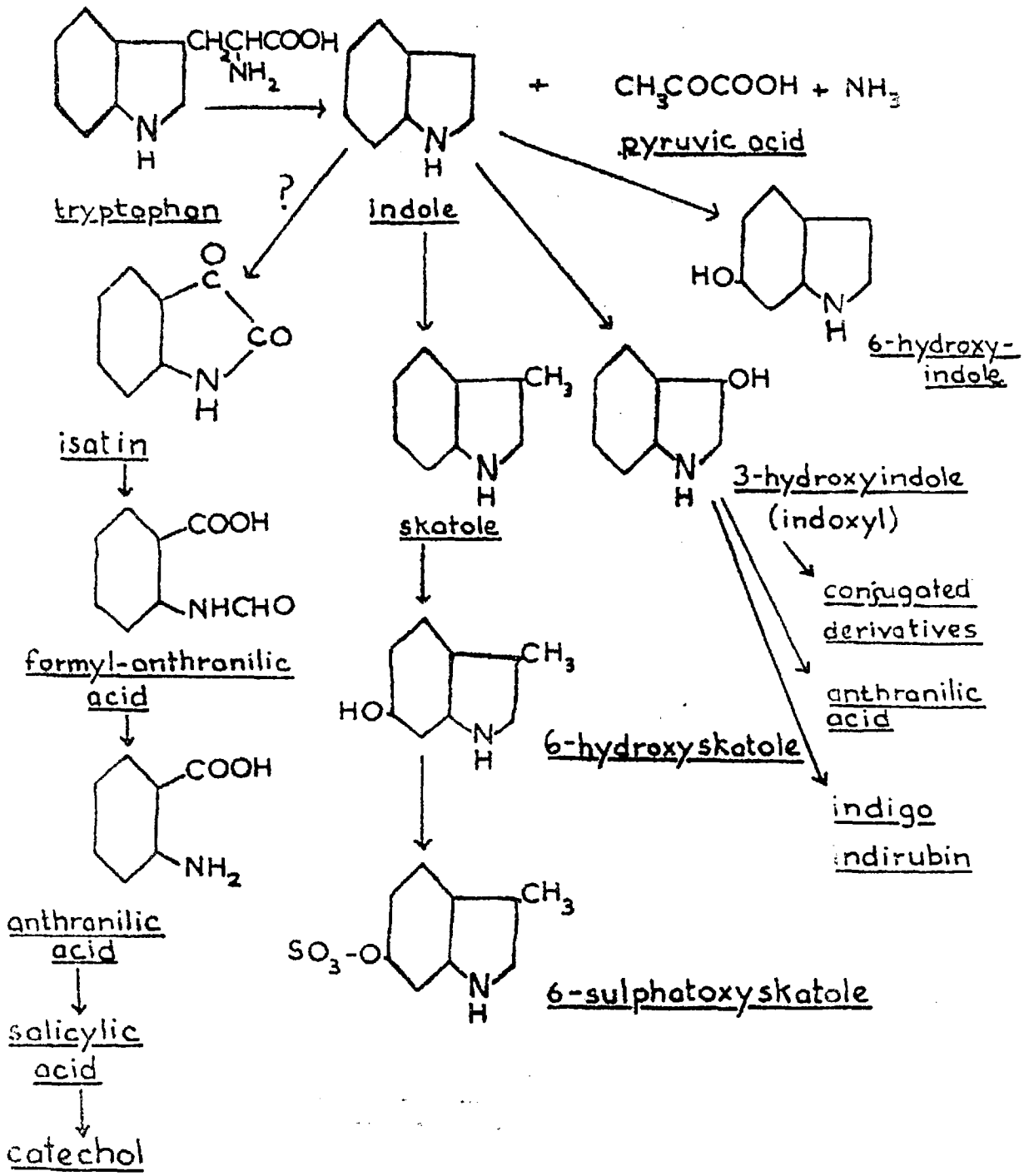


Fig. 1.10.

Zaltzman and Udenfriend, 1962) and 6-hydroxyskatole (Horning, Sweeley, Dalglish and Kelly, 1959). The excretion of 6-hydroxyskatole is similarly reduced after gut sterilisation (Nakao and Ball, 1960).

CHAPTER 2

Theory of gas-liquid chromatography

In their original paper on partition chromatography Martin and Synge (1941) pointed out the possibility of substituting gas for a liquid mobile phase. The response to this suggestion was slow and GLC did not become properly established until the early 1950's. The number of papers on the subject published since that time is very large and continues to grow; the technique is now well established as a powerful analytical tool. The availability of a wide variety of detectors and the opportunity of combining mass spectrometry, infra-red spectrometry and nuclear magnetic resonance analysis with gas chromatography have greatly increased its scope and application.

A detailed study of the dynamics of gas chromatography involves a number of highly mathematical considerations which are, in general, beyond the scope of this thesis. Some acquaintance with this background however is essential for a proper understanding of the technique. Of the two types of gas chromatography, gas-solid and gas-liquid, the latter only will be considered.

In GLC, the mobile gaseous phase is usually an inert gas and the stationary phase a non-volatile material coated

either on the surfaces of a solid support (packed columns) or on the walls of capillary tubes (capillary columns). Separation of a mixture of substances is achieved by employing differences in their partition coefficients, in one of three ways (Purnell, 1962):

i. Displacement development technique

ii. Frontal development technique

iii. Elution technique

Both techniques (i) and (ii) depend upon or involve sorptive competition, a process which introduces much uncertainty into theoretical consideration of the methods and which, in practice, may be expected to act adversely with regard to effectiveness of separation (Purnell, 1962). For these reasons, techniques (i) and (ii) have never been seriously exploited. In principle, the elution technique is free from these drawbacks and may thus be expected to offer a practical approach to separation. Thus all work performed in connection with this thesis employs elution GLC and discussion will be confined to a review of its underlying principles.

Separation and the theoretical plate concept

If one considers a mixture of two components with different partition coefficient (K) values passing down a GLC column, each component will be distributed and

redistributed between the two phases during its progression, that with the higher K value tending to remain longer in the stationary phase. If passage is prolonged, complete separation will be achieved and the components will emerge separately from the column. The processes involved are very similar to those occurring during fractional distillation; it is helpful to picture the column as consisting of a number of small sections where equilibrium takes place. Separation is dependent on the number of such sections; a small number would provide partial separation and a large number complete separation. This argument introduces the theoretical plate concept. A theoretical plate is defined as a hypothetical section of a column where unit equilibrium of solute between mobile and stationary phase occurs. The number of theoretical plates "n" is used to measure column efficiency and hence is a term of column performance.

Thus if the column length is L and the theoretical plate has a width or height H, then

$$n = \frac{L}{H} \qquad 2.1$$

or

$$H = \frac{L}{n} \qquad 2.2$$

Ideal linear chromatography

The theoretical plate concept with respect to certain functions may be simplified by considering an ideal system which is linear and where all laws of vapours and gases are perfectly obeyed i.e. a linear ideal chromatography. Here certain assumptions are made which may not be completely valid in practice, but are necessary in order to derive certain fundamental formulae generally used for the determination of column efficiency, separation factors and resolution of peaks, from measurable parameters. These assumptions are (Purnell, 1962):

1. The partition coefficient (K) is constant throughout the column and is independent of concentration.
2. Equilibrium of solute between the gaseous and liquid phase is rapid compared with the rate of flow.
3. Diffusion along the length of the column in either phase is negligible.
4. The column is considered to consist of a number of identical volume elements in each of which one equilibrium occurs. Each volume is referred to as a plate volume (v).
5. Flow of mobile phase can be regarded as discontinuous, i.e. it consists of a step-wise addition of volumes of mobile phase from one plate to another.

By considering the passage of a solute plug through

a column, the first fundamental equation relating the retention volume V_R (Ambrose, James, Kaulemans, Kovats, Rook, Rouit and Strosse, 1959) with fixed parameters of the column is given by

$$V_R = V_G + KV_L \qquad 2.3$$

where V_G = the volume of empty spaces in the column; it does not include the volume of solid support.

V_L = volume of liquid phase.

and K = partition coefficient of the solute.

If V_g and V_l are the volumes of the gaseous and the liquid phases in a plate, then equation (2.3) may be written as

$$V_R = n (V_g + KV_l) \qquad 2.4$$

Equations 2.3 and 2.4 are graphically represented in Fig. 2.1.

Trailing peaks

Substances whose distribution between the gaseous and the liquid phases is uniform give symmetrical peaks; for the gradual increase and decrease of concentrations on either side of the maximum are equal. If they are not equal, non-symmetrical peaks arise (Fig. 2.2).

When the solute enters the liquid phase more slowly

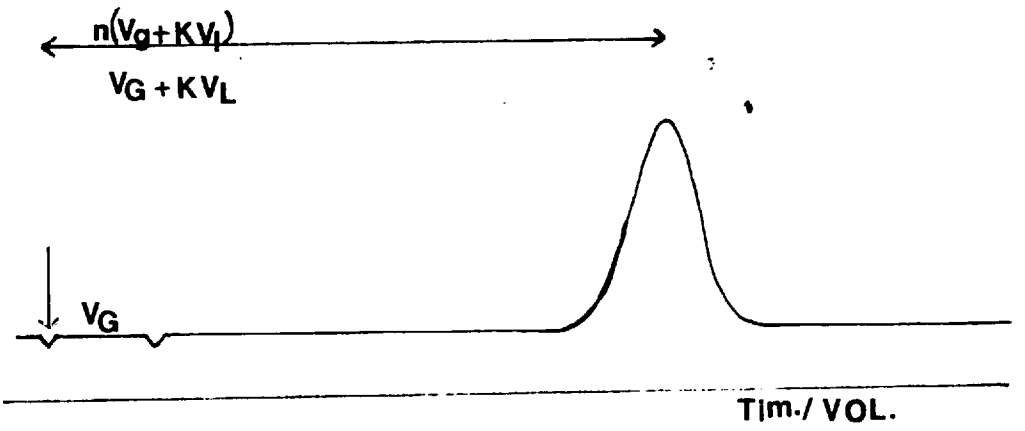


Fig. 2.1. Graphical representation of retention volume.

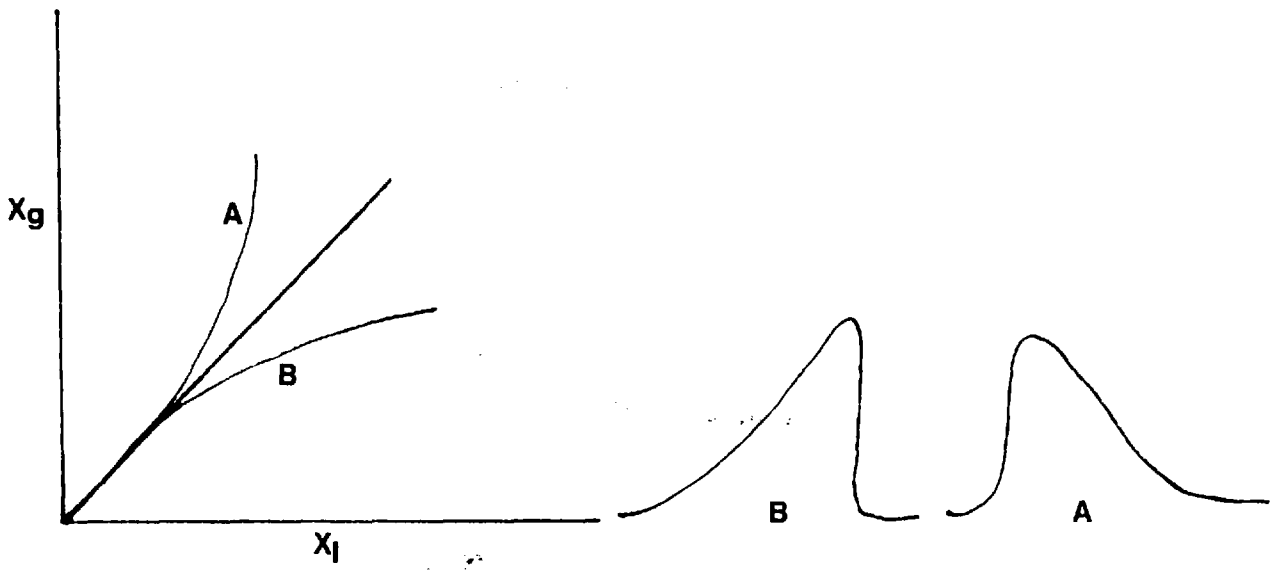


Fig. 2.2. Hypothetical representation of a plot of X_l (concentration of solute in the liquid phase) against X_g (concentration of solute in the gaseous phase) together with an illustration of elution profile accompanying the non-linear curves.

than it emerges X_g/X_l follows curve A in Fig. 2.2, where X_g and X_l are the concentrations of solute in gaseous and liquid phases respectively. This phenomenon is often caused by low temperatures or by non-uniform interaction of solute and liquid phase. When, conversely, the solute tends to remain longer in the liquid phase, X_g/X_l follows curve B. This finding is commonly due to adsorption. The elution peak which results from this variation is also shown on Fig. 2.2.

Resolution of mixtures

If two compounds are well enough separated to permit a satisfactory estimation of the peak width, and the peaks are approximately Gaussian, the resolution may be expressed by (Ambrose et al, 1959)

$$\text{Resolution, } R = 2x \frac{(\text{Differences between retention volumes})}{(\text{Sum of peak widths})} \qquad 2.5$$

At unit resolution therefore $V_{R2} - V_{R1} \simeq$ the peak width of the first peak (x) Fig. 2.3.

The difference between adjacent peaks are often referred to as the peak coefficient; hence at unit resolution two peaks are said to have a peak coefficient equal to the peak width of the first. In deriving the general equation (Fig. 2.3) where

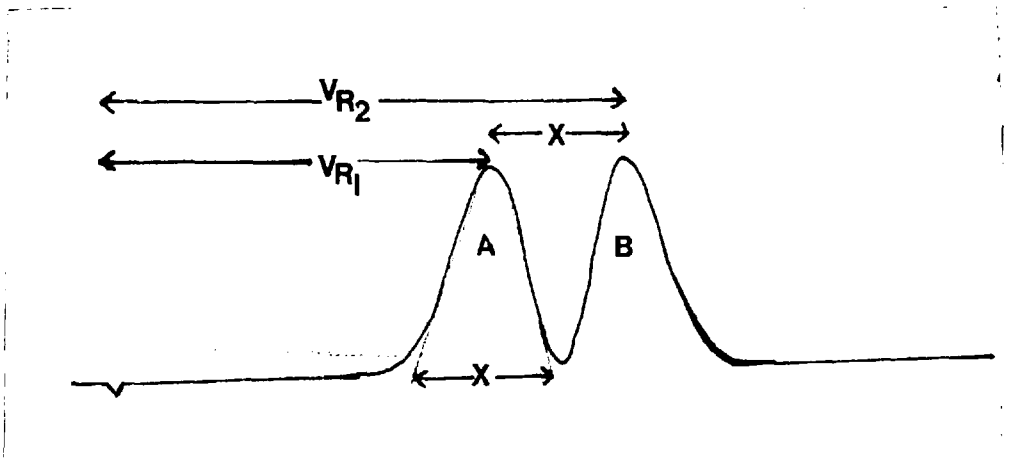


Fig. 2.3. Separation of two peaks at unit resolution, (V_{R_1} is the retention volume of substance A and V_{R_2} that of substance B).

peak coefficient = $V_{R2} - V_{R1}$

substituting from equation 2.3

peak coefficient = $(V_G + K_B V_L) - (V_G + K_A V_L)$

$= (K_A - K_B)V_L$ 2.6

$= \Delta K V_L$ 2.7

where K_A, K_B and ΔK are respectively the partition coefficients of substances A and B, and their differences.

From equation 2.7 therefore, the peak coefficient, and hence resolution, is proportional to ΔK and V_L i.e. the amount of stationary phase. Since ΔK values of any two substances are constant the only changeable parameter affecting resolution as far as equation 2.7 is concerned is therefore VL.

Relationships of peak width to retention volume and column performance

In figure 2.4 if x = peak with

y = retention volume i.e. $(V_G + KV_L)$

m = true retention volume i.e. KV_L

h = peak height

x_1 = peak width at point of inflection

and z = peak height at point of inflection

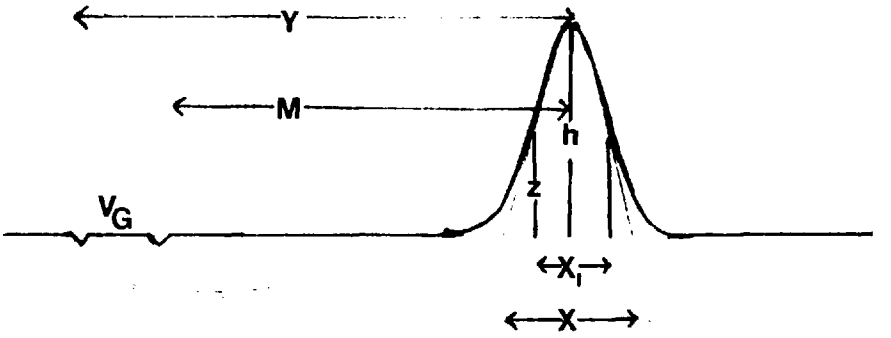


Fig. 2.4. Illustration of peak heights, widths and retention volume. (see text).

The peak width at the point of inflection is related to n by

$$x_1 = \sqrt{n} \qquad 2.8$$

converting equation 2.8 in terms of retention volume

$$x_1 = 2\sqrt{n} \cdot (V_g + KV_1) \qquad 2.9$$

Hence $$\frac{2_y}{x_1} = \frac{2n\ (V_g + KV_1)}{2\sqrt{n} \cdot (V_g + KV_1} = \sqrt{n} \qquad 2.10$$

Therefore $$n = \frac{4y^2}{x_1^2} \qquad 2.11$$

When n (number of theoretical plates) is large as is the case with most GLC, the peak width is twice x_1 and s/h is usually referred to as being equal to two standard deviations i.e. S = 2

$$\frac{y}{x} = \frac{n(V_g + KV_1)}{4\ n\ (V_g + KV_1)}$$

$$= \frac{\sqrt{n}}{4} \qquad 2.12$$

therefore $$\sqrt{n} = \frac{4y}{x}$$

or $$n = \frac{16y^2}{x^2} \qquad 2.13$$

Equation 2.13 is usually employed to determine column efficiencies.

Determination of n in terms of retention volume at unit resolution

Consider two substances A and B as shown on Fig. 2.3 At unit resolution

$$4\sqrt{n}.(V_g + K_A V_1) = n (V_g + K_B V_1) - n (V_g + K_A V_1)$$

Dividing throughout by $(V_g + K_A V_1)$

$$4 n = n\left[\frac{V_g + K_B V_1}{V_g + K_A V_1}\right] - 1 \qquad 2.14$$

If $\frac{V_g + K_B V_1}{V_g + K_A V_1} = R_A B$, which is the relative retention volume of A with respect to B

Then $4\sqrt{n} = n (R_A B - 1)$

and $$n = \frac{16}{(R_A B - 1)^2} \qquad 2.15$$

From equation 2.15 one can calculate the required efficiency of a column which will give a unit resolution for the separation of any two substances once their retention relative to one another is known.

Separation factor

The concept of separation factor provides a theoretical approach to separation independent of column parameters. It

is given by the equation (Purnell, 1962):

$$S = 36 \left[\frac{\alpha}{\alpha - 1}\right]^2 \qquad 2.16$$

where α = $\frac{\text{true retention volume of A}}{\text{" " " " B}}$

When the true retention volumes of A and B are much greater than the column free space V_G, S is equal to n. In practice, this situation cannot often be achieved and S is related to n by

$$n = S\left[1 + 2\left(\frac{VG}{V_{RB}}\right) + \left(\frac{VG}{V_{RB}}\right)^2\right] \qquad 2.17$$

Linear non-ideal chromatography

In the linear ideal chromatograph, certain assumptions were made which are not true in practice. Such an approach is helpful for determining column performance and resolution in terms of measurable functions where these assumptions do not greatly affect the final results. When assessing the contribution of column parameters to performance, a more realistic approach is needed. Of three possible deviations from the linear ideal condition; linear non-ideal, non-linear ideal and non-linear non-ideal, only the first is likely to apply to GLC (Purnell, 1962).

The underlying principles of linear non-ideal

chromatography were first explored by Lapidus and Amundson (1952) and elaborated by many other workers among whom Klinkenberg and Sjenitzer (1956) and Van Deemter, Zuiderweg and Klinkenberg (1956) are prominent. The basic approach is statistical and makes use of the theorem that for independent statistical processes occurring simultaneously, the total variance is the sum of individual variances, while the mean is the sum of individual means. Here, equations for the theoretical plate height H are deduced individually in terms of each of the main factors involved; a final equation for H is then written taking all the factors into account by adding up the different individual equations. The factors include diffusion and resistance to mass transfer and are themselves determined by such parameters as pressure, temperature, flow rate, particle size, etc.

Band spreading is caused mainly by diffusion in the gaseous phase. In packed columns, diffusion manifests itself in two ways:

1. Diffusional spreading due to the presence of the coated particles which tend to form obstacles to longitudinal diffusion and therefore enhance lateral diffusion.

2. Eddy diffusion due to the presence of channels of varying size among the particles. Since the pressure drop is the same at any plate, the time taken for solute molecules to pass through narrower channels is less than that for wider

ones; thus diffusion takes place in all directions, both laterally and longitudinally.

H is related to diffusional spreading by (Purnell, 1962)

$$H = \frac{2 \gamma \bar{D}_g}{U} \qquad 2.18$$

γ = a constant, usually having a value between 1 and 0.5.

$\bar{D}g$ = average column pressure

U = average linear velocity of gas

and H is related to eddy diffusion by

$$H = 2 \lambda dp \qquad 2.19$$

During elution through a column, solute molecules dissolve in the liquid phase repeatedly before emerging. This process occurs, in part at least, by diffusion which is subject to a certain degree of resistance as the solute diffuses in and out of solution. Resistance to mass transfer in the gas phase is therefore likely to be considerably less than in liquids, although this can often be offset by making the liquid a very thin film in which the distances to traverse are much smaller than in the gas phase.

H is related to resistance to mass transfer in the liquid phase by

$$H = \frac{2}{3} \cdot \frac{aK}{(a+K)^2} \cdot \frac{df^2}{D_l} \cdot U \qquad 2.20$$

$a = \frac{V_g}{V_l}$, K = partition coefficient,

df = the effective thickness of the liquid phase,

D_l = diffusion coefficient of solute in liquid phase.

The relationship between H and resistance to mass transfer in the gas phase is

$$H = \frac{1}{100} \cdot \frac{aK}{(a+K)^2} \cdot \frac{df^2 U}{D_g} \qquad 2.21$$

Combining equations 2.18, 2.19, 2.20, and 2.21 for the final equation of H we get

$$H = 2\,dp + \frac{D_g}{U} * \frac{2}{3} \cdot \frac{aK}{(a+K)^2} \cdot \frac{df^2 U}{D_l} + \frac{1}{100} \cdot \frac{aK}{(a+K)^2} \cdot \frac{df^2 U}{D_g} \qquad 2.22$$

Equation 2.22 may be simplified as

$$H = A + \frac{B_o}{U_o} + C_l U + C_g{}^o U_o \qquad 2.23$$

<u>Theoretical dependence of H upon experimental parameters</u>

In GLC, every attempt should be made to attain the minimum value of the theoretical plate height H_{min}, by interchanging the various parameters which contribute towards equation 2.23. This concept may be better understood by

considering each variant in the equation on its own.

Eddy diffusion i.e. (A):

"A" is a function of packing and particle size. The smaller the particle size the smaller is H, but only up to a certain point after which increasing channelling starts to increase B_o. Thus there is a minimum particle size which usually lies between 85/100 mesh and 100/120 mesh, as will be discussed in the next chapter. Once the particle size is selected however, H can be further reduced through careful and uniform column packing.

Diffusional spreading i.e. B_o/U_o:

Here H diminishes with increased gas velocity and, through reduction of D_g^o, with increased molecular weight of carrier gas. H is also reduced by increasing column outlet pressure.

Gas phase mass transfer i.e. $C_g^o U_o$:

The effect of molecular weight of carrier gas and outlet pressure on C_g^o is exactly opposite to their effect on B_o. Therefore for any type of column, the terms are exactly opposed and their combined contribution to H can only be evaluated experimentally. This being so, C_g^o is inversely proportional to K for which high values are to be favoured through keeping C_g^o low.

K is increased or C_g^o decreased by increasing the

solvent/support ratio and by reducing the column temperature. These changes should be brought about with care, taking into account their effects on other variances viz. A, B_o and C as well as certain practical limitations. For example there is a limit to the increased solvent/support ratio above which excessive column bleeding is inevitable.

Liquid phase mass transfer i.e. $C_l U$:

The contribution of C_l to H depends on the mode of distribution of the liquid phase which may be either of a true film or droplet type. C_l diminishes rapidly with reduced particle size and is inversely proportional to the interdiffusional coefficient (D_f) - see equation 2.22. Little can be done to control (D_f) except that high temperatures and solvents of low viscosity are to be preferred. This is perhaps one of the reasons why the silicon oil group of stationary phases, e.g. F60 are slightly better than the silicon gum group, e.g. SE30, under some circumstances (Karoum, Ruthven and Sandler, 1968).

As for the relationship between C_l and K, while C_l is proportional to K in the film type of distribution, in the droplet a plot of C_l against K rises to a maximum and then falls Fig. 2.5.

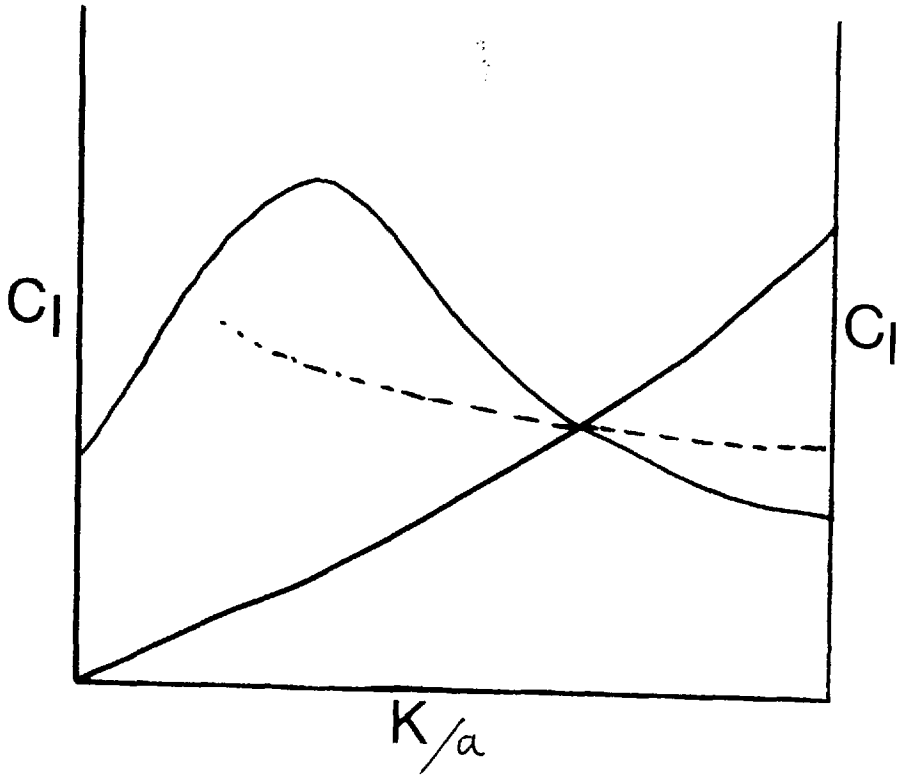


Fig. 2.5. Illustration of a hypothetical type of dependence of C_1 upon K in the two types of liquid phase coating. The upper curve is that of a droplet coating, and the lower that of a film coating. The broken line curve is the resultant of the two types when occur together, a most likely situation with supports coated below 20%. After Purnell, (1962.)

In practice the likely distribution of solvent is a combination of the two types of distribution, depending on solvent/support ratio. For example at high ratios of solvent/support the film type of distribution predominates while at low ratio the droplet distribution is observed. The precise effect cannot be predicted, but the transition form will acquire a curve ~~similar to the broken line curve~~ similar to the broken line curve shown in Fig. 2.5.

Approach to fast analysis :

While it is desirable to obtain maximum efficiency, Hmin, from any column, it is equally important to aim for the GLC arrangement offering minimum analysis time.

It can be shown that every time a condition of maximum efficiency of a column is reached, there is a minimum corresponding analysis time which also corresponds to a maximum temperature (T_{max}). At temperatures below T_{max}, the column efficiency as well as the values of K and C_1 do not change substantially, while at higher temperatures, the efficiency falls greatly.

Where column efficiency is far in excess of what is needed for a particular performance, n can be exchanged for time by increasing the temperature above T_{max}, but it should be emphasised here that the separation factors S

and α are also reduced (see equation 2.16). In practice, the best approach in such situations is to alter other parameters without changing T_{max}.

Parameters which are usually changed to increase or decrease n include solvent/support ratios, column length and internal diameter, flow rates, temperatures, inlet pressures and particle size, in order of importance. It should, however, be stressed that whatever column is used, it must be operated at conditions optimum for flow rate, packing and temperature.

CHAPTER 3

GLC theory into practice

For the analysis of complex mixtures such as urine, the general aim is to obtain the best separation in the shortest time. A sound understanding of GLC theory greatly assists in defining a set of properties which characterise an appropriate column. In this chapter a discussion of theoretical considerations will precede, at each stage, the experimental methods by which the writer has been able to convert them into practice.

A practical approach to obtaining a suitable GLC procedure may be listed under three main headings:

1. Establishing the form in which the solute is introduced into the column e.g. with or without prior treatment.

2. Choice of stationary phase.

3. Choice of appropriate conditions offering the best result in the shortest time and permitting accurate and reproducible quantitation.

Relative merits of isothermal and temperature programming analysis

Structurally related substances with similar molecular weights and boiling points, usually have similar retention volumes also. Biologically important phenolic and indolic compounds fall within this category so that optimal GLC separation is needed if accurate quantitation is to be achieved.

On the other hand, when a mixture of wide boiling range is analysed there frequently occurs a broad spread of retention times or volumes; because compounds of increasing retention volumes are increasingly diluted, a progressive diminution of detector sensitivity towards components results. Quantitative measurement of peaks appearing late may thus be difficult if not impossible. Ideally therefore one needs a column arrangement where different components of a mixture are well separated but which yet allows easy and accurate quantitation. The two most common ways of overcoming this difficulty are by using temperature programming which has been successfully employed for qualitative and semi-qualitative analysis of urinary aromatic acids (Dalgliesh et al., 1966; Horning et al., 1966; Horning, Boucher and Moss, 1967; Coward and Smith, 1968) or by employing more than one column under isothermal conditions (Sweeley and Williams, 1961; 1961a, 1962; Williams, 1962; Williams and Leonard, 1963; Williams Dorin and Greer, 1963a; Williams and Greer, 1963b; Williams and Sweeley, 1964; Williams and Greer, 1965; Wilk, Gitlow, Mendlowitz, Franklin, Carr and Clarke, 1965; Horii, Makita, Takeda, Tamura and Ohnishi, 1965; Bondurant, Greer and Williams, 1966; Mendes and Stevenson, 1966; Karoum, Ruthven and Sandler, 1968; Karoum and Sandler, 1968a; Karoum, Anah, Ruthven and Sandler, 1969).

In spite of great instrumental improvements, temperature programming (Drew and McNesby, 1965) does not offer resolution

as great as isothermal analysis (Purnell, 1962). Other things being equal, in a temperature programming system a gradual drop in efficiency occurs as the temperature rises, because of a fall in K and α (see equations 2.7 and 2.16). Thus a pair of substances which are well separated isothermally at the lowest temperature of a programme, are brought closer together by temperature programming unless a different column with greater separation efficiency is used. This advantage of isothermal analysis coupled with the fact that it is easy, quick and simple to operate makes it more useful than temperature programming especially when routine GLC analysis of large quantities of urine as during screening examinations for abnormal excretion of phenolic and indolic metabolites (Karoum et al., 1968, 1969) is desired.

Temperature programming, however, has its proper place as a research procedure during investigations where qualitative information on a wide range of compounds is desired and in preliminary setting up of GLC methods prior to selecting optimal chromatographic conditions for isothermal operation (see chapter 2). In such situations, the employment of isothermal systems requires the use of multiple columns, not always a practical approach.

Because accurate quantitative data were always the goal during the present project isothermal analysis was carried out at every stage of this work.

Choice of derivatives

For effective GLC separation, the plug of solute should be introduced to the column in as compact a band as possible. One of the first prerequisites for achieving this aim is that the injected solute should vapourise completely and immediately; low vapourisation leads to band distortion and low resolution. Therefore, for the chromatography of substances of high boiling points (low vapour pressures), temperatures considerably higher than their boiling points are needed for quick vapourisation to be achieved.

High temperature chromatography does not often offer the best solution, for it suffers from two important drawbacks. Firstly the substances undergoing analysis may not be stable at such high temperatures and secondly, as discussed earlier, the partition coefficient K of a substance diminishes as column temperature increases, with consequent reduction in column efficiency. Thus unless the solute is present in pure solution or is sufficiently different from other components to be well separated, quantitation may be affected.

Although some phenolic (Mendes et al, 1966) and indolic (Stone and Sohilke, 1964) compounds have been successfully separated by GLC in their natural state, in general these compounds are not sufficiently volatile and are in some cases thermally unstable. They are best chromatographed by converting them chemically to suitable volatile derivatives. Chemical

conversion often necessitates the preparation of ether and/or ester derivatives resulting in changes in polarity as well as volatility of the parent compound. These qualities play important roles in the solute-solvent molecular interactions which affect retention volumes and, in most cases, separation characteristics (Vanderheuvel, 1966).

Among the different types of derivative proposed for the GLC of phenolic (Williams, 1962; Williams et al., 1964; Williams, 1965; Wilcox, 1966) and indolic (Powell, 1964; Judith, Biggs, St.John and Anthony, 1967; Grunwald, Vendrell and Stone, 1967) compounds, methyl ester/trimethylsilyl ethers (ME/TE) and trimethylsilyl ether/esters (TE/E) have proved most suitable for the analysis of urinary metabolites (Dalgliesh et al., 1966; Horning et al., 1966; Wilcox, 1967; Karoum et al., 1968, 1968a).

Experimental findings concerning the preparation of ME/TE and TE/E derivatives.

During the preparation of methyl ester derivatives certain findings emerged concerning the O-methylation of ring hydroxyl groups of phenolic and indolic compounds. While all monoaromatic acids studied were completely esterified within one minute on exposure to ethereal diazomethane (see chapter 4), dihydroxy-phenolic acids underwent varying degrees of O-methylation also. The tendency for O-methylation to occur was found to be highest among the 3,4 and 3,5- and lowest among compounds with a 2,5-

substitution. The effect decreased with the number of $-CH_2-$ groups in the side chain; thus 3,4-dihydroxybenzoic acid formed the corresponding vanillic acid ester more readily than 3,4-dihydroxyphenylacetic acid formed homovanillic acid ester. By reducing esterification time to 30 seconds for dihydroxy-phenolic compounds, adventitious O-methylation was eliminated without seriously reducing the yield of methyl esters of monohydroxy compounds. In contrast, mono and dihydroxyindoles did not undergo appreciable O-methylation even after 15 min. in a solution of diazomethane. With the exception of phenylpyruvic acid, exposure of ketoacids to diazomethane for 2 min. rather than 1 min. considerably reduced the tendency to form subsidiary peaks (Karoum et al., 1969).

During the preparation of TE/E and ME/TE derivatives, complete silylation (as will be described in chapter 4) was obtained within 1 hr. as indicated by the clear supernatant resulting from centrifugation of the reaction mixture.

Solvent for trimethylsilylation

Pyridine was the solvent employed by many early workers for the preparation of trimethylsilyl (TMS) derivatives (Hedgley and Overend, 1960). It has subsequently been widely used for this purpose prior to GLC (Langer, Pentage and Wender, 1958; Smith and Carlsson, 1963; Bentley, Sweeley, Makita and Wells, 1963; Sen and McGeer, 1963; Freedman and Charlier, 1964; Horii

et al., 1965; Williams et al., 1965; Dalgliesh et al., 1966). Other solvents have also been used including dioxane (Mendes et al., 1966; Karoum et al, 1968, 1968a, 1969), carbon disulphide (Rowland and Kiegelman, 1967) ethyl acetate, triethylamine, hexamethyldisilazane, acetone (Coward et al., 1968a) and tetrahydrofurane (Horning, Horning, Vanden Heuvel, Knox, Holmstedt and Brooks, 1964). The solvent of choice has to satisfy three prerequisites (apart from acting as a medium in which chemical reaction takes place). These are listed below:

i. It should not interfere with the production of a derivative or destroy it in any way.

ii. It should have a short elution time, with minimal trailing.

iii. It should be easy to handle, convenient to use and pleasant to work with.

All the solvents mentioned above fulfil prerequisite i, although pyridine possessed the added advantage in that it may act as a catalyst for the silylation process (Dalgliesh et al., 1966). The introduction of trimethylchlorosilane (TMCS) as an additional additive to catalyse the reaction (Horii et al., 1965) robs pyridine of its pre-eminence. As pyridine possesses an offensive smell and tends to cause headaches if repeatedly inhaled, an inevitable consequence during these manoeuvres, a more satisfactory solvent was sought. Carbon disulphide was not investigated because of its toxicity even though it has been

claimed to possess a short elution time on a QF1 column (Rowland et al., 1967).

Amongst the solvents mentioned above (with the exception of carbon disulphide) dioxane showed the shortest elution time with least trailing. As it is also odourless it appeared to be the solvent of choice. Coward et al., (1968) criticised dioxane as giving rise to most variation compared with other solvents after chromatography of urine extracts. This criticism has been difficult to substantiate; derivatives of all compounds studied during the present work unless otherwise stated gave single symmetrical peaks when dioxane was used. Dioxane has never been found to interfere in any way with the production of a derivative.

Choice of stationary phase

The selection of an appropriate phase is the most important step in any GLC procedure, for it is the means by which the actual separation takes place. Unlike other factors, it is often difficult to predict its behaviour. As a general guide however, the principle of "like dissolve like" should be adopted. For instance non-polar solutes are best separated by non-polar solvents while polar solutes are best dealt with using polar solvents. In some cases when a solute is slightly polar, columns with a mixture of two solvents, one polar and the other non-polar e.g. 7% silicone oil plus 1% ethylene glycol succinate-phenylmethylsiloxane, F-60-z (Brooks and Horning, 1964) or a

slightly polar solvent such as QF1 (Greer and Williams, 1967) may be required to obtain effective separation.

Because there are a few hard and fast rules to guide the choice of solvent, it is necessary to rely mainly on trial and error.

Experimental findings

For the choice of a satisfactory stationary phase, fourteen different arrangements of phases were investigated (Table 3.1), applying to the column the TE/E derivatives of a mixture of closely related phenolic acids. An experimental arrangement was selected for each column in accordance with its optimum temperature and flow as described later. Comparison of solvents was carried out by assessing the separation of the mixture, especially of 3,4-dihydroxybenzoic and 3,4-dihydroxyphenylacetic acids. The non-polar solvents F60, SE30, SE52 and SE54 proved to be the most satisfactory, although all were shown later to possess certain drawbacks with regard to quantitation of urinary VMA (Karoum et al., 1968). The best separation was obtained with F60 followed by SE52, SE30 and SE54.

A similar comparison was carried out of the four non-polar phases mentioned above using ME/TE derivatives. Comparison of liquid phase was assessed by measuring the degree of separation of 3,4-dihydroxyphenylacetic and homogentisic acids. The best separation was obtained with the SE group of phases Fig. 3.1

TABLE 3.1.

Stationary phase	Polarity	Comment
1% and 10% polyethylene glycol (PEG)	Strongly polar	Not satisfactory. Fast elution of phenolic acid mixture of derivatives.
5% QFI	Slightly polar	Not satisfactory. Fast elution of phenolic acid derivatives.
5% silicone elastomer (polydimethylsiloxane)	Non polar	Not satisfactory. Long retention time of phenolic acid derivative. Poor resolution.
10% methyl silicone gum (SE30)	Non polar	Satisfactory. Good resolution of derivatives.
10% methyl phenyl silicone gum (SE52)	Non polar	Satisfactory. Good resolution of derivatives.
10% methyl phenyl vinyl silicone gum (SE54)	Non polar	Satisfactory. Good resolution of derivatives.
10% silicone oil (F-60)	Non polar	Satisfactory. Resolution of derivatives better than the SE groups.
10% Apiezon Grease	non polar	Not satisfactory. Poor resolution of derivatives.
7% F-60 with 1% ethylene glycol succinate-phenylmethylsiloxane (F60-z), (Brooks and Horning, 1964).	Slightly polar	Satisfactory. Not as good as the SE groups.
0.6% methylsiloxane polymer (JXR) and 0.2% cyclohexanedimethanol succinate polyester (HIEFF-8BP).	Polar	Not satisfactory. Poor resolution of compounds.

TABLE 3.1 (Cont.)

Stationary phase	Polarity	Comment
5% silicone elastomer and 5% F-60.	Non polar	Not satisfactory. Long retention time of phenolic acid derivatives. Poor resolution of derivatives.
5% F-60 plus 5% SE54	Non polar	Satisfactory. No improvement on when either used alone.
10% F-60 plus 5% SE52	Non polar	Satisfactory. No improvement on when either used alone.
7.5% F-60 plus 2.5% QFI	Very slightly polar	No improvement on F-60 when used alone.
7.5% SE52 plus 2.5% QFI	Very slightly polar	No improvement on SE30 when used alone.

Summary on the characteristics of a group of stationary phases investigated for the separation of phenolic acids when converted to their TE/E derivatives. Phases were coated on 85/100 mesh siliconised celite and packed into 7 ft. U-shaped glass columns. For condition of G.L.C. analysis see text.

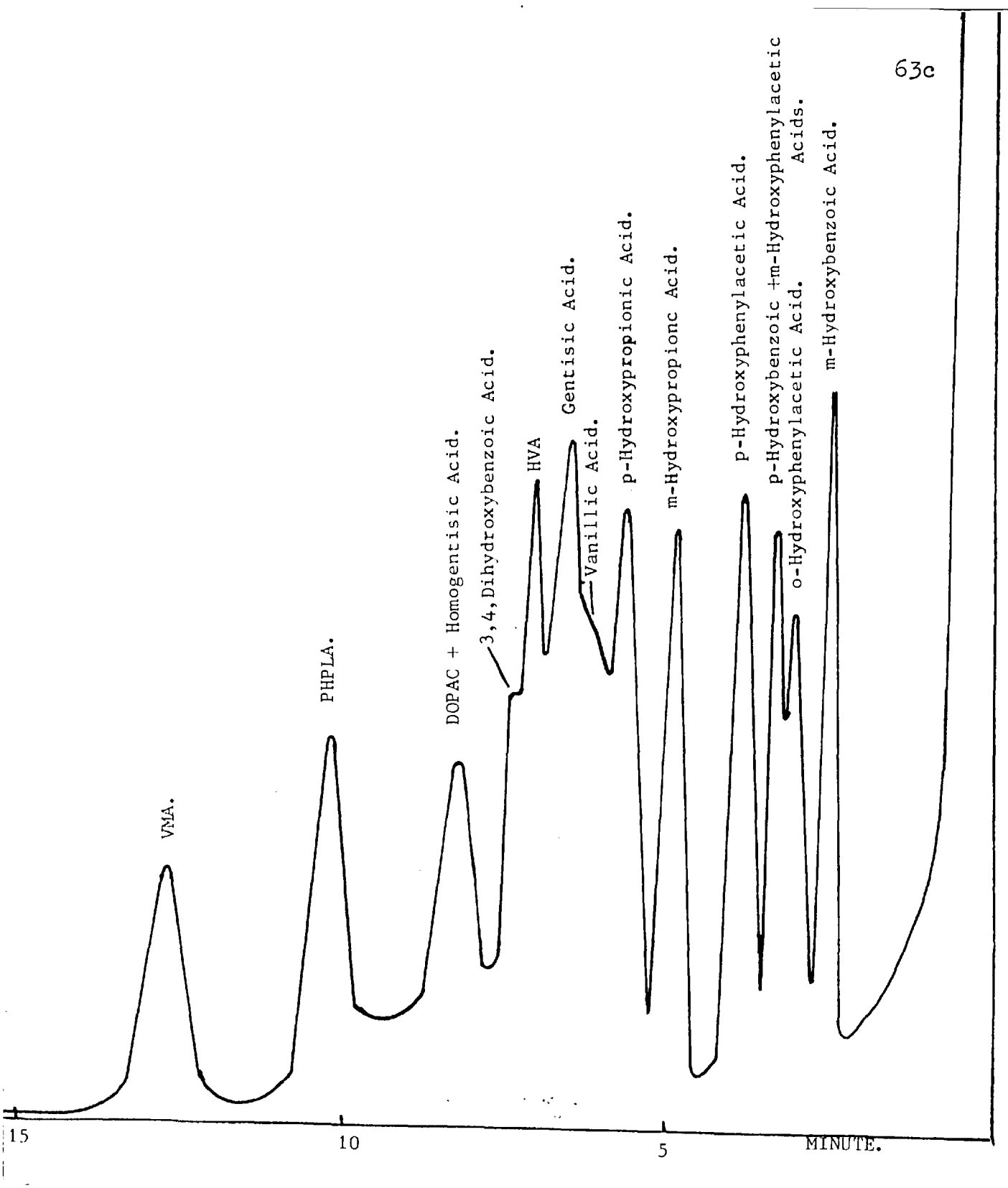


Fig. 3.1. Separation of phenolic acids as their TE/TE on a 7 ft. 10% SE52 condition of GLC as described in the text.

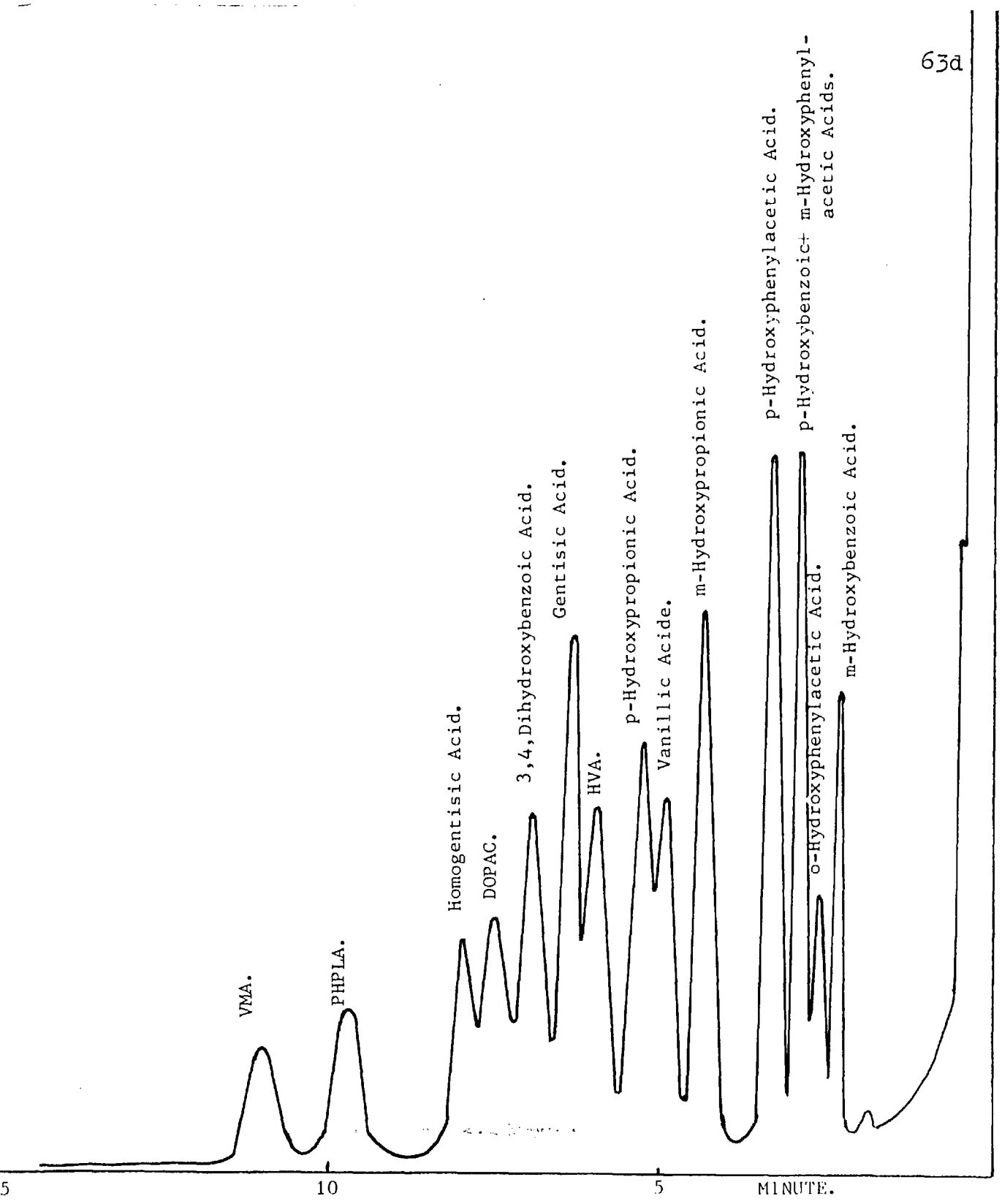


Fig. 3.2. Separation of phenolic acids as their ME/TE on a 7 ft. 10% SE52 column. Condition of GLC as described in the text.

and 3.2. The ME/TE derivative was found to be the most suitable for the analysis of urinary phenolic acids (Karoum et al, 1968, 1968a) as it permits the measurement of both HVA and VMA on the same chromatogram. Among the SE group, SE52 was found to be the most suitable.

Employing the same four non-polar stationary phases, indolic acids showed similar characteristics to phenolic acids with respect to derivatives and solvents; ME/TE derivatives chromatographed on SE52 appeared to provide the best separation (Karoum et al, 1968a, 1969).

A TE/E derivative was of necessity used for phenolic and indolic alcohols. Whilst there was little to choose between the four non-polar phases, SE52 was selected for convenience, as it had previously been adopted as the liquid phase of choice for the analysis of phenolic and indolic acids.

Type and size of support particle

The principal function of a solid support in GLC is to provide an inert framework for the stationary phase. A suitable support should possess the following physical properties (Purnell, 1962):

1. It should be able to absorb large volumes of viscous liquid; even so, its constituent particles should run freely and pack into a column offering maximum permeability to gas flow.
2. It should be separable into fractions of well defined

particle size within the approximate range of diameters 0.05 to 0.5 mm.

3. The particles should be sufficiently robust to withstand the usual procedures of preparation and column construction without deterioration.

4. It should offer a large surface area per gram of weight, but nevertheless be completely non-adsorbing and chemically unreactive.

Materials approximating to these requirements include glass powder and microspheres, low activity carbon Pelletex, certain crystalline salts and diatomaceous earth products such as Celite, Dicalite, Possilsil and Chromosorb and the fire bricks, Sil-o-cel and Sterchamol (Purnell, 1962). The diatomaceous earth products Celite, Chromosorb and Sil-o-cel are the most popular

In practice, adsorption of solute by support is not infrequently encountered, and has been attributed to the presence of metal (Fe and AL) (Liberti, 1958) and free hydroxyl radicals on the surfaces of the support (Purnell, 1962). Washing with acid and alkali (Liberti, 1958) removes the metals, but does not provide a complete answer. Silylation of the support with trimethylchlorosilane (TMCS) (Kiselev, 1958) and hexamethyl-disilazane (Bohemen, Langer, Perrett and Purnell, 1960) which attack the free hydroxy radicals provides more satisfactory results than acid and alkali washing. Celite is the most inert

of the diatomaceous earths, and siliconisation improves further this property (Figs. 3.3 and 3.4).

In general column efficiency improves with diminishing particle size because of the effects of this characterisation on eddy diffusion (term 2λdp in equation 2.19) where reduction in dp (particle size) reduces "H". Although this is true for particles over 100 mesh, the reverse is seen with smaller particles (Purnell, 1962). Thus, for any support, there exists an optimum particle size, usually lying between 60 and 120 mesh.

Experimental findings

The particle sizes often referred to in the literature lie between 60 and 120 mesh (Purnell, 1962). A comparison has been made of column performance of 5% and 10% SE52 coated on 85/100 mesh and 100/120 mesh siliconised celite, employing ME/TE and TE/E derivatives. The results, which are qualitative, but point quite distinctly to the superiority of 85/100 rather than 100/120 mesh, are summarised as follows:

1. Less crumbling was observed with 85/100 mesh when both were coated with 5% or 10% SE52.
2. Coated 85/100 mesh support runs freely during packing and unpacking but 100/120 mesh did not.
3. Less solvent trailing was observed with coated 85/100 than 100/120 mesh.

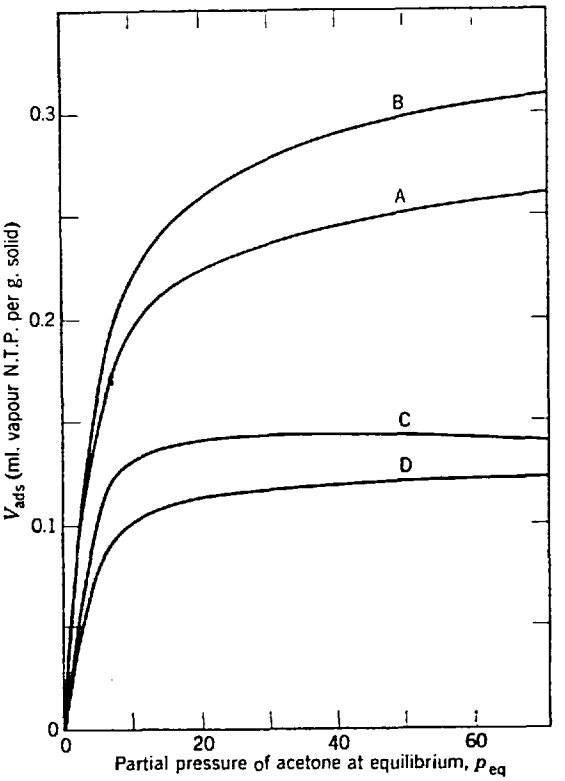


Fig. 3.3. Isotherms for the adsorption at 50°C. of acetone vapour by A, Sil-O-Cel and Chromosorb; B, acid washed Chromosorb; C, Chromosorb-W; D, Celite 545. After Purnell (1962).

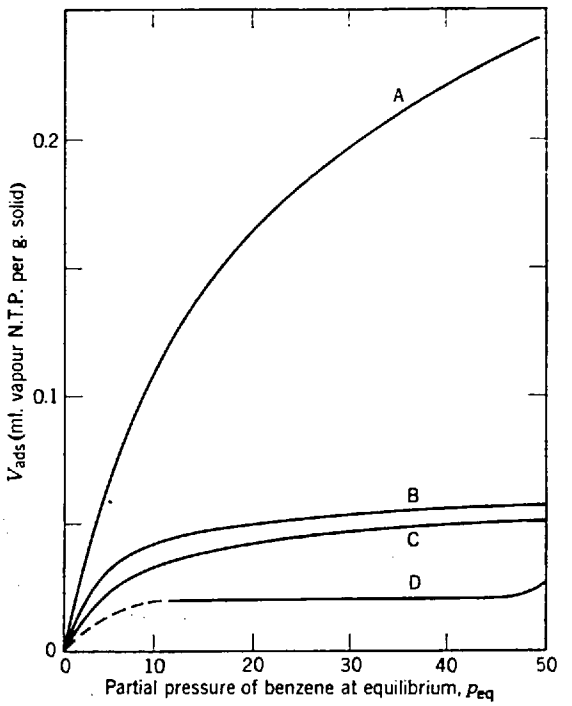


Fig. 3.4. Isotherms for the adsorption at 50°C. of benzene vapour, first by Sil-O-Cel (A, before and C, after treatment with hexamethyldisilazane) and second by Celite 545 (B, before and D, after treatment). After Purnell, (1962).

4. The overall performance as judged from elution of peaks, steadiness of baseline and flow of carrier gas, was better with 85/100 than 100/120 mesh celite.

Thus it was decided to use 85/100 mesh siliconised celite in preference to any other grade.

Choice of optimum parameters from studies on "height equivalent to theoretical plate" (HETP)

During the stage of searching for a suitable stationary phase, little attention was paid to the selection of parameters offering the best performance with respect to analysis time, separation factors and quantitation. These parameters include flow rate, U, % of solvent to support, column length and temperature.

As mentioned in chapter 2, a column should ideally be operated under condition of maximum efficiency whilst at the same time allowing effective separation to be carried out in the shortest possible time. The practical approach to solving this problem is to plot H against U (and in some cases against H/U) as shown in Fig. 3.5. Here H drops rapidly to a minimum, H_{min}, as U increases and then rises gradually. For rapid high efficiency operation, low values of H_{min}/U_{min} or $(H/U)_{min}$ should be sought.

From a consideration of the simplified Van Deemter equation (equation 2.23), minimum values of H and U for any

column and solute (Purnell, 1962) are given by

$$H_{min} = A + 2\sqrt{B}\,C_1 \qquad 3.1$$

and $$U_{min} = \sqrt{B}/C_1 \qquad 3.2$$

(A and B being constants and C_1 a factor corresponding to amount of stationary phase)

Hence $$\frac{H_{min}}{U_{min}} = A\sqrt{\frac{C_1}{B}} + 2C_1 \qquad 3.3$$

On the other hand, writing the simplified Van Deemter equation as (see equation 2.23)

$$\frac{H}{U} = \frac{A}{U} + \frac{B}{U^2} + C_1 \qquad 3.4$$

$(\frac{H}{U})_{U_{min}}$ is approximately equal to C_1 when values of U are high. This is shown graphically in Fig. 3.5. where the slope $\frac{H_{min}}{U_{min}}$ is approximately twice $(\frac{H}{U})_{min}$ as predicted from equation 3.3 and 3.4

For rapid high efficiency column operation therefore, there exist two main approaches; either to run the column at its U_{min} value or, better, at high values of carrier gas flow i.e. U_g; but here, if the curve BC in Fig. 3.5 is not a plateau, there will be some loss of theoretical plates, n, which will have to be compensated for if a particular efficiency is needed, by increasing the column length. Thus, if one uses a column

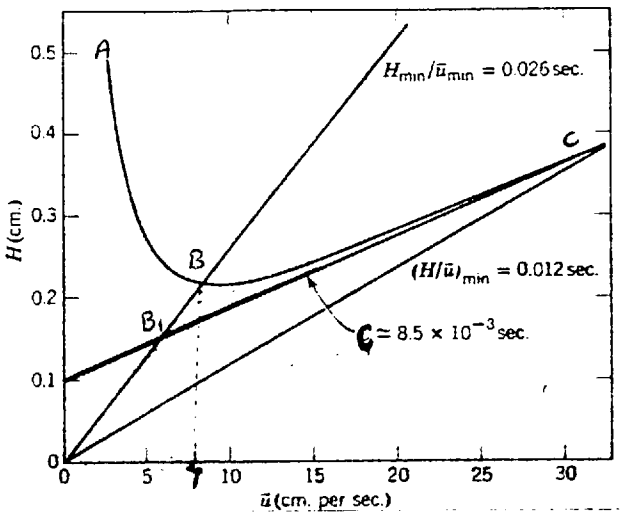


Fig. 3.5. Plot illustrating the difference between $(H/\bar{u})_{min}$ and $(H_{min}/\bar{u}_{min})$. After Purnell, 1962.

with characteristics shown in Fig. 3.5, it should be operated for a given value of n with a length L cm at 7 cm sec $^{-1}$ ($\frac{H_{min}}{U_{min}}$ or, better, with a length 1.7 L cm at 30 cm sec $^{-1}$.

For fast analysis, where H/U $C \simeq C_1 + \frac{C_g}{f}$ (Purnell, 1962) f being a constant, the time of elution of the second component of an exactly separated pair of substances is given by

$$t = 36 \left(\frac{\alpha}{\alpha - 1}\right)^2 \times C \left[\frac{(1 + k')^3}{(k')^2}\right] \qquad 3.5$$

α = relative retention volume of the second component to the first

$k' = K\frac{V_R}{V_d}$ or $K\frac{V_R}{V_G}$

if C = a constant which is equal to the slope B_1C in Fig. 3.5. Hence t can be varied by changing α or k'. Since in GLC high values of α are important, it is usually the practice to change k' to attain t_{min}.

By differentiating equation 3.5, it will be seen that t_{min} corresponds to k' = 2. For rapid analysis therefore, the solvent/support ratio and temperature should be chosen so that k' = 2 for the second of a pair of substances most difficult to separate well in a mixture. It is desirable to keep α

high, usually by keeping the temperature low (high k'); hence once a suitable temperature is selected, as described below, k' is usually monitored by changing the solvent/support ratio. To keep k' values low and near 2, therefore, a low percentage of solvent is to be preferred to a high one. Whenever possible therefore, column efficiencies are best increased by increasing column length while keeping the percentage of solvent to a minimum.

The optimum temperature for a particular solvent/solute system may be determined approximately by plotting H against temperature, at constant U_{min}. The temperature which corresponds to H_{min} is within $\pm$ 5°C of T_{max}. This factor can then be evaluated precisely by studying column performance with a mixture of compounds at temperatures varying between $\pm$ 5°C from that determined from the above experiment. T_{max} is selected as that which offers the best separation of peaks yet permitting easy quantitation of all substances chromatographed.

Experimental findings

From HETP studies on 7 ft columns of 10% F60 and SE52, as described above, the optimum temperatures for the TE/E derivatives were found to be 195°C and 190°C for F60 and SE52 columns respectively, and 190°C for both columns when the ME/TE derivative was used. Fig. 3.6 represents plots of H

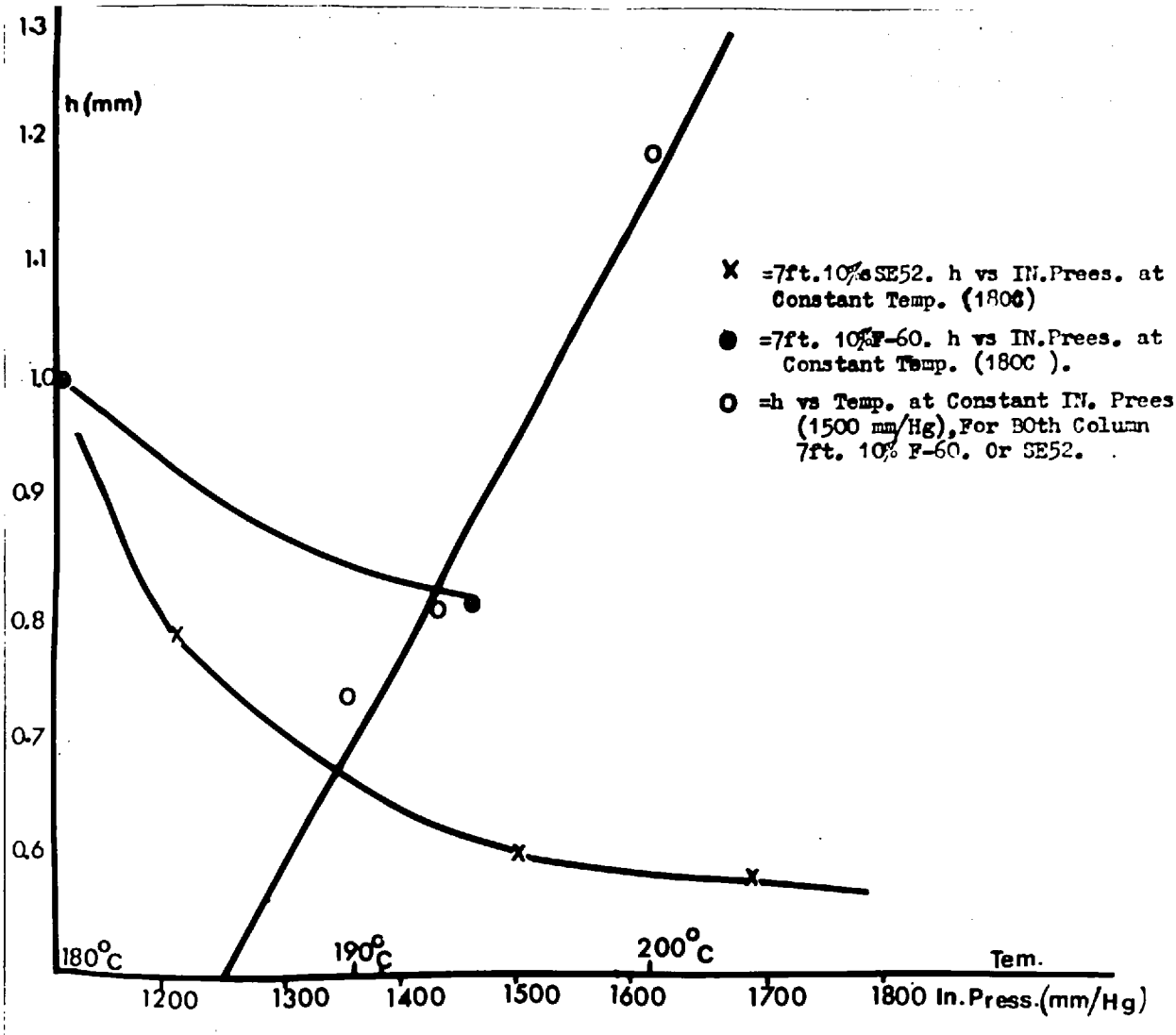


Fig. 3.6. Plots of h (plate height) against inlet pressure at constant temperature, and against temperature at constant inlet pressure for two 7 ft. columns packed with coated 10% SE52 and 10% F-60.

against inlet pressures at constant temperature, and H against temperature at U_{min}, from which the optimum temperatures were approximately determined.

Having determined the optimum temperatures for SE52 and F60 columns, the next task was to assess the appropriate efficiences required for the quantitative analysis of urinary metabolites, and the types of columns able to produce these efficiences. It was found that urinary metabolites of interest during the present investigation fell into two groups, the first including most of the important phenolic compounds e.g. HVA, VMA etc., and the second, hydroxyindolic compounds e.g. 5HIAA and 5HTOH, possessing longer retention times than the first group. For their quantitative analysis, metabolites belonging to the first group required column efficiency between 2500 and 3500 plates while those of the second need efficiencies of about 1500 plates; higher efficiencies produce broad peaks which are difficult to quantitate accurately at concentrations normally present in the urine.

In an attempt to find SE52 columns providing these efficiencies, 3 ft., 5 ft., 7 ft. and 9 ft. columns packed with 3%, 5%, 10% and 15% SE52 on 85/100 mesh siliconised celite were examined, employing mixtures of phenolic and indolic compounds both as their ME/TE and TE/E derivatives. The results were judged qualitatively taking into account solvent

elution, recorder pen return to baseline, solute elution and general column performance. For phenolic compounds it was established that a 7 ft. 10% SE52 column was optimal, while for hydroxyindolic compound a 5 ft. 5% column appeared to be the most suitable.

Summary: An optimum GLC arrangement taking into account theoretical and experimental considerations

The features discussed above which have been established as most appropriate for GLC analysis of phenolic and indolic compounds of biological origin are summarised below:

Choice of derivative	ME/TE for acidic compounds. TE/E for alcoholic compounds.
Column dimension	i) For phenolic compounds, 7 ft. x 5 mm (internal diameter) siliconised U-shaped glass tube prepared as described in chapter 4. ii) For hydroxyindolic compounds, 5 ft x 5 mm siliconised U-shaped glass tubes as in (i)
Support	85/100 mesh siliconised celite.
Carrier gas flow and pressure	1500 mm/Hg inlet and atmospheric outlet pressures, producing an average carrier gas flow corresponding to 60 - 100 cm sec^{-1}.

Stationary Phase	i)	For phenolic compounds, 10% SE52.
	ii)	For hydroxyindolic compounds, 5% SE52.
Column temperature	190°C ± 3°C.	

Injection volumes

For high efficiency analysis, it is essential that solute bands occupy the smallest possible number of plates if accurate and reproducible results are to be obtained. This is achieved by injecting small compact volumes of solute, making sure that the temperature of the injection head is high enough to bring about immediate vaporisation of solute. Were large volumes of solute to be injected, vaporisation would be impeded, particularly if the volume of solute vapour, at the temperature employed, is larger than the volume of the injection chamber. The importance of injecting small volumes created a demand for sufficiently sensitive detectors, of which several are now available on the market (Fowlis, Maggs and Scott, 1963).

Macro argon ionisation detector

This detector is based upon a principle formulated by Lovelock (1958, 1958a). Argon carrier gas molecules become

excited as they pass through a radiation chamber (^{90}Sn). The metastable molecules have a sufficiently long life to allow them to collide with other molecules to which they transfer their energy and fall to their ground levels. The energy transferred (11.6 electron volts) is enough to ionise a large variety of organic substances. These ions are then collected on an electrode placed inside the ionisation chamber with voltage applied across it. The current produced is measured after amplifying it several thousand times and fed to a suitable recording device.

The detector possesses a high degree of sensitivity while being relatively unaffected by changes in temperature, pressure and gas flow. Owing to its high sensitivity, however, it is easily contaminated (by deposited SiO_2) particularly when there is excessive bleeding from the column or if large volumes of solute are frequently injected (personal observation); such contamination is characterised by increased base line "noise" and may be followed ultimately by complete destruction of the detector.

Linearity of the detector

The detector is linear at one operating voltage only. Fortunately it is usually possible to arrange for the applied voltage to decrease with increase of current through the

voltages above a certain minimum (detector linearisation). For quantitative work, it is essential to calibrate the detector and to determine the appropriate voltage with its linear range of response for the type of analysis involved (Fowlis, Maggs and Scott, 1964). For more detailed information, see Evans and Scott (1963). The recommended voltage range at different detector temperatures, whereby linearity may be expected, is given in the table below, (PYE Panchromatograph manual 1964):

Table 3.2

Temperature	Typical voltage range
50°C	550 - 1500
75°C	550 - 1250
100°C	550 - 1250
150°C	550 - 1000
200°C	380 - 1000
225°C	380 - 770
250°C	380 - 770

Amplifier ranges

The detector is normally used with the following amplifier ranges.

1	x	10^{-8}	Amp.	full	scale
3	x	10^{-9}	"	"	"
1	x	10^{-9}	"	"	"

Experimental findings

The most convenient applied voltage and operating temperature for the detector were found to be 1000 V and 200°C respectively. Operating at high temperatures and voltages, although offering increased detector sensitivity, dramatically shortens its working life from over six months to less than two months.

The linearity of the detector was assessed at three applied voltages 800V, 1000V and 1250V, at a constant temperature of 200°C. Different amounts of a mixture of the TE/E derivatives of HVA, VMA, DOPAC and DHMP were injected into a 7 ft. 10% SE52 column maintained under "optimal conditions" (see earlier) with applied voltages of 800V and 1000V (Fig. 3.7 and 3.8) and repeated using a C_{18} normal alkane standard at applied voltages of 1000V and 1250V (Fig. 3.9). Quantitative results were obtained by integrating peak areas with a Technicon integrator calculator and by measuring peak heights.

As seen from Figs. 3.7 and 3.8, at applied voltages of 800 and 1000 V the detector response is linear for amounts of up to 1.5 μg of parent phenolic compound converted to its TE/E derivative. At an applied voltage of 1250V, however, the upper

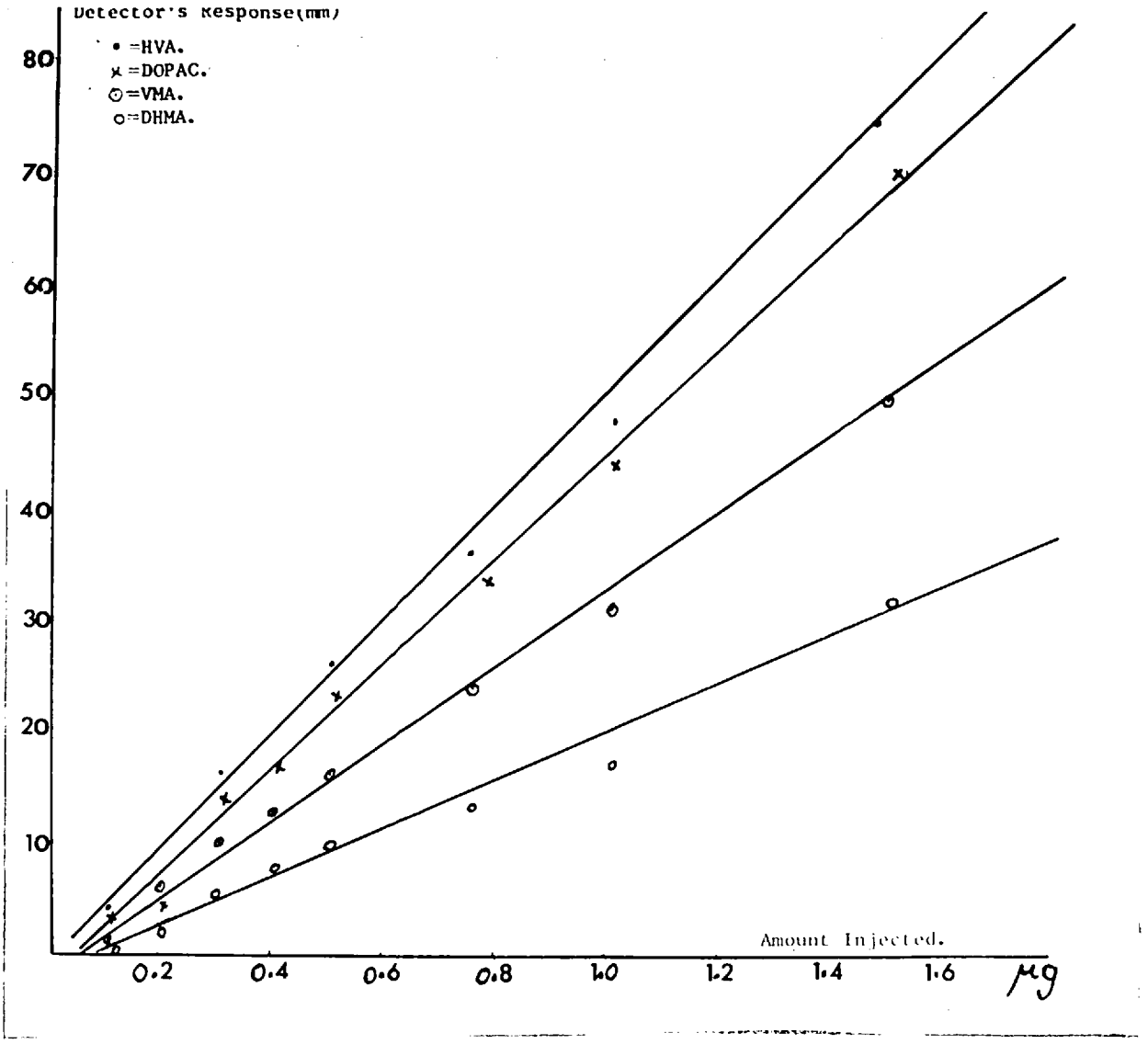


Fig. 3.7. Plots of different quantities of phenolic acid as their TE/E derivatives against detector response measured in terms of peak heights at an applied voltage of 800 V.

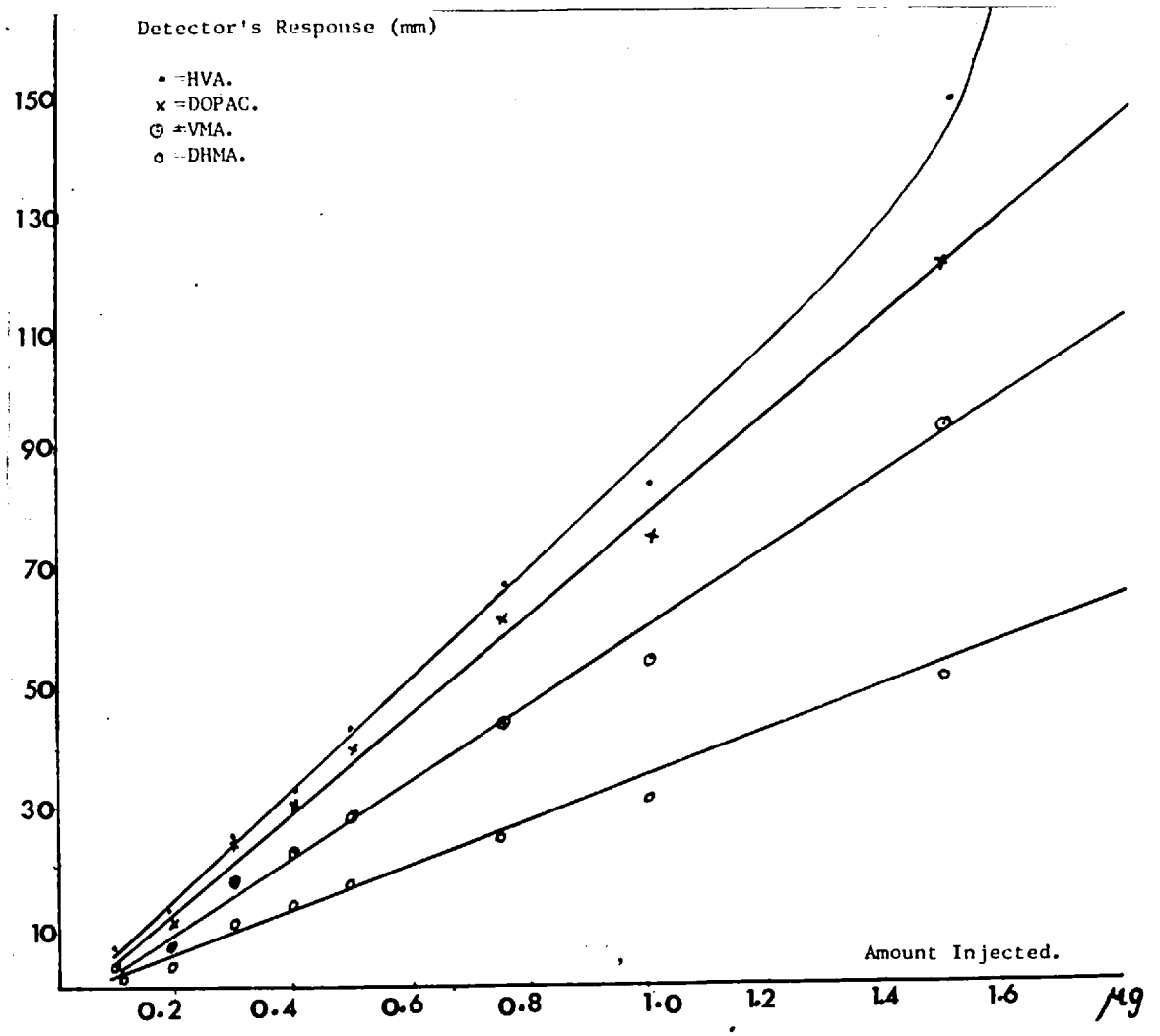


Fig. 3.8. Plots of different quantities of phenolic acids as their TE/E derivatives against detector response measured in terms of peak heights at an applied voltage of 1000 V.

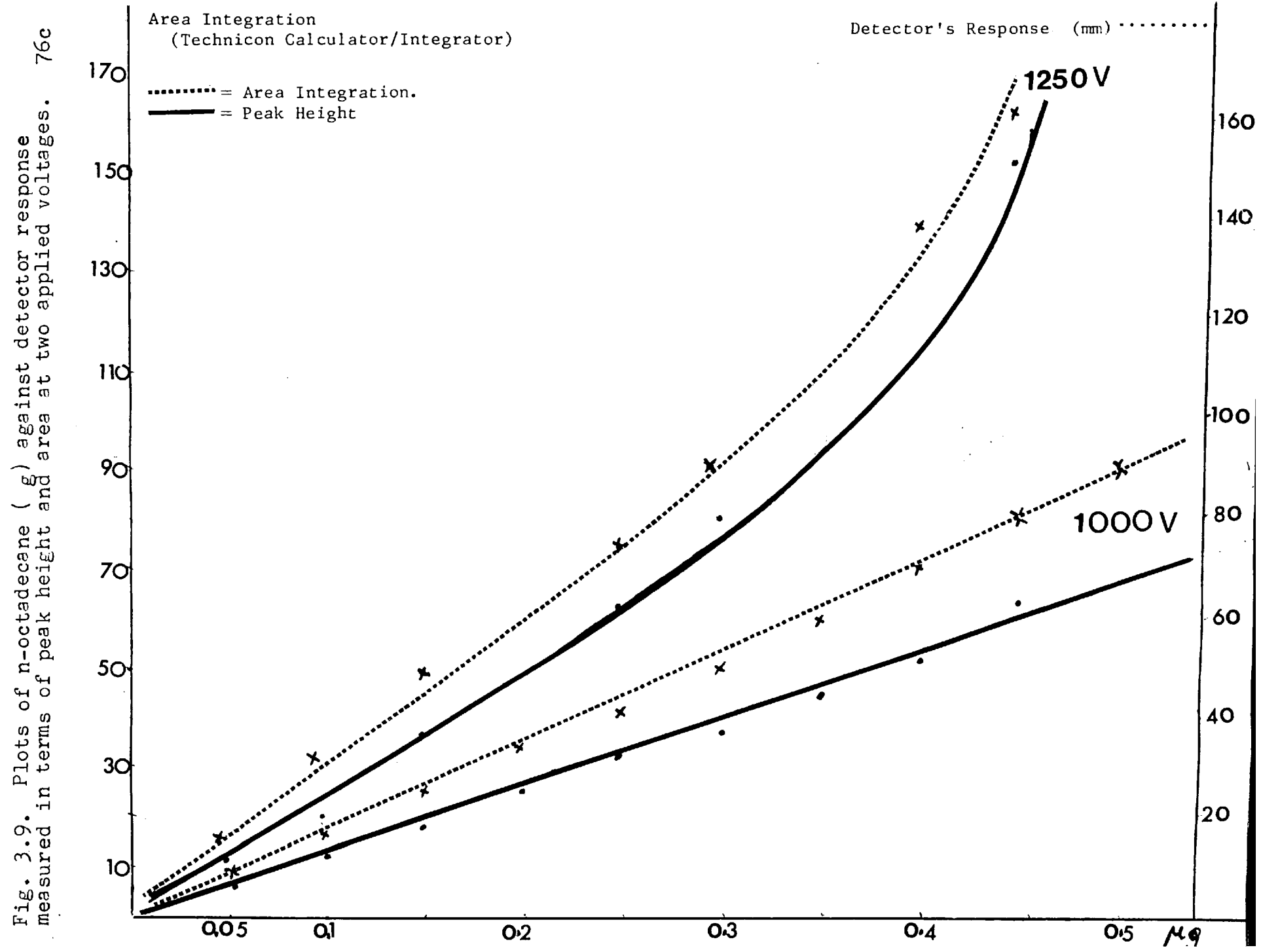


Fig. 3.9. Plots of n-octadecane (g) against detector response measured in terms of peak height and area at two applied voltages.

limit of linearity is reduced to about 0.5 μg parent phenolic compound, as shown in Fig. 3.9. For the reasons noted above, an operating detector temperature of 200°C and an applied voltage of 1000V were selected as optimal and employed for all subsequent work.

Quantitation of peaks

For accurate quantitative analysis it is necessary to eliminate all sources of instrumental error and employ accurate, reliable and consistent means of peak measurement.

To eliminate instrumental technical error, it is necessary to bear the following points in mind (Evans et al., 1963):

1. The sample should be stored correctly.
2. A "hypodermic syringe" method of injection should be employed.
3. Adsorption on to the support should be reduced to a minimum, (adsorption is manifested by asymmetrical peaks).
4. The detector should be operated at the minimum sensitivity commensurate with other column requirements. When small changes and very high sensitivities have to be employed, calibration should be carried out to account for the effects of adsorption or other factors affecting peak symmetry.

5. The detector used should have been proved to have a linear response over the sensitivity range of operation. For detectors with significant non linearity, mass/peak area calibration must be carried out for each substance under the column conditions to be used for analysis. Any change in the type of compound or column conditions will necessitate recalibration.

With regard to peak measurements, there are basically two approaches, measurements of peak height and of peak area. Peak height may be measured directly from the differential record; peak area can be obtained by geometrical measurement or by the use of an automatic integrator. Measuring peak height, although simple (Purnell, 1962; Evans et al., 1963) possesses the disadvantage of being dependent on constant column operating conditions. Thus, peak height measurements are more suited to isothermal analysis than temperature programming. Furthermore, the precision of the measurement is not merely dependent on the mass of substance present, but on its retention volume. Peaks representing equal masses of substances with different retention ratios may have widely different heights. This effect may be overcome either by using internal standards for each substance to be measured or employing one standard and multiplying by an appropriate relative response factor. This is determined from the ratio

of the detector response for equal quantities of a standard to that of other compounds. The accuracy of any result obtained by peak height measurement also depends upon the peak being symmetrical or gaussian.

Experimental findings

The experimental arrangement for GLC analysis throughout this work was carefully controlled to eliminate any source of error deriving from the apparatus or from the mode of injection. Thus a) during the period of analysis column conditions were kept constant, b) adsorption of solute by stationary phase was known to be negligible as manifested by the production of symmetrical peaks, c) precision syringes were used for injection and the amount of any individual constituent in a mixture to be injected did not exceed 1 - 1.5 μg. and d) as shown in Figs. 3.7 and 3.8 (and discussed earlier), the detector was used within its linear range of response.

During the present work, peak height and peak area measurements did not differ appreciably. Apart from its rapidity the former technique is probably more suitable for the analysis of urinary metabolites where return of the recorder pen to base-line is slow because of solvent trailing. Further support for the validity of peak height measurement was provided by observations on the excretion ranges of certain

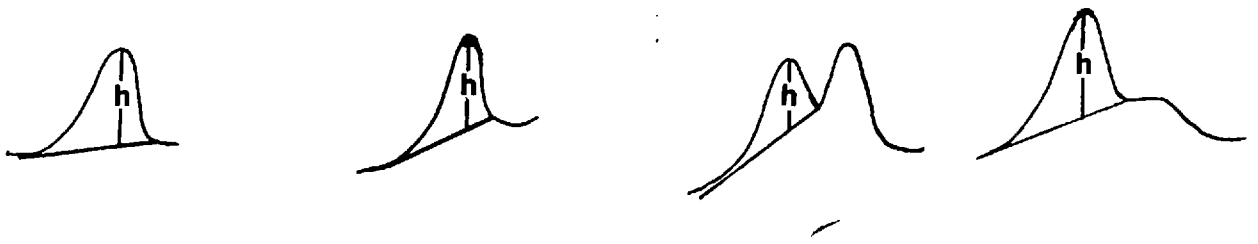


Fig. 3.10. An illustration of four main types of peaks encountered in urinary GLC analysis showing how their heights would be measured for quantification.

TABLE 3.3

Compounds compared	Solvent	PH	Derivative	Relative detector reponse
P-HPAA/HVA	Ethylacetate	2	ME/TE	4.15
HVA/3CPAC	"	"	"	1.7
HVA/VMA	"	"	"	2.4
P-HPLA/VMA	"	"	"	1.4
VMA/DHMA	"	"	"	1.8
VMA/VLA	"	"	"	2.5
VMA/P-HPPA	"	"	"	2.0
P-HPE/HMPG	"	8	TE/E	8.0
HMPE/HMPG	"	"	"	2.2
HMPG/DHPG	"	"	"	1.7

Relative response values of pairs of phenolic compounds when extracted and chromatographed through exactly the same procedure as they would be treated for urinary analysis of such metabolites.

urinary metabolites which were found to be comparable with those obtained by other methods. (see chapter 5). The four main types of peak encountered and the manner in which their heights were measured are shown in Fig. 3.10. Table 3.3 contains relative response values of some phenolic compounds encountered in the course of this work.

Peak characterisation

Successful analysis of any mixture depends upon three factors, devising a soundly based experimental procedure involving the use of dependable apparatus, unequivocal identification of all components to be analysed and finding means of accurate determination of each eluted component. The first factor has been adequately dealt with; peak identification and measurement will be the concern of the remainder of this chapter.

Of the methods which have been employed for peak identification (Purnell, 1962) those based on relative retention data are the most commonly used. They employ the principle that the true retention volume V_{TR} of different substances under the same conditions are dependent on their partition coefficients, as shown in the equation

$$V_{TR} = KVL \qquad 3.6$$

where VL is the volume of stationary phase and K the partition

coefficient.

Alternatively the ratios of the true retention volumes of two substances, within certain ranges of temperatures, are constant; thus

$$\frac{V_{TR1}}{V_{TR2}} = \frac{K_1}{K_2} \qquad 3.7$$

This relationship between true retention volumes of substances and their partition coefficients has been extensively applied in characterising peaks on chromatograms where the retention data are recorded in relation to an arbitrary standard. Such results are usually reported as relative retention times, RRT, or volumes. Anomalies usually associated with RRT are mainly due to the commonly committed error of using apparent retention volumes or times rather than true retention volumes or times. The apparent retention volume VR is related to K by

$$V_R = V_G + KVL \quad \text{(see chapter 2)}$$

where V_G is the free space in the column, sometimes referred to as the dead volume; V_G has therefore to be calculated and subtracted from the apparent retention volume V_R.

Recently (Dalgliesh et al., 1966; Karoum et al., 1968) the use of methylene units (M.U.) in the characterisation of peaks has been reported. These have the advantage over RRT in

that they are not dependent on true retention volumes or times and that they can be easily and accurately measured.

The methylene unit value of a substance is measured by comparing its position on the chromatogram with those of two even numbered normal alkane standards between which it appears. Thus the M.U. value of a particular peak is calculated from the formula.

$$MU = n + \frac{2y}{x} \qquad 3.8$$

where y is the distance (mm) from the peak to that of a neighbouring reference standard (Cn) and x is the distance (mm) between the peak of this standard and that of a second hydrocarbon of chain length Cn+2.

Experimental findings

Variation of column temperature by ± 5° from the optimum and of stationary phase by ± 5% from the preferred concentration of 10% (Karoum et al., 1968) was found to produce changes of M.U. values of less than 0.04 in four non-polar columns, a finding within the overall experimental error of the method. The methylene unit values of a number of phenolic and indolic compounds are given on tables 3.4, 3.5 and 3.6.

Apart from using M.U. measurements, the identity of some compounds of possible metabolic importance was confirmed in

TABLE 3.4

NAME	ME/TE	TE/E
Phenylacetic Acid	13.05	13.46
Salicylic Acid	14.28	15.20
p-hydroxybenzaldehyde	14.22	-
m-hydroxybenzoic Acid	14.63	15.68
p-hydroxybenzoic Acid	15.09	16.29
o-hydroxyphenylacetic Acid	14.79	15.84
m-hydroxyphenylacetic Acid	15.16	-
p-hydroxyphenylacetic Acid (P-HPAA)	15.54	16.39
3,4-dimethoxybenzoic Acid	16.07	17.11
3,4-dimethphenylacetic Acid	16.29	17.18
2,5-dimethoxyphenylacetic Acid	16.28	-
3,4-dimethoxymandelic Acid	18.16	-
3,4-dihydroxybenzoic Acid	17.29	18.24
3,4-dihydroxyphenylacetic Acid (DOPAC)	17.50	18.35
2,5-dihydroxybenzoic Acid (Gentisic)	17.04	17.85
2,5-dihydroxyphenylacetic (Homogentisic)	17.66	18.38
Mandelic Acid	14.11	14.72
β-phenyllactic Acid	14.85	16.04
Phenylpyruvic Acid	-	17.27
Ferulic Acid	19.49	-
Hippuric Acid	17.27	18.44
p-hydroxymandelic Acid	17.27	17.92
p-hydroxyphenyllactic Acid (p-HPLA)	18.18	19.05
p-hydroxyphenylpyruvic Acid (P-HPPA)	19.57	20.51
3,4-dihydroxycinnamic Acid	21.47	-

TABLE 3.4 (Cont.)

NAME	ME/TE	TE/E
3-methoxy-4-hydroxyphenylacetic Acid (HVA)	16.84	17.78
3 methoxy-4-hydroxymandellic Acid (VMA)	18.43	18.77
3,4-dihydroxymandelic Acid (DHMA)	18.87	19.30
3 methoxy-4-hydroxyphenylpyruvic Acid (VPA)	21.15	-
3 methoxy-4-hydroxyphenyllactic Acid (VLA)	19.41	20.31
m-hydroxypropionic Acid	16.14	-
p-hydroxypropionic Acid	16.46	17.63
3,4-Dihydroxyferulic Acid	-	17.70
3 methoxy-4-hydroxybenzoic Acid (vanillic)	16.53	17.63
5 methoxy-2-hydroxygentisic Acid	16.94	-
3,4 dihydroxy-5-methoxyphenylacetic Acid	18.82	-
p-hydroxycinnamic Acid	18.03	19.37

R.U. of a number of aromatic acids as their TE/E and ME/TE derivatives characterised on a 7 ft. 10% SE52 column.

TABLE 3.5.

SUBSTANCE	TE/E
p-hydroxyphenylethanol (P-HPE)	15.81
3 methoxy-4-hydroxyphenyethanol (HMPE)	17.04
3,4-dihydroxyphenylethanol (DHPE)	17.79
3-methoxy-4-hydroxyphenylglycol (HMPG)	18.37
Dihydroxyphenylglycol (DHPG)	18.82
p-hydroxyphenylglycol	17.26
2-(3,4-dimethoxyphenylethanol)	16.54

R.U. of a number of aromatic alcohols as their TE/E derivatives characterised on a 7 ft. 10% SE52 column.

TABLE 3.6.

SUBSTANCE	MB/TB	TB/E
Indolacetic Acid	18.55	19.15
Indolacetaldehyde	18.17	-
Indolpyruvic Acid	22.92	
5-hydroxyindoleacetic Acid	21.45	22.16
5-hydroxy-6-methoxyindol-2-carboxylic Acid	22.16	-
5,6-dihydroxy-2-carboxylic Acid	22.51	-
Indole	(15.65)	-
5,6-dihydroxyindoleacetic Acid	24.14	-
Tryptophol	18.42	-
5-hydroxytryptophol	21.30	-

M.U. of a number of Indolic compounds on a 5 ft. 5% OV52 column.

the following way:

1. By preparing two derivatives of the unknown, ME/TE and TE/E, and comparing M.U. values with those of an authentic reference compound. This approach has also been used to distinguish between compounds with and without carboxylic groups which become methylated during the formation of a ME/TE derivative (Karoum et al., 1968, 1969).
2. By combining either column and/or thin layer chromatography (TLC) with GLC.
3. By combining gas chromatography and mass-spectrometry, as described in chapter 8.
4. By demethylation of methoxy aromatic compounds to the hydroxy configuration followed by GLC identification of the product (see chapter 4 for details).

Preliminary extraction prior to GLC

A material of biological origin such as urine contains in solution a mixture of a very large number of compounds. As the detector on the apparatus will not discriminate between any compound presented to it, it is usually necessary to limit the large number of potential peaks by separating compounds of like physico-chemical characteristics by organic extraction procedures. Thus urinary phenolic and indolic acids and alcohols may be suitably separated in this way.

Experimental findings

A survey of the extraction characteristics of a number of phenolic and indolic compounds at different pH values was carried out with the two organic solvents most widely used in this field, ethyl acetate and ether. The recoveries of a number of phenolic and indolic compounds from aqueous solution using these two solvents are shown in table 3.7.

Whilst, in general, both ethyl acetate and ether were found suitable for phenolic compounds, the indolic metabolites 5HIAA and 5HTOH were more readily extracted with ether, which was about twenty times more efficient than ethyl acetate. Ether was also found to be a more suitable solvent for certain phenolic acids, p-hydroxymandelic, phenyllactic, dihydroxyphenylacetic and homogentisic acids.

Information obtained from these studies on the behaviour of compounds during extraction, summarised on table 3.7, was extensively employed during the analysis of urinary metabolites from both normal and pathological subjects. For example, although pH values of either 1 or 2 are suitable for the extraction of most urinary phenolic acids into ethyl acetate, pH 2 was selected, as hippuric acid, which may interfere with certain GLC analyses (Karoum et al., 1968, 1968a) is less readily extracted at this pH. For indolic acids, on the other hand, pH 3.5 was selected, as extraction with ether

TABLE 3.7

pH value of aqueous phase	Percentage Recovery									
	Ethyl acetate extract					Ether extract				
	1	4	6	8	10	1	4	6	8	10
Phenyllactic acid	30	30	20	N.D*	N.D	75	12	N.D	N.D	N.D
p-Hydroxyphenyllactic acid	73	31	24	13	N.D	84	25	N.D	N.D	N.D
p-Hydroxyphenylacetic acid	90	70	58	N.D.	N.D	100	81	12	N.D	N.D
Phenylpyruvic acid+	50	N.D	N.D	N.D	N.D	50	5	N.D	N.D	N.D
p-Hydroxyphenylpyruvic acid	100	5	N.D	N.D	N.D	35	N.D	N.D	N.D	N.D
4-Hydroxy-3-methoxymandelic acid (VMA)	85	10	N.D	N.D	N.D	45	N.D	N.D	N.D	N.D
Homovanillic acid (HVA)	100	100	23	23	-	79	23	10	N.D	N.D
3,4-Dihydroxyphenylacetic acid	10	5	3	N.D	N.D	75	40	3	N.D	N.D
p-Hydroxymandelic acid	23	8	1	N.D	N.D	68	19	N.D	N.D	N.D
3,4-Dihydroxymandelic acid	63	N.D	N.D	N.D	N.D	13	N.D	N.D	N.D	N.D
Homogentisic acid	N.D	N.D	N.D	N.D	N.D	71	N.D	N.D	N.D	N.D
Gentisic acid	97	28	34	8	8	97	49	13	15	N.D
Hippuric acid	64	N.D	N.D	N.D	N.D	47	N.D	N.D	N.D	N.D
4-Hydroxy-3-methoxyphenylglycol (HMPG)+	99	72	68	58	51	7	29	47	50	29
4-Hydroxy-3-methoxyphenylethanol+	-	-	79	70	-	-	-	-	-	-

TABLE 3.7 (Cont.)

	Percentage Recovery									
	Ethyl acetate extract					Ether extract				
pH value of aqueous phase	1	4	6	8	10	1	4	6	8	10
Indolylacetic acid	53	53	26	N.D	N.D	80	53	26	N.D	N.D
Tryptophol +	N.D	28	34	48	44	97	97	97	100	100
5-Hydroxyindolylacetic acid (5HIAA)	5	N.D	N.D	N.D	N.D	76	94	9	N.D	N.D
5-Hydroxytryptophol+	-	-	-	-	-	94	109	103	106	110
Dihydroxyphenylethanol	-	-	N.D	N.D	-	-	-	80	80	-

Recoveries of phenolic and indolic compounds extracted with ethyl acetate or ether from aqueous solution at pH values between 1 and 10. Except where stated, ME/TE derivatives were prepared and chromatographed on a 7 ft. 10% SE52 column apart from 5HIAA and 5-hydroxytryptophol where a 5 ft. 6% SE52 column was used.

Note: *N.D = not detected +The TE/E derivative was prepared.

leaves behind most phenolic compounds at this pH. Phenolic and indolic alcohols and other neutral compounds were extracted with one or other appropriate solvent at pH 8, which effectively leaves most acidic compounds in the aqueous phase.

Preservation of urine

As it is not practicable to analyse all urine samples freshly after voiding, it is obviously important that they should be effectively preserved, preventing degradation of metabolites by chemical or bacterial action.

Experimental findings

Both free and conjugated phenolic compounds appeared to be satisfactorily preserved at pH 1 and 2 when stored at 4°C. No changes in apparent concentrations of HVA, VMA or HMPG were observed in urine samples of normal or pathological origin collected and stored in this way when analysed within one month and again after two years. Free and conjugated 5HIAA and 5HTOH were both found to be unstable at low pH however, as shown from the following experiment; // 50 g of 5HIAA and 5HTOH respectively were added to three duplicate portion of 10 ml of freshly collected urine one of which was adjusted to pH 1 with HCl, one to pH 3.5 with glacial acetic and one remained at pH 5.5 preserved with only a few drops of chloroform. One set of duplicates was left at room

temperature for two days and 5HIAA and 5HTOH were then measured by the GLC method described in the next chapter employing standards of 50 g of 5HIAA and 5HTOH evaporated from a freshly prepared methanolic solution (taking these to represent 100% recoveries). The other set was stored at 4°C for 5 weeks and similarly analysed. The overall recoveries obtained compared with authentic standards, are given in table 3.8. In another experiment, two 24 hr. urine samples collected from a carcinoid patient in the same week were analysed for 5HIAA and 5HTOH as described in chapter 4 and 7: one was preserved with 25 ml ⅙NHCl and the other with 25 ml glacial acetic and stored for over six months at - 18°C before analysis. Both 5HIAA and 5HTOH were significantly lower in the sample preserved with HCl compared with that preserved with glacial acetic, values being 78 mg 5HIAA/24 hr and 0.3 mg 5HTOH/24 hr in the former compared with 304 mg 5HIAA/24 hr and 7.45 mg 5HTOH/24 hr in the latter. These differences may be physiological, but the possibility that the preservative employed is responsible for such differences cannot completely be excluded.

From these experiments, it was concluded that for urinary analysis of 5HIAA and 5HTOH, 24 hr urine collections are best stored frozen either with a small amount of glacial acetic (not more than 10 ml) to prevent bacterial growth

TABLE 3.8

Compound	Natural pH (5-6)	pH3 (acetic acid)	pH1.5 (Hcl)
A - 5HIAA	100%	70%	70%
A - 5HTOH	94%	75%	37%
B - 5HIAA	Less than 10%	Less than 10%	Less than 10%
B - 5HTOH	"	"	"

Recoveries of 5HIAA and 5HTOH at three different PH values compared against authentic standard (representing 100% recovery) after incubating at: A - room temperature for 48 hr and B - at 4°C for 5 weeks.

or better, at normal acid or neutral urine pH with a little chloroform or toluene (5 ml) as a preservative.

CHAPTER 4

EXPERIMENTAL

Material, equipment, preparation and methods

Material

Siliconised, acid-washed celite, 85/100 and 120/100 mesh; stationary phases, silicon oil (F60 or DC550), Apiezon grease, polyethylene glycol 20M (PEG20), trifluropropylmethyl silicone fluid (QF1), diethylene glycol succinate (DEGS), methyl silicone gum (SE30), methylphenyl silicone gum (SE52), methylphenylvinyl silicone gum (SE54); 3 ft, 5 ft and 7 ft. U-shaped glass tubes: all obtained from W.G. Pye Ltd., Cambridge, England. Silicone elastomer (E301) was from May & Baker, Ltd., Dagenham, England.

7% F60 plus 1% ethylene glycol succinate-phenylmethyl-siloxane copolymer (EGSS-z), on 100/120 mesh chromosorb P (F60-Z), and 0.2% cyclohexane-dimethanol succinate polyester (CHDMS) plus 0.6% methylsiloxane polymer (JXR) on 100/120 mesh chromosorb P (0.6% JXR and 0.2% HMPF-8BP), were purchased from Applied Science Laboratories, Inc., P.O. Box 440, State College, Pennsylvania, U.S.A.

n-Alkane reference standards, from n-dodecane to n-eicosane (C_{12}, C_{14}, C_{16}, C_{18} and C_{20}) were obtained from

British Drug Houses, Ltd., Poole, Dorset, England (BDH) and n-docosane and n-tetracosane (C_{22} and C_{24}) from Koch-Light Laboratories, Ltd., Colnbrook, Bucks, England (Koch-Light).

1,4-Dioxane (specially dried) was obtained from BDH trimethylchlorosilane (TMCS) from Hopkin & Williams Ltd., Chadwell Heath, Essex, England (Hopkin & Williams), hexamethyldisilazane (HMDS) from Sigma Chemical Company, 12 Lettice St., London S.W.6, England (Sigma) and p-tolylsulphonylmethylnitrosoamide (pure) from Koch-Light.

Authentic aromatic and phenolic compounds listed in tables 3.4, 3.5 and 3.6 were purchased from commercial sources, with the exception of VPA and VLA which were gifts from Dr. P. Smith and 3,4-dihydroxyphenylglycol from Dr. J. Axelrod.

Ethereal diazomethane (approx. 10 mg/1 ml) was prepared as described below under "methods".

All other reagents were of analytical grade.

Equipment

An "Evapo-mix" was purchased from Buchler Instruments, New York 31, N.Y., U.S.A.; 1µl Hamilton syringes from Pye; and a Honeywell chart recorder with an electronic continuous balance potentiometer (Honeywell Controls, Ltd.) by Pye.

GLC analysis was carried out with a Pye Panchromatograph fitted with an argon ionisation detector (AID) (see chapter 3). The detector was maintained at a temperature of 200°C with an

ionising potential of 1000V. The sensitivity was varied between 10^{-8} to 3×10^{-9}, a full scale deflection. A column was not connected until the detector had reached the operation temperature.

Preparation of columns

Glass U-shaped columns were siliconised before packing by allowing them to stand overnight with a mixture of petroleum ether (B.P 40/60°) - TMCS - HMDS (100:4:2); they were then rinsed with petroleum ether, and dried in a stream of argon at 200°C. Obstinate deposits of NH_4Cl were removed by burning over a bunsen flame until the column appeared clean and transparent.

Columns were packed evenly with the appropriate coated support. Freshly packed columns were preheated for 48 hr at 250° in a stream of argon. This procedure was repeated for at least 24 hr whenever a fresh preheated column was used.

Collection of urine samples

Urine samples from newborn male infants were collected by attaching Paul's tubing to the penis. The tubing was emptied at 6 hr intervals and the contents were deep-frozen, later to be combined in 24 hr collections. The volume of the collection was estimated approximately and the specimen was

acidified with a volume of 6M HCl equal to about 1% of the total volume. The volume was then accurately measured and the urine returned to the deep-freeze to await analysis.

For the analysis of urinary phenolic acids and alcohols in adult subjects, 24 hr. urine samples were collected into containers with 25 ml 6M HCl, and kept at 4°C until assayed.

For the analysis of 5HIAA and 5HTOH in normal and carcinoid subjects, urines were collected into 10 ml glacial acetic (in some cases into 25 ml glacial acetic acid) and stored in the deep freeze (see chapter 3).

Urine extraction

Neonatal urine specimens (15 ml if the 24 hr volume was greater than 100 ml or 10 ml if less), or urine samples from normal adults (5 or 10 ml) or from patients with catecholamine secreting tumours, tyrosyluria, phenylketonuria, alkaptonuria, or carcinoid disease (1 to 5 ml), made up to 10 ml with water, were adjusted to an appropriate PH value and saturated with NaCl, after which they were extracted with either ethyl acetate or ether as indicated on table 4.1.

Extraction was carried out twice by vortex mixing ("Whirlimixer") for 1 min, with the appropriate solvent (25 ml). Aliquots (20 and 25 ml) of the organic phases were separated by centrifugation, combined, and evaporated to dryness at about

TABLE 4.1

Compound	Recommended pH	Solvent	Comment
Phenyllactic, homogentisic and dihydroxyphenylacetic acids	2	Ether	-
All phenolic acids except those mentioned above	2	Ethyl acetate	-
HMPG and HMPE	8	Ethyl acetate	Hydrolysed urine*
Dihydroxyphenylethanol	8	Ether	Hydrolysed urine*
Indolic acids	4	Ether	-
Indolic alcohols	8	Ether	Hydrolysed urine*

Appropriate pH and solvent for the extraction of urinary phenolic and indolic compounds prior to GLC.

* For hydrolysis of urine, see methods section.

40°C in vacuo ("rotary evapo-mix").

For the quantitation of DOPAC, urine aliquots as above were extracted with ether and the TE/E prepared.

For p-hydroxymandellic acid urines were extracted with ethylacetate at PH 4.5, the PH then lowered to 1 with 0.5 ml of 6 NHCl, then extracted with ether. The TE/E was prepared.

Chromatographic arrangement for GLC analysis

Phenolic and indolic compounds with M.U. values between 14.0 and 19.0

Column: 7 ft. 10% SE52 on 85/100 siliconised celite.

Column temperature: 190°C

Inlet pressure and flow: 1500 mm/Hg, corresponding to 50-100 ml/min.

Detector temp: 200°C

Applied voltage: 1000 V.

Derivative: ME/TE or TE/E, depending on the type of analysis, as discussed in relevant sections of this thesis

Injection volumes: 0.1 to 0.5 l of clear supernatant (Hamilton syringe)

Recorder chart speed: 1 cm/1 min.

Phenolic and indolic compounds with M.U. values over 19.0

Column: 5 ft. 5% SE52 on 85/100 mesh siliconised celite.

Column temperature: 190°C

Inlet pressure: 1500 mm/Hg.

Detector temp: 200°C

Applied voltage: 1000 V

Derivatives: ME/TE or TE/E, depending on type of analysis.

Injection volume: 0.1 to 0.5 l of clear supernatant.

Recorder chart speed: 1 cm 1 min.

Methods

Preparation of ethereal diazomethane (approx. 10 mg./1 ml)

Reagents

p-tolylsulphonylmethylnitrosamide.

Potassium hydroxide (KOH)

Absolute ethanol

Ether (A.R)

Procedure

The procedure is a modification of that described by Vogel, 1961.

All work was carried inside a fume cupboard with the extraction fan switched on.

48 g of p-tolylsulphonylmethylnitrosoamide were dissolved in 675 ml of ether, kept ice cold in a 5L round bottom flask following which a solution of 9 g KOH in about 200 ml of 96% (V/v) ethanol was added; the flask was then stoppered and mixed. Further 96% ethanol (about 100 ml) was

added, with constant mixing, until the yellow precipitate which had formed just redissolved. The solution was left at room temperature with the flask well stoppered for 5 min. After five minutes, the ethereal diazomethane was distilled off by heating the flask in a water bath, gradually from 30°C to 50°C, keeping at that temperature for 10-15 minutes and then gradually raising the temperature to 73°C. This temperature was maintained until the distillate became almost colourless. Ethereal diazomethane distillate was collected in a flask cooled by acetone in solid carbon dioxide (app. -60°C) and stored in brown bottles at below - 14°C.

Ethereal diazomethane was found to last over one month if the bulk of the preparation is kept in the deep-freeze. A portion of about 30 ml in another separate bottle kept also in the deep-freeze was assigned for regular use. When in use the ethereal diazomethane bottle was kept cold in ice-cold water and returned back to the deep-freeze as soon as possible.

Preparation of methyl ester derivatives (ME) (Karoum et al, 1968)

Esterification was carried out by dissolving the dried urine extract or authentic reference compound in methanol (0.2 ml) and ethereal diazomethane (1.5 ml), mixing for 1 minute and then immediately evaporating under a stream of nitrogen. Dihydroxyphenolic compounds were, however, exposed

to ethereal diazomethane for only 30 sec, to reduce O-methylation (Karoum et al, 1969).

Preparation of trimethylethers and esters, ME/TE and TE/E (Karoum et al, 1968)

Esterified acidic compounds as described above (ME), or untreated phenolic and indolic compounds either as urine extracts or reference compounds were silylated by the addition of 0.3 ml of a 1:2 mixture of HMDS and dioxane, followed by 0.05 ml of TMCS. For the preparation of the trimethylsilylether/ester of a urinary extract the dry residue was reconsituted in about 0.3 ml of methanol and dried under a stream of nitrogen, before silylation. The silylated mixture is kept for 15-20 min and then transferred with a pasteur pipette into 2 ml test tubes, left for about 30 min. and then centrifuged. The end of silylation reaction is indicated by the production of a clear supernatant after centrifugation.

Demethylation of phenolic compounds (Ruthven and Sandler, 1964)

Reagents

Concentrated HBr freed of bromine by extracting several times with carbon tetrachloride.

Glacial acetic acid.

HBr - acetic acid mixture: prepared by mixing 2 parts of HBr with 5 parts of glacial acetic acid. Stable indefinitely at

room temperature.

Demethylation procedure

2 ml of HBr - acetic acid mixture is autoclaved at 15 lb/in^2 with the methylated compound for 1 hr. This procedure was employed for the demethylation of both HVA and HMPE. The latter may however give rise to some brominated derivative of DHPE. This could substantially be reduced, however, by treating HMPE with HBr - acetic at room temperature for 12-15 hr.

Thin layer chromatography

Plates coated with microcrystalline cellulose (19 microns) were used.

Solvents: i. Isopropyl alcohol: ammonia: water 8:1:1.

ii. n-Butanol: acetic acid: water 4:1:1

Ion-exchange column for the absorption and elution of HVA and HMPE

Reagent:

Silica gel (Davison U.S.A. grade 923, 100-200 mesh; or Davison U.S.A. grade 950, 60-200 mesh).

Dichloromethane, redistilled (BDH)

Procedure:

2 g of silica gel was added to dichloromethane in a small

beaker and then transferred to a column (0.5 cm internal diameter) with a pasteur pipette, always maintaining the level of dichloromethane above the gel. Urine extracts of dichloromethane are run through the column which absorbs both HIPA and HVA.

HIPA was eluted by a 2% ($^{V}/_{V}$) methanol in dichloromethane mixture, and HVA by a 5% ($^{V}/_{V}$) methanol in dichloromethane mixture.

Hydrolysis of conjugated metabolites

Urine aliquots were adjusted to about pH 6.0 and then incubated with 0.2 ml of Helix pomatia enzyme preparation for at least 17 hours with the container tubes loosely stoppered. After incubation the pH was adjusted to 8 and the mixture extracted as described earlier.

Quantification

This was carried out by adding an internal standard to one specimen in each batch of seven analyses. The peak height equivalent to the added standard was then used to quantify other peaks presumed to consist of the same substance by virtue of the rate at which they migrate. In some cases the peak height of one particular standard was used to quantify another peak. The final result however, was corrected by multiplying by an

appropriate relative response factor. This was obtained from the ratio of detector response (peak heights) of one substance to another when equal amounts of the two were analysed by the same procedure. For example, if S was the response equivalent to 0.1 mg. of substance A and the relative response of A to substance B is R, and if the peak height corresponding to B on the chromatogram was H, then the amount of substance B equivalent to a peak height of H is equal to

$$\frac{H}{S} \times 0.1 \times \frac{1}{R} \text{ mg.}$$

The relative response values of some metabolites against HVA and VMA and MHPG as shown on the table 3.3.

CHAPTER 5

Excretion of phenolic and indolic acids and alcohols in normal adult humans and rats

Of the many phenolic (Armstrong et al., 1956; Mathieu and Revoll, 1958) and indolic (Armstrong et al., 1958; Karoum et al., 1968) acids and alcohols identified in mammalian urines, those derived from endogenous biologically active monoamines are particularly interesting because of their physiological implications. By measuring these endogenous metabolites one is also able to make some quantitative assessment of the rate of metabolism of these amines in a variety of pathological conditions. In order to evaluate variations from the normal however, it is necessary to possess methods sufficiently sensitive to measure relatively low concentrations of metabolites excreted under normal conditions and even detect small fluctuations within the normal range. The GLC procedures developed in the course of this work have proved suitable for these purposes.

Subjects and material

Adult humans

Urine from normal healthy adults (2 males and 3 females) aged between 25 and 45 years, were analysed for a variety of

phenolic and indolic compounds. No restrictions on diet or activity were imposed during the time of urine collection.

For assay of phenolic compounds, 24 hr. urine samples were collected into bottles containing 25 ml. 6N HCl, and stored at 4°C for periods varying from one week to 5 years until analysed.

For assay of indolic compounds, 24 hr. urine specimens were collected into bottles containing 10 ml. glacial acetic acid and stored frozen (-15°C) for periods of up to two years until analysed.

Rat

Rats (Wister, male, 280-350 g in one experiment and latter when 55-775 g) were placed separately in cylindrical (26.5 cm. diameter) perspex metabolic cages fitted with floors of stainless steel mesh resting on large glass funnels. The mesh was siliconised by treating it with polymethylhydrogen siloxane (Hopkin and Williams, Ltd.) to prevent possible catalytic oxidation of catecholamines on contact with the metal. Faeces were separated from urine by interposing a conical glass bulb, apex downwards, between the tip of the large glass funnel and the urine collection vessel. Urine was collected for 24 hr. into 6N HCl (1 ml.) during which time the animal was fasted from solid food but allowed water ad libitum. At the end of

collection period any urine on the funnel was washed into the collection vessel with distilled water. Specimens were stored at - 15°C until required.

Methods

A detailed description of all the procedures mentioned below are given in chapter 4. The concentration of substances other than those added as internal standards were calculated from these standards by multiplying by an appropriate relative response factor.

A sample to which internal standard had been added was included in every batch of seven human urines; each rat urine was assayed with a duplicate to which internal standard had been added.

Phenolic acids and alcohols were analysed on a 7 ft. 10% SE52 column, while indolic acids and alcohols were analysed on a 5 ft. 5% SE52 column.

Phenolic acids

Urine aliquots (10 ml. of human urine when the 24 hr. volume was over one litre or 5 ml. urine + 5 ml. H_2O when less, or 5 to 10 ml. rat urine) were adjusted to pH2 and then extracted twice with ethyl acetate. The TMS/TE was prepared. 0.1 mg. VMA and 0.05 mg. HVA was used as internal standards in human urine and 0.02 mg. VMA and 0.01 mg. HVA in rat urine.

Total phenolic alcohols

Similar urine aliquots were hydrolysed and extracted twice at pH8 with ethyl acetate. The TE/E was prepared. 0.1 mg. HMPG was included as internal standard in human urine and 0.02 mg. in rat urine.

Indolic acids (5HIAA)

Similar aliquots were extracted twice with ether at pH 3.5. The ME/TE was prepared. 0.1 mg. 5HIAA was included as internal standard in human urine.

Total indolic alcohols (5HTOH)

Similar aliquots were hydrolysed and extracted twice with ether at pH8. The TE/E was prepared. 0.1 mg. 5HTOH was included as internal standard in both human and rat urine.

Result and Discussion

24 hour excretion values of some metabolites in adult human and rat urine together with their means, observed ranges and standard error, are summarised on tables 5.1 and 5.2. Other metabolites, either occasional peaks from compounds of dietary origin or those excreted in insufficient quantity for accurate measurement by the procedure employed without special modification are also considered (table 5.4).

Excretion values of VMA, HVA, HMPG and 5HIAA in normal

TABLE 5.1

NAME	VMA	HVA	HMPG	5HIAA	p-HPAA
N	5.0	3.9	-	-	-
C.R.J.	5.8	3.9	-	4.4	-
Wh.	3.6	5.1	-	-	-
B	5.5	4.4	-	-	-
H	8.8	3.4	-	-	-
P	4.9	3.5	-	-	-
C	4.0	2.3	-	-	-
Ch	5.9	7.1	-	-	-
L	-	-	2.5	3.4	-
F.K	4.3	-	2.2	6.8	-
M	-	-	-	3.1	-
L	-	-	-	4.1	-
Pl	4.2	4.2	1.5	2.3	15.7
Stk.	3.1	3.6	1.3	-	6.7
M.S	4.2	3.0	2.9	-	5.5
My	5.9	4.6	2.5	-	25.2
R	6.1	4.7	3.9	-	7.5
Chm.	6.7	5.8	1.9	-	38.2
P.C	5.6	4.6	2.7	-	12.1
Ne	6.0	1.8	2.5	-	11.3
Fr	-	-	-	5.2	-
Plk	4.8	6.0	-	-	9.7
So	1.8	1.3	0.5	-	4.8

TABLE 5.1 (Cont.)

NAME	VMA	HVA	HMPG	5HIAA	p-HPAA
J	3.0	2.2	-	-	-
Lm	-	-	-	3.5	-
Mean	5.0	3.9	2.2	4.0	13.7
± standard error (SE)	0.5	0.3	0.3	0.6	3.5
Observed Range	(1.8-8.8)	1.8-6.0)	(0.5-3.9)	(2.3-6.8)	(5.5-38.2)

Normal excretion of five biologically important monoamines metabolites in adult humans. All values are expressed in ~~±~~ mg/24 hr.

TABLE 5.2

No. of rat		HVA µg/24 hr	HMPG µg/24 hr	5HTOH µg/24 hr
1.	Group A	34.1	-	-
2.	"	39.3	-	-
3.	"	38.0	-	-
4.	"	44.2	-	-
5.	"	61.5	-	-
6.	"	49.6	-	-
mean		44.77	-	-
S.D.		± 4.05	-	-
range		(34.1-61.5)	-	-
1.	Group B	34.45	42.93	32.2
2.	"	39.14	108.49	31.2
3.	"	36.16	70.40	19.8
4.	"	37.32	77.14	22.4
5.	"	36.16	53.46	22.4
6.	"	-	41.79	16.0
mean		34.77	65.70	24.0
(S.E)		± 2.07	± 9.48	± 2.6
range		(34.5-39.5)	(41.79-108.49)	(16-32.2)

The excretion of HVA, HMPG and 5HTOH in normal rats (see text). In group A the rats weighed 280-350g and in group B 455-775g.

TABLE 5.4

SUBSTANCE	COMMENT
m-hydroxyphenylacetic Acid.	Origin obscure, probably from gut flora. Could be formed from p-dehydroxylation of DOPA, dopamine and DOPAC (Calne et al., 1969a). 24 hr excretion varies from 100 µg to 2 mg.
o-hydroxyphenylacetic Acid	Its excretion is increased in phenylketonuria (Armstrong, Shaw and Robinson, 1955). Derived probably from dietary o-tyrosine (Armstrong and Shaw, 1955). It was less frequently detected than m-hydroxyphenylacetic acid. As judged from their relative peak heights, o-hydroxyphenylacetic acid is excreted in amounts about 1/3 of the m-isomer.
p- and m- hydroxypropionic Acids	These two substances are infrequently detected. Their probable daily excretion is not more than 0.5 mg/24 hr.
Vanillic Acid	Derived from a variety of vegetables, food additives and caffeic acid (Booth, Emerson, Jones and De Eds, 1957; Dirscherl and Wirtzfeld, 1964). It could be formed endogenously as a minor product of DOPAC (Alton and Goodall, 1969), NA and A (Imaizumi, Yoshida and Kita, 1958). Excreted in values varying from 100 µg to 10 mg/24 hr.
p-hydroxycinnamic Acid	It is occasionally observed in normal chromatograms and in some cases the excretion may be as high as 2 mg/24 hr. Normal output however rarely exceeds 0.5 mg/24 hr.
Hippuric Acid	Always detected in acid extracted urines. When ME/TE derivatives are prepared it masks peaks corresponding to DOPAC and dihydroxybenzoic acid. Its normal excretion is greater than 2 µg/24 hr in majority of cases.

TABLE 5.4 (Cont)

SUBSTANCE	COMMENT
p-hydroxymandelic acid	Its excretion by the modified procedure described in chapter 4, ranges between 1 and 3 mg/24 hr. Excretion between 1-4 mg/24 hr have been reported by Williams and Sweeley, 1964.
phenylpyruvic, p-hydroxyphenylpyruvic, phenyllactic and p-hydroxyphenyllactic acids.	Output of each rarely exceed 1 mg/24 hr.
LOPAC	Difficult to detect in normal urines unless a large volume of urine (20-40 ml) is extracted (see chapter 4). Excretion is less than 0.5 mg Free LOPAC/24 hr.
DHMA	Difficult to detect in normal urine. Output rarely exceeds 500 µg/24 hr. Excretion reported in the literature are 300-400 µg/24 hr. (Lequattro, Nybenga, von Studnitz and Brunjes, 1964) and 720 ± 320 µg/24 hr (Miyake, Yoshida and Imaizumi, 1962; Brujan, Alvarez and Borges, 1966).
VLA, VPA	Difficult to detect in normal urine. Excretion rarely exceeds 500 µg/24 hr.
HMPE, DMPE	Excreted in quantities less than 100 µg/24 hr.
3-methoxy-4-hydroxybenzylalcohol (HMBA)	Occasionally a small peak corresponding to this compound was noticed. Excretion is less than 100 µg/24 hr.

A summary on the excretion of certain aromatic acids and alcohols that were encountered during urine analysis of normal adult humans, together with comments.

adults (table 1) are not too dissimilar from those obtained by other methods (Pisano, Crout and Abraham, 1962; Wilk, Gitlow, Clarke and Paley, 1967; Udenfriend, Titus and Weissbach 1955), (table 5.3)

p-Hydroxyphenylacetic acid is the end metabolite of tyramine which is derived mainly from decarboxylation of tyrosine by tissue and intestinal bacteria L-amino acid decarboxylase (Asatoor, 1968); large amounts may also be present in some foodstuffs, especially cheese (Sen, 1969). Although the presence of p-hydroxyphenylacetic in normal urines has been recognised since the classical report on paper chromatography of phenolic acids by Armstrong et al., (1956) no previous attempt has apparently been made accurately to measure its excretion by any more precise assay procedure.

In the rat, the major metabolite of NA and A is HMPG (Kopin et al., 1961). Ceasar, Ruthven and Sandler (1969) obtained values of 67.73 ± 3.73 µg HMPG/24 hr. and 31.97 ± 4.07 µg HVA/24 hr. which are not very different from those shown in table 5.2. It is interesting to note the higher output of HVA in the young rats (group A) compared with the older ones (group B in table 5.2) perhaps implying an increased dopamine turnover in the former. In the human also, at least on a weight for weight basis, urinary NA and A, dopamine and VMA output (Voorhess, 1967) tend to be higher before than after

TABLE 5.3

Substance	Mean and/or upper limit of normal excretion.	S.E and/or range	References
VMA	Below 7 mg/24 hr	-	Sandler & Ruthven (1963)
"	3.6 mg/24 hr	±0.66 mg/24 hr	Voorhess (1967)
"	-	2-4 mg/24 hr	Armstrong, McMillan & Shaw (1957)
"	4.8 mg/24 hr	±0.1 mg/24 hr (3.2-6.4)	Von Studnitz (1960)$_a$
HVA	Approx. 6 mg/24 hr (3.6 µg/mg creatinine)	-	Williams and Sweeley (1961)
"	Below 10 mg/24 hr	-	Sandler & Ruthven (1963)
"	5.4 mg/24 hr	±1.1 mg/24 hr	Ruthven & Sandler (1966)
"	5.4 mg/24 hr	±1.4 mg/24 hr (3.7-7.5 mg/24 hr	Sato
"	6.0 mg/24 hr	±1.1 mg/24 hr (2.5-9.0 mg/24 hr	Ruthven (1962)
MHPG	3.0 mg/24 hr	±0.8 mg/24 hr	Ruthven and Sandler (1965)
"	Approx. 2 mg/24 hr (0.86 µg/mg creatinine)	-	Wilk, Gitlow, Clarke and Paley (1967)
5HIAA	-	2.10 mg/24 hr	Lembeck (1956) Sjoerdsma, Weissbach & Udenfriend (1956) Dalgliesh (1958) Davis & Rosenberg (1961) Oates and Sjoerdsma (1962)
"	Less than 8 mg/24 hr	-	Udenfriend, Titus and Weissbach (1955)

A summary on the normal excretion of VMA, HVA, MHPG and 5HIAA in adult man reported in the literature.

puberty. The observed difference in the excretion of HVA in the two groups of rat could be related to changes in the activities of the two major enzymes involved in the destruction of catecholamines, COMT and MAO. In the rat liver the activities of both enzymes are increased between 9-35 weeks compared with those over 35 weeks old (Prange, White, Lipton and Kinkead, 1967), (group A falls within the age of 9-35 weeks and group B over the age of 35 weeks) whilst in the kidney COMT decreases with age (Prange et al, 1967). Cardiac MAO activities also increase with age (Novick, 1961; Horita, 1967; Prange et al 1967). Thus it is possible that the higher urinary excretion of HVA in the younger rats is mainly a reflection of the higher kidney and liver COMT activities in this group. To test the validity of this hypothesis it would be interesting to compare the excretion of DOPAC in rats of similar age to groups A and B, and thereby investigate whether or not the excretion of DOPAC follows an opposite pattern to that of HVA.

Unfortunately, the 24 hr. urine volumes of the rats were almost completely used up for the quantification of HVA, MHPG and 5HTOH, and as the urines were collected sometime ago before the analysis was undertaken, it was not possible to determine 5HIAA in these series. 5HTOH has been reported as a major metabolite of administered labelled 5HT in rats (Kveder et al,

1962). If however, the 5HTOH shown on table 5.2 is compared with the 5HIAA excretion value of 48.4 ± 8.93 g/24 hr. reported by Caesar et al (1969) it is likely that in rats about 30% of the aldehyde deriving from oxidative deamination of 5HT follows a reductive route of metabolism. In humans on the other hand, 5HTOH excretion cannot exceed 100 µg/24 hr., as is evident from many unsuccessful attempts to quantify its excretion, even after the extraction of 50 ml. of urine, no 5HTOH peak could be obtained gas chromatographically although internal standard carried through the procedure could easily be identified down to 2 µg when added to a urine aliquot. Thus 5HTOH may be quantitatively a more important metabolite in the rat than in human an observation in keeping with the relatively major role of hMPG.

CHAPTER 6

The urinary excretion of phenolic acids and alcohols in the newborn infant

Introduction

The importance of early detection of inborn errors of metabolism is well known (Lancet, 1969). Although obvious, it is perhaps still worth stating that in order to identify an abnormality, the full range of the normal must first be defined. The urinary excretion pattern during the neonatal period of compounds relevant to this study often differs from that found in later life (Voorhess, 1967; Partington, 1968; Zeisel and Keische, 1959). It is likely that such differences may be ascribed to the effect of enzymatic (Boehm and O'Brien, 1963) and renal (McCance and Widdowson, 1954; McCance, 1959) immaturity and to differences between the infant and adult diet.

Characteristic and grossly abnormal phenolic acid excretion patterns in the fully developed case of phenylketonuria (Knox, 1966), tyrosinosis (LaDu, 1966_a) or alkaptonuria (LaDu, 1966_b), for example, are quite immistakeable. There occur cases of these, or other inborn errors of metabolism however where the biochemical defect shades off into a transitory though abnormal urinary excretion pattern associated with a purely temporary enzyme immaturity (Avery, Clow, Mankes, Nomas, Scriver, Stern and Wasserman, 1967).

Typical examples are "tyrosyluria" (Bloxam, Day, Gibbs and Woolf, 1960; Woolf, 1965; Wong, Lambert and Komrower, 1967; Partington, 1968) or "hyperphenylalaninaemia" (Menkes and Avery, 1963) where immaturity of p-hydroxyphenylpyruvic oxidase and phenylalanine hydroxylase respectively may be difficult to distinguish from a similar biochemical pattern associated with absence of the enzyme. It is thus important to try to delimit each condition. It is similarly important for other reasons to define the normal range of catecholamine metabolite output. Catecholamine secreting tumours are known even in the newborn period of life (Brett, Oppé, Ruthven and Sandler, 1964).

Although some quantitative information exists on the excretion of VMA in the neonate (von Studnitz, 1960_a; Gjessing 1966_a; Nicolopoulos, Agathopoulos, Danelatou-Athanassiadou and Bafataki, 1968) comparable data for HVA, MHPG and HMPE and metabolites of phenylalanine and tyrosine are scanty, due no doubt to the complexity of the methods available up to now for their determination. This difficulty has now been overcome by using relatively simple GLC techniques. With their aid the excretion of a number of phenolic acids and alcohols has been studied quantitatively in infants during the first week of life.

Material and methods

Forty-four infants, mostly males (38 infants) were classified into four groups:

Group 1 - Full-term non-jaundiced (FNJ); period of gestation 39 weeks or over; weight at birth greater than 5.5lb (2,500 g); peak serum total bilirubin concentration not exceeding 5 mg/100ml.

Group 2 - Full-term jaundiced (FJ); as group 1, except that peak serum total bilirubin concentration was over 5mg/100ml.

Group 3 - Premature non-jaundiced (PNJ); period of gestation under 37 weeks; birth weight less than 5.5 lb (2,500 g); peak serum total bilirubin range as in group 1.

Group 4 - Premature jaundiced (PJ); As group 3, except that peak serum total bilirubin range was the same as that in group 2.

Measured 24 hr. urine collections were made on most of the days of the first week of like in the infants. A few collections were made between the end of the first week and the end of the third. The urine samples were acidified (2 ml 6N HCl) and stored frozen. All specimens were tested for keto acids using "phenistex" and ferric chloride (Perry, Hansen and MacDougall, 1966).

For examination of phenolic acids, urine aliquots

(10 ml if the 24 hr. volume was less and 15 if it was greater than 100 ml.) were extracted with ethylacetate and the ME/TE prepared. For urine specimens which turned out to be tyrosyluric (high excretion of p-HPLA), 2 to 5 ml urine volumes were employed for definitive analysis. Internal standard was added to a duplicate of every urine sample as follows: 0.1 mg VMA, 0.1 mg p-HPLA and 0.05 mg HVA. Other metabolites were quantified using the relative response of one of the internal standards chosen as described previously (see chapter 3).

Alcoholic metabolites (MHPG and HMPE) were determined in hydrolysed urine aliquots as above. The TE/E derivatives was prepared. MHPG (0.1 mg) was added as internal standard to a duplicate of every urine specimen. Where small chromatographic peaks only were obtained for metabolites under investigation, the analysis was repeated on a larger volume using in some cases two levels of internal standards.

Serum total bilirubin was estimated daily from birth to the 7th day of life by the method of Lathe and Ruthven (1958). Urine creatinine was estimated by the method of Jere (1950).

Results

"Phenistex" and ferric chloride tests were negative for all urine samples except for two specimens which gave a weak

positive reaction - a pale green colour - with ferric chloride, although "phenistex" was negative.

Creatinine concentration, estimated in 20 infants during the first week of life, was variable with values ranging from 8 - 300 mg/24 hr; the lower values were found in premature babies.

Quantitative analytical data from the four groups (FNJ, FJ, PNJ and PJ) are compared in a number of ways in table 6.1. The tendency for metabolite excretion values to be higher in premature than full-term, when expressed in terms of μg/mg creatinine, but not when expressed as μg/24hr arises from the low levels of creatinine excreted by premature infants. Histograms of the mean 24 hr. excretion values of HVA, VMA and p-HPLA are shown on figures 6.1 and 6.2.

In a few infants in the PJ group estimations of urine p-HPLA and total serum bilirubin were made up to the age of one month. Although serum total bilirubin concentration dropped to values about or below 5 mg/100 ml within the first week of life, p-HPLA excretion continued to be elevated for up to 2 or 3 weeks, although occasionally, lower values (less than 1 mg/24 hr) were encountered during this time.

Other metabolites detected are listed and commented on in table 6.2.

	Full-term non-Jaundiced (39)		Full-term Jaundiced (13)		Premature non-Jaundiced (23)		Premature Jaundiced (25)		All groups combined (100)	
	Mean	Observed range	Mean	Observed range	Mean	Observed range	Mean	Observed range	Mean	Observed range
p-HPAA μg/24 hr	671	95-1070	422	70-775	638	40-1640	96	10-200	463	10-1640
HVPG μg/24 hr									78.5	10-252

The overall mean excretion of HVA, VMA, p-HPIA, p-HPPA, HVPG, p-hydroxyphenylacetic acid (p-HPAA) during the first week of life.

	Full-term non-Jaundiced (39)		Full-term Jaundiced (13)		Premature non-Jaundiced (23)		Premature Jaundiced (25)		All groups combined (100)	
	Mean	Observed range	Mean	Observed range	Mean	Observed range	Mean	Observed range	Mean	Observed range
HVA µg/24 hr	265	38-1350	195	84-1010	217	15-575	231	46-490	260	10-1350
HVA µg/mg creatinine	13.2	0.7-29.8	-	-	17.6	10-45.2	28.8	8.3-101		
HVA µg/ml	6.9	2-18	2.5	1.1-6.4	5.0	0.6-11.2	6.3	2.2-23.2		
HVA µg/Kg/24 hr	120.8	61.1-400	90.8	38.2-376	124.9	53.6-163	126.3	60-233		
VMA µg/24 hr	208	24-1230	152	40-450	93	15-975	109	50-500	156	10-1230
VMA µg/mg creatinine	8.0	0.5-20.8	-	-	8.0	2.7-29.8	18.2	5-49.5		
VMA µg/ml	6.5	0.5-18	1.6	0.5-10	2.8	0.5-25	2.1	10-10		
VMA µg/Kg/24 hr	126.5	23.9-365	55.4	15.4-132	66.0	15.6-159	64.0	5.7-133		
p-HPLA µg/24 hr	227	10-975	639	54-4166	453	10-4100	2377	155-7800		
p-HPLA µg/mg creatinine	13.7	1-45	-	-	27.8	0.9-95	367.4	92-530		
p-HPLA µg/ml	5.7	1-10	8.5	1.5-40.5	9.2	1-44.4	51.3	1.3-166		
p-HPLA µg/Kg/24 hr	-	-	-	-	-	-	-	-		
p-HPPA µg/24 hr	50	10-100	-	-	148	10-570	1299	155-7800		
p-HPPA µg/mg creatinine	-	-	-	-	-	-	-	-		
p-HPPA µg/ml	-	-	-	-	3.7	1-20.8	14.5	10-43.5		
p-HPPA µg/Kg/24 hr	-	-	-	-	-	-	-	-		

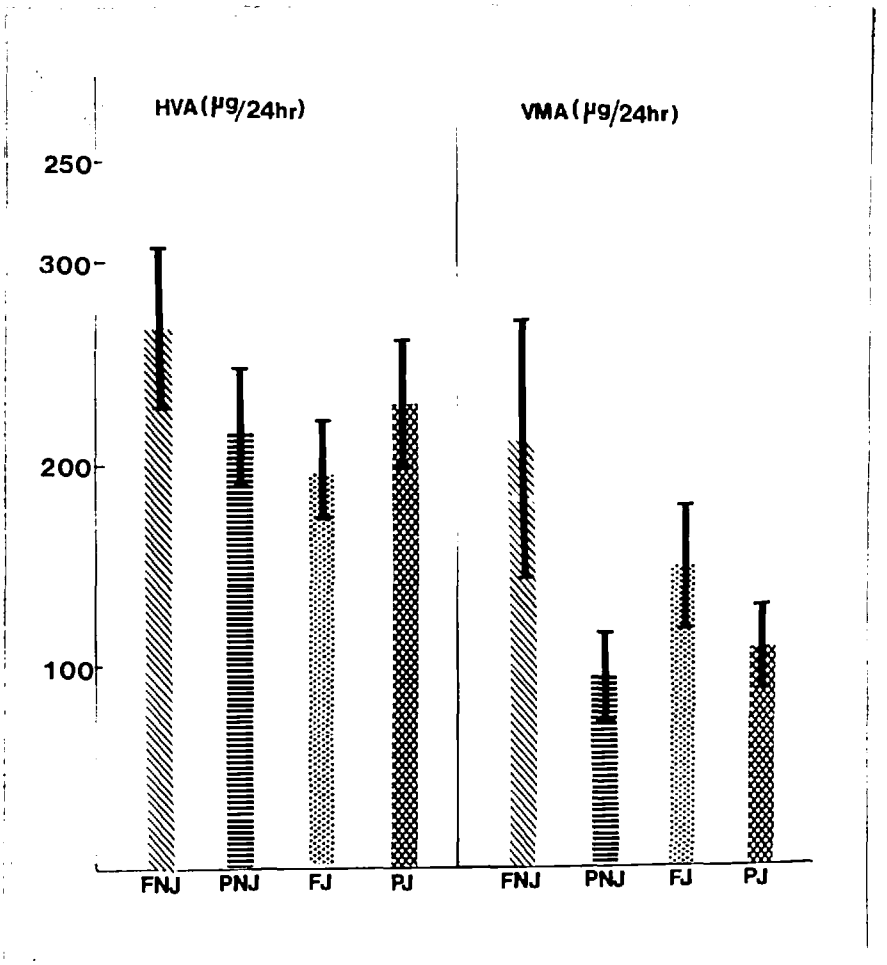


Fig. 6.1. Histogram representation of HVA and VMA excretion in the four groups of neonates as classified in the text.

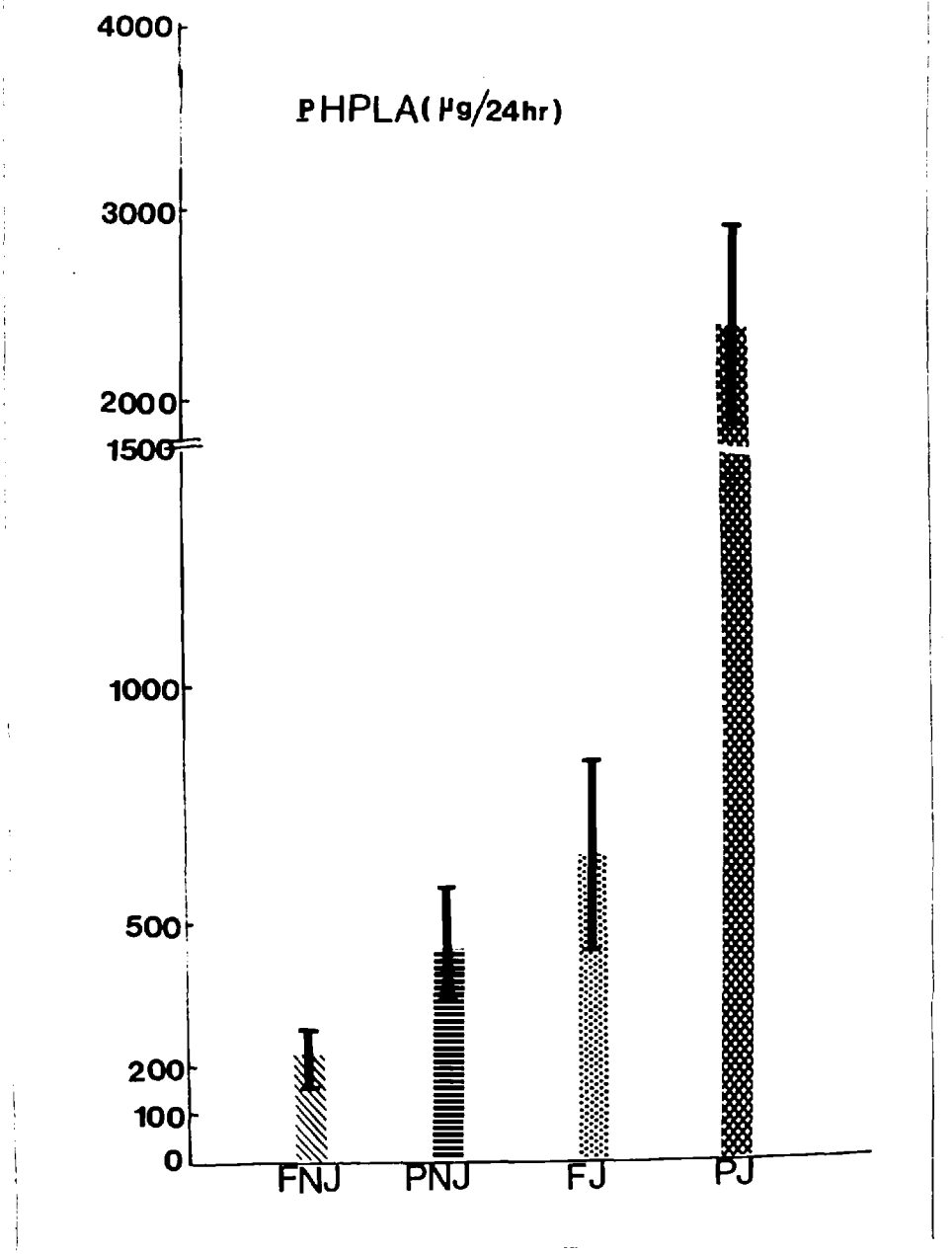


Fig. 6.2. Histogram representation of P-HPLA excretion in the four groups of neonates as classified in the text.

Table 6.2.

Substance	Comment
m-Hydroxybenzoic acid	Not detected
p-Hydroxybenzoic acid	Detected only in trace amounts. Less than 100 µg/24 hr.
o-Hydroxyphenylacetic acid	Detected in most cases in amounts varying from 10 - 100 µg/24 hr.
m-Hydroxyphenylacetic acid	Detected in most cases in amounts varying from 10 - 100 µg/24 hr.
Vanillic acid	Detected in about 30% of cases in amounts varying from 5 - 1000 µg/24 hr.
HVPA	Excretion less than 50 µg/24 hr.
Hippuric acid	Detected in trace amounts in most cases.
p-Hydroxymandelic acid	Its excretion in most cases is parallel to that of p-HPTA and p-HPPA. It cannot be accurately quantified under present conditions, since it is only poorly extracted by ethyl acetate. For accurate quantitation the modified procedure for this compound should be used (see chapter 4).

Comments on the occurrence and excretion of some free acids in neonatal urine.

Statistical tests.

Statistical analysis of results was based on the logorithms of the recorded data, as this transformation appeared to create greater similarity of variance within groups.

The mean HVA excretion values per 24 hr. for each group, compared between groups showed no statistically significant differences. A similar comparison of the mean VMA excretion values also showed no significant differences except between the following pairs, PHJ,& PHJ, PHJ & PJ, and PHJ & PJ (table 6.3). When, however, the HVA excretion was expressed in terms of concentration, the following two pairs PHJ & PJ, and PHJ & PHJ were now significantly different. VMA output on the other hand, whether expressed in terms of concentration or excretion per 24 hr, ~~was~~ had essentially the same significance between all groups (table 6.3).

A similar statistical analysis was carried out for mean p-HPLA excretion and the results are summarised in table 6.3. Comparing the differences of the mean 24 hr excretion or concentration between groups for p-HPLA and either HVA or VMA, statistically bigger differences were observed for p-HPLA (smaller "p" values) (table 6.3).

Mean 24 hr. excretion values of HVA, VMA and p-HPLA for each group in the first day of life were compared with the corresponding mean pooled values over the remainder of

Table 6.3.

Metabolite	Values Compared	Between Groups (number of cases)	Probability
HVA	Mean 24 hr excretion	PNJ (39) v PJ (13)	$p > 0.1$
"	" "	" v PNJ (23)	"
"	" "	" v PJ (25)	"
"	" "	PNJ (23) v PJ (13)	"
"	" "	" v PJ (25)	"
"	" "	PJ (13) v PJ (25)	"
HVA	Mean urine concentration	PNJ (39) v PJ (13)	$0.01 > p > 0.005$
"	" "	" v PNJ (23)	$0.1 > p > 0.05$
"	" "	PNJ (39) v PJ (25)	$p > 0.1$
"	" "	PNJ (23) v PJ (13)	"
"	" "	" v PJ (25)	"
"	" "	PJ (13) v PJ (25)	"
VMA	Mean 24 hr excretion	PNJ (39) v PNJ (23)	$0.01 > p > 0.005$
"	" "	" v PJ (25)	$0.05 > p > 0.025$
"	" "	PJ (13) v PNJ (23)	$p < 0.001$
"	" "	PNJ (39) v PJ (13)	$p > 0.1$
"	" "	PNJ (23) v PJ (25)	"
"	" "	PJ (13) v PJ (25)	"
VMA	Mean urine concentration	PNJ (39) v PNJ (13)	$0.005 > p > 0.001$

Table 6.3 Cont:

Metabolite	Values Compared	Between Groups (number of cases)	Probability
VMA	Mean urine concentration	PNJ (39) v PJ (25)	$0.01 > p > 0.005$
"	" "	" v FJ (13)	$p > 0.1$
"	" "	PNJ (23) v FJ (13)	"
"	" "	" v PJ (25)	"
"	" "	FJ (13) v PJ (25)	"
p-HPLA	Mean 24 hr urine excretion	PNJ (39) v FJ (13)	$0.02 > p > 0.01$
"	" "	" v PJ (25)	$p < 0.001$
"	" "	PNJ (23) v PJ (25)	$p < 0.001$
"	" "	FJ (13) v PJ (25)	$0.05 > p > 0.025$
"	" "	PNJ (39) v PNJ (23)	$p > 0.1$
"	" "	PNJ (23) v FJ (13)	"
"	Mean urine concentration	PNJ (39) v PJ (25)	$p < 0.001$
"	" "	PNJ (23) v PJ (25)	$p < 0.001$
"	" "	FJ (13) v PJ (25)	$0.2 > p > 0.01$
"	" "	PNJ (39) v FJ (13)	$p > 0.1$
"	" "	" v PNJ (23)	"
"	" "	PNJ (23) v FJ (13)	"

Statistical evaluation of urinary output of metabolites between groups. Comparisons were made on the logorithms of recorded data (see text).

the first week. Although the differences were not statistically significant ($P>0.1$), the mean excretion of HVA and VMA was higher on the first day than during the rest of the week in the PNJ group; the reverse relationship was observed for the prematures (PNJ and PJ) (table 6.4).

The correlation between urine volume and metabolite excretion expressed in terms of both 24 hr. metabolite output and concentration was examined (table 6.5). It was established that urine output is positively correlated with HVA and VMA excretion and negatively with their concentrations. The correlations were stronger for HVA than VMA. No such correlation could be obtained for p-HPLA where the probability was larger than 0.2 in all groups whether expressed as mg/24hr or as urine concentration.

Discussion

Catecholamine metabolites

The merits of expressing the output of urinary metabolites on the basis of creatinine excretion as opposed to 24 hr. collections has been the subject of some debate (Vestergaard and Leverett, 1958; Cramer, Cramer and Selander, 1967; Applegarth, Hardwick and Ross, 1968; Gitlow, Mendlowitz, Wilk, Wilk, Wolf and Bertani, 1968). The convenience of the former method cannot be denied, particularly where a complete collection of the specimen is difficult, as in young children

Table 6.5.

Metabolite	values compared	Group (number)	Correlation Coefficient(R)	Sign of Slope	Probability
HVA	24 hr urine excretion	PNJ (39)	0.470	+ve	$0.01 > p > 0.001$
	" "	PNJ (23)	0.637	"	$p < 0.001$
	" "	PJ (13)	0.577	"	$0.05 > p > 0.01$
	" "	PJ (25)	0.359	"	$0.1 > p > 0.05$
VMA	" "	PNJ (39)	0.271	+ve	$0.1 > p > 0.05$
	" "	PNJ (23)	0.511	"	$0.05 > p > 0.01$
	" "	PJ (13)	0.377	"	$0.2 > p > 0.1$
	" "	PJ (25)	0.359	"	$0.1 > p > 0.05$
HVA	urine concentration	PNJ (39)	0.236	-ve	$0.05 > p > 0.01$
	" "	PNJ (23)	0.314	"	$p > 0.1$
	" "	PJ (13)	0.190	"	$p < 0.001$
	" "	PJ (25)	0.230	"	$0.01 > p > 0.001$
VMA	" "	PNJ (39)	0.342	"	$0.05 > p > 0.01$
	" "	PNJ (23)	0.385	"	$0.1 > p > 0.05$
	" "	PJ (13)	0.598	"	$0.05 > p > 0.01$
	" "	PJ (25)	0.445	"	$0.05 > p > 0.01$

Correlations results of urine 24 hr volume with metabolite excretion per 24 hr and with urine concentration.

TABLE 6.4

Metabolite	Values compared	Groups (Number of cases)	Probability
HVA	24 hr urine excretion	FNJ (1st day 8, rest 31)	p 0.1
"	" "	FJ	-
"	" "	PNJ (1st day 5, rest 18)	p 0.1
"	" "	PJ (1st day 6, rest 19)	0.025 p 0.02
"	urine concentration	FNJ (1st day 8, rest 31)	p 0.1
"	" "	PNJ (1st day 5, rest 18)	"
"	" "	FJ	"
"	" "	PJ (1st day 6, rest 19)	"
VMA and p-HPLA	24 hr urine excretion	FNJ, PNJ, FJ, PJ	p 0.1
VMA and p-hPLA	urine concentration	FNJ, PNJ, FJ, PJ	p 0.1

The excretion of metabolites in day one compared statistically with rest of the first week of life. Comparisons were made on the logorithms of recorded data (see text).

(Gitlow et al, 1968). Unfortunately, however, in this age group creatinine concentration is less reliable as an index of total urinary volume than any other time (Applegarth et al., 1968). Renal function (Berlyne, 1965) and disturbances in normal feeding accompanied by weight loss (Pscheidt, Berlet, Spaide and Himwich, 1966) have been reported to influence creatinine excretion in the adult. Renal dietary and metabolic factors undoubtedly exert an even greater effect on urinary creatinine levels in the new born (Smith, 1959). Therefore, in these subjects particularly, it seems desirable to express metabolite excretion in terms of a timed collection, or as concentration per unit volume (Cramer et al., 1967) if this is not possible, rather than link it to the highly variable creatinine output.

There have been a number of reports on the urinary excretion of VMA in the first week of life (Von Studnitz, 1960_b; Zeisel, 1961; Boehm and O'Brien, 1963; Nicolopoulos et al., 1968). All values compared closely with the present observations apart from those of Nicolopoulos et al.,(1968) which are somewhat higher. Corresponding data for HVA in the neonatal period do not appear to be available in the literature, although excretion values of both HVA and VMA in infants from the age of one month to one year have been reported (von Studnitz, 1962; Gjessing 1966_a; Gitlow et al., 1968); these values range from 1.2 to 35 μg HVA/mg creatinine

and 1.4 to 15 µg VMA/mg creatinine. When comparisons are made on the basis of creatinine excretion, these levels are broadly similar to those found for the FNJ infants and PNJ, but they are lower than those observed in the PJ infants (table 6.1) (creatinine was not determined in the full-term jaundiced infants).

The reasons for the observed statistically significant higher urine output of HVA (FNJ and PNJ, FNJ and PJ) and of VMA (FNJ compared with all groups) in the FNJ than all other groups (table 6.3) and the implications of the jaundiced state are not readily forthcoming. They probably indicate a more efficient renal clearance and more mature catecholamine metabolising enzyme system in the FNJ compared with the other groups. The lower output of VMA in the premature compared with the full-term infants agrees with the report by Zeisel (1961) of comparatively low adrenaline (A) and noradrenaline (NA) production in the former.

After the first few days of life NA and A excretion is higher on a weight for weight basis in the newborn than in adults (Zeisel and Kuschke, 1959), although the ratio of dopamine to NA remains proportionately the same (Stern, Greenberg and Lind, 1961). These observations therefore point to a raised output of dopamine in the newborn compared with the adult, an impression which has recently received direct support from Voorhess (1967). In the light of the

consistancy in the relationship between the two amines irrespective of age, the tendency towards an age dependent alteration in ratios of their major acidic metabolites HVA and VMA (see earlier) may be a further example of a difference between neonatal and adult renal excretion mechanisms and/or rate of metabolism. Voorhess (1967) speculated that the greater output of dopamine and NA per unit body surface area which she noted during the first year of life, might be related to rapid growth and maturation of the sympathetic nervous system at this time. The same argument might apply to the increase of catecholamines per unit body weight observed by Zeisel and Kuschke (1959) in the first year of life. However in all work of this kind, including that described in this thesis, it is not immediately obvious whether variations in output stem from alterations in catecholamine synthesis, change in rate of release from storage granules or alterations in degradation pathways. The higher HVA to VMA ratio observed in the neonates (table 6.1), with a mean of 1.6 and a range between 1 and 3, which are considerably higher than the values of 0.5 to 1, found in normal adults (see chapter 5), strongly indicate a somewhat higher dopamine turnover than A and NA.

The direct association between urine volume and both HVA and VMA output observed during the first week of life is

a further illustration of a functional difference between the neonate and older child or adult, a difference which presumably derives from immaturity of the neonatal kidney (Smith, 1959). Using a colorimetric procedure (Pisano et al., 1962) to estimate VMA, Ruthven (1965) also observed a significant correlation ($P < 0.01$) between VMA excretion and urine volume in the neonate.

von Studnitz (1960), in a study of a small number of newborn infants, observed an increased VMA excretion on the first day of life compared with the third day. Although results obtained here point to a higher mean excretion of both HVA and VMA in the first day compared with the rest of the first week of life in the FNJ group, the differences are not statistically significant (table 6.4). As suggested by von Studnitz (1960) for VMA, an argument which may apply equally to HVA, the high levels excreted after birth in the FNJ group might reflect a rise in catecholamine production following the stress of birth. This view is in fact supported by the indirect evidence of Howard, McDevitt and Stander (1964) who observed a significant increase in catecholamine excretion in the first urine specimen passed after birth by infants delivered by forceps compared with those delivered spontaneously by a caesarean section.

HMFG excretion (table 6.1) bears approximately the same

proportionate relationship to VMA excretion as in adults when mean values are compared with those of adults, although the individual measurements are much more widely scattered in the neonate.

Tyrosine metabolites

A reduced activity of p-hydroxyphenylpyruvic acid oxidase (p-HPPA oxidase) is not uncommon in the newborn period of life especially in the premature infant (Kretchmer, Levine, McNamara and Barnett, 1956; Kretchmer and McNamara, 1956; Bloxam et al, 1960). This phenomenon is caused either by a deficiency of the enzyme (Kretchmer et al., 1956; Kretchmer and McNamara, 1956) or by substrate inhibition (Zannoni and LaDu, 1959; Bloxam et al., 1960) and may usually be corrected by the administration of ascorbic acid (Levine, Marples and Gordon, 1939; Levine, Gordon and Marples, 1941_a; Levine, Dann and Marples, 1943; Woolf and Edmunds, 1950: Nitowsky, Covan and Gordon, 1953), 2,6-dichlorophenolindophenol (Hager, Gregerman and Knox, 1957) and folic acid (Nitowsky et al., 1953; Menkes and Avery, 1963). The condition is manifested by raised blood tyrosine concentration (tyrosinaemia) (Mathews and Partington, 1964; Wong et al., 1967; Partington, 1968) and increased excretion of both tyrosine and its metabolites p-HPLA, p-HPPA and p-hydroxyphenylacetic acid (tyrosyluria) (Bloxam et al., 1960; Woolf 1965; Partington, 1968).

Reports on the incidence of tyrosyluria and its association with tyrosinaemia vary, principally because of the greater sensitivity of methods used to estimate blood tyrosine compared with those employed to diagnose tyrosyluria. In a survey of 1276 infants aged 3 to 8 weeks, Gibbs and Woolf (195) found 14 cases of tyrosyluria using ferric chloride, while in another survey of 120 infants, screening urines by paper chromatography which is more sensitive than ferric chloride, Woolf (1965) detected 26 cases of tyrosyluria. Early reports by Levine and his co-workers (Levine et al., 1939; Levine et al., 1941a,b; Levine et al., 1943) that tyrosyluria occurs only in the premature baby were later found to be incorrect (Wong et al., 1967). Partington (1968) in a survey of 123 neonates under the age of four weeks, found that the incidence of tyrosyluria is closely related to tyrosinaemia if the blood tyrosine is greater than 10 mg/100 ml, but that the relationship is less constant when the tyrosine level is below this value. It is surprising that the correlation between these two conditions is not stronger since one is a direct consequence of the other. Although tyrosinaemia has been quantitatively defined (Partington, 1968), tyrosyluria has not been, due to limited quantitative information on p-HPLA and p-HPPA excretion. Woolf (1965) reported values of 1200 and 3500 μg p-HPPA/mg creatinine in cases classified as

tyrosyluria, whilst Coward and Smith (1968_b) gave values of p-HPLA greater than 500 μg/mg creatinine in a study of six similar patients. These values are greater than most of those observed in the present series, where the upper end of the range was 530 μg/mg creatinine (table 6.1). By virtue of its stability compared with p-HPPA, p-HPLA is a more useful index of tyrosyluria. An excretion of 1 mg p-HPLA /24 hr is tentatively proposed here as the upper limit of normal since this level was not exceeded in the FTJ group of infants.

The incidence of tyrosyluria (p-HPLA excretion greater than 1 mg/24 hr) in infants during the first week of life was found to be higher in the present series than any previously reported study, about 27% of the neonatal population. This figure was calculated on the assumption that the infants studied were representative of the population from which they were drawn, which is probably valid since they were chosen in a random manner. The cause of the discrepancy is the higher sensitivity of the GTJ procedure employed here compared with other methods available for the detection of tyrosine metabolites in urine. For example a parallel survey on these babies using conventional screening tests (Perry, Hensen and MacDougal 1966) showed that the majority would have been classed as non-tyrosyluric.

The association between jaundice and an increased

output of tyrosine metabolites has not previously been reported. Recently Partington (1968) observed a bimodal distribution of plasma tyrosine levels in newborn infants, more marked in the premature than the full-term baby. A comparable distribution more pronounced in premature than full-term infants could be observed on the basis of p-HPLA excretion. The distribution was strongly influenced by the presence of jaundice, which also may have been an important factor in the bimodel distribution observed by Partington (1968). It is possible that high serum bilirubin levels irreversibly inhibit p-HPPA oxidase and that the lag observed between the disappearance of jaundice (usually within the first week) and tyrosyluria or tyrosinaemia (usually about 6 weeks) (Partington, 1968) represents the time required for the production of fresh enzyme. However a tendency for p-HPPA oxidase activity to be low in common with other neonatal enzymes e.g. uridine glucuronyl transferase (Brown and Luelzer, 1958; Lathe and Walker, 1958) is a more likely explanation of tyrosyluria in the newborn.

Some increase in p-hydroxyphenylacetic acid excretion has been suggested in tyrosyluria (Bloxam et al., 1960). This observation is supported indirectly by the detection of an increased output of its precursor, p-tyramine, in severe tyrosinaemia (Bremer, Jaenicke and Leopold, 1969). It is possible that an insufficient degree of tyrosinaemia was

present in the present series to bring about a significant increase in p-hydroxyphenylacetic acid.

Because of the low sensitivity of the GLC assay for p-hydroxymandelic acid (table 6.2) it could only be estimated satisfactorily by the procedure employed here when present in large amounts (see table 6.2). This metabolite probably arises from octopamine by oxidative deamination (see chapter 1). Octopamine may be derived from tyramine by the action of dopamine β-hydroxylase (see chapter 1). Nevertheless a peak corresponding with p-hydroxymandelic acid was detected in many tyrosyluric urines but not in non-tyrosyluric, agreeing with the findings of Woolf (1965).

CHAPTER 7

Studies on 5-hydroxyindole metabolites in carcinoid disease

Although 5HT has yielded pride of biochemical place to the kinins (Oates, Melmon, Sjoerdsma, Gillespie and Mason, 1964) and more recently, the postaglandins (Sandler, Karim and Williams, 1968) its overproduction must still be considered "a hallmark of the carcinoid syndrome" (Engelman, Lovenberg and Sjoerdsma, 1967). Whilst possible interactions between all these pharmacologically active agents should not be neglected (Sandler, 1968_a) as having a possible modifying effect on 5-hydroxyindole pathways, most useful information can still be obtained from quantitative studies of this group of compounds in carcinoid disease. 5HT is derived from tryptophan by 5-hydroxylation followed by decarboxylation (Hagen and Cohen, 1966) as discussed in chapter 1. It is mainly metabolised through oxidative deamination by MAO to 5-hydroxyindolacetaldehyde which is then further oxidised by aldehyde dehydrogenase to 5HIAA (Sjoerdsma et al., 1955; Weissbach et al., 1957). A small proportion of this aldehyde intermediate is however reduced to the alcoholic metabolite, 5HTOH, as described earlier in chapter 1. In normal subjects, the excretion of 5HTOH does not exceed 100 µg/24 hr (see chapter 5, a level very comparable with the urinary output

of 5HT (Rodnight, 1956; Rodnight and McIlwain, 1956; Sjoerdsma et al., 1956; Pernow and Waldenström, 1957; Noble, 1961; Cates and Sjoerdsma, 1962). Apart from the observation of one group (Davis et al., 1966), no information is available on the pattern of excretion of 5HTOH in carcinoid disease. Following an early observation (Smith, Nyhus, Balgliesh, Dutton, Lennox and MacFarlane, 1957) that ethanol ingestion causes a decrease in 5HIAA excretion in patients with carcinoid, a shift in 5-hydroxyindolacetaldehyde metabolism from a predominently oxidative pathway to a reductive one (Davis et al., 1966; Davis, Brown, Huff and Cashaw, 1967_a) was identified in normal and carcinoid subjects. To provide more information on 5HTOH excretion and the effect of ethanol ingestion on it, estimations of urinary 5HIAA and 5HTOH were undertaken on a group of carcinoid patients, one of whom had ingested an alcoholic beverage.

Material and methods

Urine samples from all subject were collected into plastic bottles containing 25 ml glacial acetic acid, and were stored at - 15°C until time of analysis.

For 5HIAA analysis, urine aliquots (1 ml., except when concentration of 5HIAA were high, when 0.5 ml of urine was taken) were made up to 10 ml with distilled water. After the

pH had been adjusted to 3.5 with 0.1 N NaoH, the mixture was extracted twice with ether, by the standard procedure (see chapter 4). An internal standard of 0.1 mg 5HIAA was used for each batch of 7 estimations. The M../T. was prepared and analysis performed on a 5 ft. 5% SE52 column.

For 5HTOH assay, urine aliquots (10 ml when 5HIAA levels were less than 50 mg/24 hr or 5 ml when greater) were hydrolysed, extracted twice with ether at pH 8, and the T../. prepared. As with 5HIAA, a 5 ft. 5% SE52 column was employed. Internal standard (0.02 mg) was used with every batch of seven estimations.

Results and discussion

5HIAA and 5HTOH urinary excretion values and ratios in 13 patients with carcinoid disease are shown in table 7.1. Statistical analysis of these results revealed a positive correlation ($p < 0.01$) between 5HIAA and 5HTOH output.

The excretion of 5HIAA is usually but not invariably (von Heilmeyer and Clotten, 1958; Davis and Rosenberg, 1961) raised and the increase is usually large; thus values of 500 - 1000 mg/24 hr are not uncommon (Stracey, 1966). The range observed here (Table 7.1) is on the whole consistent with what one expects in this disease in view of the variable mass and activity of 5HT secreting tumour tissue.

TABLE 7.1

NAME	5HIAA	5HTOH	5HIAA/5HTOH
HT	304 mg/24 hr	7.45 mg/24 hr	40.8
"	125 "	4.5 "	27.7
PT	-	1.58 "	-
"	155 "	1.54 "	100.6
RDF	66.5 "	0.81 "	82.0
HAT	140 g/ml	2.1 g/ml	70.0
SHP	25.2 mg/24hr	1.33 mg/24hr	18.9
SK	18.7 "	-	
TR	18.4 mg/100ml	0.212 mg/100ml	86.7
FRD	70.5 mg/24hr	5.1 mg/24hr	13.8
HB	52.3 "	0.98 "	53.3
HAR	535 "	6.6 "	81.0
CLM	200 "	7.6 "	26.3
"	940 "	28.8 "	32.6
Range	18.7-940 "	0.81-288 "	18.9-100.6

Urinary excretion of 5HT metabolites in carcinoid disease employing a 5 ft 5% SE52 column, preparing the ME/TE for 5HIAA and the TE/E for 5HTOH.

Davis et al., (1966) in a study of two carcinoid patients reported values of 5HTOH of 0.826 mg and 0.604 mg/24 hr in one, and of 1.971 mg/24 hr in another. The present results are in general, of the same order, although the highest value obtained was 28.8 mg/24 hr.

The ratio of 5HIAA to 5HTOH were found to vary from 18.9 to 100.6 with a mean of 52.8. Although no such relationship has so far been established directly for output in the normal subject, approximate values can be inferred indirectly: thus from the reported value of 1.3% of administered ^{14}C-5HT recovered as 5HTOH in both normal and carcinoid subjects (Davis et al., 1966) and from a consideration of the ranges of 5HIAA (2-10 mg/24 hr) and 5HTOH (less than 100 µg/24 hr) excretion values in normal subjects (see chapter 5), a calculated value of 50 might not be too unreasonable.

No attempt has been made here to distinguish between the different forms of carcinoid (Williams and Sandler, 1963) or to detect whether or not there was an increased excretion of 5HT or 5HTP. It would have been of interest to have studied cases of carcinoid syndrome excreting an elevated amount of 5HTP (Sandler and Snow, 1958), with special reference to the excretion of 5-hydroxyindolelactic acid which might be formed by transamination followed by oxidation of 5HTPyA.

Urine samples from one carcinoid patient before and

after ingestion of 50 ml of brandy were analysed for 5HIAA and 5HTOH. Results before brandy were 30.4 mg 5HIAA/12 hr and 0.292 mg 5HTOH/12 hr, and after brandy 29.6 mg 5HIAA/12 hr and 0.430 mg 5HTOH/12 hr. Thus after ingesting brandy there was a slight decrease of 5HIAA, but a doubling of 5HTOH excretion. This effect was considerably smaller than that which might have been expected from a consideration of the work of Davis et al., (1966). This discrepancy is probably explained in terms of the considerably smaller dose of ethanol employed in the present work. Smith et al., (1957) have reported an actual decrease in 5HIAA after ethanol ingestion in carcinoid disease. It is therefore possible that any direct or indirect inhibitory effect of ethanol on aldehyde dehydrogenase or stimulatory effect on aldehyde reductase is dependent on the amount ingested and the individual tested.

CHAPTER 8

Studies on catecholamine-secreting tumours

Introduction

Catecholamine-secreting tumours belong to a group of neoplasms derived from cells differentiated embryologically from elements of the neural crest. Tumours of both medullary and extramedullary chromaffin cells give rise to phaeochromocytoma while those of the neuroblast and mature ganglion give rise to neuroblastoma and ganglioneuroma respectively (Käser 1966; Gjessing, 1966). For a more comprehensive classification, see Gjessing (1968). Although the characteristic feature of most of these tumours is an over production of catecholamines, the excretory pattern of the amines and their metabolites varies considerably from patient to patient (von Studnitz, 1966), especially in phaeochromocytoma (Gitlow, Mendlowitz, Kruk and Khässis, 1961; Crout, Pisano and Sjoerdsma, 1961; Crout and Sjoerdsma, 1964; von Studnitz, 1966).

Phaeochromocytoma occurs mainly in adults and is only rarely present in children. The overall incidence is about 0.5 of all hypertensive patients (Graham, 1951; Kvale, Roth, Manger and Priestley, 1956) but the problem is not quite so clear-cut as patients with this tumour may sometimes be

normotensive or even hypotensive (Moorhead, Caldwell, Kelly and Morales, 1966). In about 80% of cases the tumour arises in the adrenal gland (Gjessing, 1968) being benign in the majority; the patient has a good prognosis if it is removed early (Moorhead et al., 1966). NA, and to a lesser extent A are the main secretory products of these tumours (Sandler, 1967). The rate of production of A may reflect the age and maturity of the tumour (Robinson, 1966), since in malignant phaeochromocytoma the A content in the primary tumour (Kennedy, Symington and Woodger, 1961) tends to be higher than in the metastases (Davis, Peart and van't Hoff, 1955; Kennedy et al., 1961). The overall secretion of catecholamines is often related to the size and activity of the tumour. Small tumours tend to have higher rates of catecholamine turnover compared with larger ones in which secretion is lower and the tissue content of amines higher (Crout and Sjoerdsma, 1964; Crout, 1966). Although the presence of catecholamine-producing metastases in organs which do not normally contain chromaffin cells is the only conclusive proof of malignancy in phaeochromocytoma (Gjessing, 1968), the excretion of dopamine (McMillan, 1956; Weil-Malherbe, 1956) and its metabolites together with the excessive production of NA is often associated with malignancy (Robinson, Smith and Whittaker, 1964). The former observation lead Robinson and

his collegues (Robinson et al., 1964; Robinson, 1966) to suggest that the presence of urinary DOPA and dopamine and their metabolites might be indicative of malignancy. It has since been established however, that this is not always the case (Sato and Sjoerdsma, 1965; Sandler, 1967); but nevertheless, dopamine secretion increases the likelihood of the tumour being malignant (Kaser, 1966).

Neuroblastoma occurs far more frequently in early childhood than later in life and may be present at birth (Wells, 1940; Potter and Farrish, 1942; Bodian, 1959; Gross, Farber and Martin, 1959; King, Storaasli and Bolande, 1961; Margoen, 1962; Voorhess and Gardner 1962_a). Such early cases are more likely to undergo spontaneous remission than those occurring in the older child (Bodian, 1959; Brett, Oppe, Ruthven and Sandler, 1964). The tumours are primarily dopamine-secreting (Smellie and Sandler, 1961; Sandler and Ruthven, 1961; Voorhess et al, 1962; Rosenstein and Engelmann, 1963; Käser, Bettex and von Studnitz, 1964). The urinary output secretion of NA and A is usually low compared with phaeochromocytoma despite the fact that large amounts of β-hydroxylated catecholamine metabolites are found only a few of the subjects with neuroblastoma have elevated blood pressure (Voorhess and Gardner, 1962; Sourkes, Denton, Murphy, Chavez and St. Cyr, 1963; von Studnitz, Käser and Sjoerdsma,

1963). Presumably the production of β-hydroxylated catecholamines by the tumour is higher than in phaeochromocytoma, but degradation within the tumour is also high, resulting in a greater urinary excretion of metabolites (Sandler and Ruthven, 1966a; Calne, Karoum, Ruthven and Sandler, 1969). A greater proportion of these derive from dopamine (von Studnitz, 1960; Gjessing, 1963; Käser, Schweisguth, Sellie and Spengler, 1963; Sourkes et al, 1963; Ruthven and Sandler, 1964; Brett et al, 1964; Hinterberger and Bartholomew, 1969) although high concentrations of NA metabolites are also seen (von Studnitz, 1962; Voorhess, Pickett and Gardner, 1963; Williams and Greer, 1963; Hinterberger et al., 1968).

In ganglioneuroma a benign tumour, the biochemical features may not be as well defined as in either phaeochromocytoma or neuroblastoma, although an increased production of catecholamines has been reported (Dicke, Kingma, Radman, de Jongh and Booch, 1959; Greenberg and Gardner, 1959, 1960; Smellie and Sandler, 1961; von Studnitz, Käser and Bjoerdsma 1963; Gjessing, 1963; 1964; Rosenstein and Englemann, 1963; Sankoff and Sourkes, 1963; Greer, Anton, Williams and Echevarria, 1965; Käser, 1966) with dopamine again being more prominent than NA or A (Greenberg and Gardner, 1960; von

Studnitz et al., 1963; Gjessing, 1964). However non-secreting tumours are much more common among ganglioneuromas than in other neural tumours (von Studnitz, 1962; Kaser et al., 1964; Kaser, 1966).

Diagnostic elevations of metabolites derived from the major pathways of catecholamine metabolism (as discussed in chapter 1) are found in catecholamine-secreting tumours. Thus in phaeochromocytoma, VMA (Armstrong et al., 1957; Sandler and Ruthven, 1959_a; Crout et al., 1961; Gitlow et al., 1961) and HMMG (Axelrod, Kopin and Mann, 1959; Sandler and Ruthven, 1966_a; Milk, Gitlow, Clarke and Daby, 1967) are excreted in large amounts in a high percentage of cases (Crout, 1966) as are the O-methylated amines, normetadrenaline and metadrenaline (Crout, 1966; Sandler, 1967). In cases where DOPA and dopamine are secreted such as malignant phaeochromocytoma (Robinson et al., 1964) the excretion of dopamine metabolites is also raised (Sankoff et al., 1963; Robinson et al., 1964; Sato et al., 1965; Sandler, 1967). Other metabolites reported in phaeochromocytoma urines which have not so far been evaluated diagnostically include vanillic acid (Engelman and Sjoerdsma, 1964) protocatechuic aldehyde (Herrlich and Sekeris, 1963), N-methylmetadrenaline (Itoh, Yoshinaga, Sato, Ishida and Wada, 1962; Robinson and Smith, 1962), and N-acetylated dopamine (Karlson, Sekeris and

Herrlich, 1963).

As already stated, the overproduction of catecholamines in neuroblastoma is accompanied by a wide urinary spectrum of metabolites usually higher in concentration and more varied than in phaeochromocytoma. The major metabolites include HVA (Greenberg and Gardner, 1959; von Studnitz, 1960, 1966; Brett et al., 1964; Hinterberger and Bartholomew, 1969) and DOPAC (von Studnitz, 1960; Sourkes et al., 1963; Williams and Leonard, 1963) deriving from DOPA and dopamine, and β-hydroxylated metabolites of NA and A (LaBrosse and Karon, 1962; Gjessing, 1963; Sandler and Ruthven, 1966_a; Kaser, 1966; Hinterberger and Bartholomew, 1969). The following metabolites have also been detected in neuroblastoma: 4-hydroxy-3-methoxyphenylalamine (von Studnitz, 1961), 4-hydroxy-3-methoxy phenylpyruvic acid (Gjessing and Borud, 1964_a; Smith, 1965) and 4-hydroxy-3-methoxyphenyl lactic acid (Gjessing, 1963_a 3,4-dihydroxymandelic acid (De Quattro, Mybenga, von Studnitz and Brunjes, 1964) and 3,4-dihydroxyphenylpyruvic acid (Gjessing, 1964_a).

In view of the great variation in the pattern of catecholamines metabolite excretion in these tumours, it is often useful to estimate at least two metabolites to help to differentiate them (Voorhess and Gardner, 1961; Gjessing, 1963).

Chromatographic procedures are usually more suitable for this purpose because of their simplicity compared with other procedures (for review, see Sandler and Ruthven, 1969). Among the different types of chromatography GLC is the only approach capable of providing accurate quantitative estimations (Karoum et al., 1968, 1969); other types are at best semiquantitative (Sandler and Ruthven, 1966_a). During its early stages of development however, GLC of aromatic compounds was beset with many problems connected with peak trailing, purity of eluted components and difficulty of separation (Williams and Greer, 1962, 1965) which have now been almost completely overcome, at least for the assay of most of the important phenolic and indolic acids and alcohols of biological interest (Dalgliesh et al., 1966; Horning et al., 1966; Karoum et al., 1968,1969).

In this chapter the excretion of a wide range of phenolic acids and alcohols is reported on a group of patients with catecholamine secreting tumours.

Material and method

Urine samples (24 hr.) were collected into 25 ml 6 N HCl and stored at 4°C until analysed.

For the estimation of phenolic acids, urine aliquots (1 to 5 ml) were made up to 10 ml with distilled water; the

pH was adjusted to 2 and they were extracted with ethyl acetate. Ether was substituted for ethyl acetate in the estimation of DOPAC (see chapter 4). The ME/TE derivatives were prepared for all compounds except DOPAC where the TE/E was formed. Internal standards were included in every batch as follows: 0.1 mg VMA, 0.1 mg DOPAC and 0.05 mg HVA. Other metabolites were quantified by comparing their relative response with that of either VMA or HVA (see chapter 3.)

Alcoholic metabolites were analysed by extracting with ethyl acetate at pH 8 (see chapter 4) after preliminary hydrolysis (5 ml). HMPG (0.1 mg) was employed as internal standard and, with appropriate relative response corrections, was used to quantify HMPE and dihydroxyphenylglycol (see chapter 3).

The identity of HMPE was checked by direct gas chromatography-mass spectrometry (GC-MS) using an AEI MS-12 mass spectrometry in cooperation with Dr. J. Chapmen of Associated Electrical Industries, Manchester, to whom grateful thanks are due. Three techniques were employed: scanning the peak corresponding to HMPE with a total ion monitor to obtain a complete mass spectrum; monitoring the current at $^{m}/_{e}$ 312 (the molecular ion of HMPE), done on the assumption that any impurity would not contribute an intense peak at

$^{m}/_{e}$ 312; to obtain a more precise characterisation of HMPE the accelerating voltage of the MS-12 was switched rapidly to monitor $^{m}/_{e}$ 297 and 312 alternately every 0.5 sec. Both mass peaks are characteristic of HMPE, but are unlikely to be present in the spectra of other compound, particularly in the correct relative intensity (Fig. 8.1.)

Results

Urine samples from 19 patients were analysed, 9 with benign and 4 with malignant phaeochromocytoma, and 6 with neuroblastoma. Individual output values of the major and minor metabolites are given in tables 8.1 (benign phaeochromocytoma), 8.2 (malignant phaeochromocytoma) and 8.3 (neuroblastoma). Table 8.4 provides information about the excretion of other metabolites which were detected in concentrations too low to allow accurate measurement to be performed. Figures 8.2, 8.3, 8.4 and 8.5 illustrate patterns of phenolic acid and alcohol excretion in typical cases of phaeochromocytoma and neuroblastoma. Figure 8.7 shows a summary of the mean excretion of five important metabolites in the three types of catecholamine secreting tumours discussed here.

The identity of HMPE was confirmed by GC-MS in all neuroblastoma and in 4 malignant phaeochromocytoma patients (Fig. 8.1). The identities of HVA, VMA and HMPG in two urine

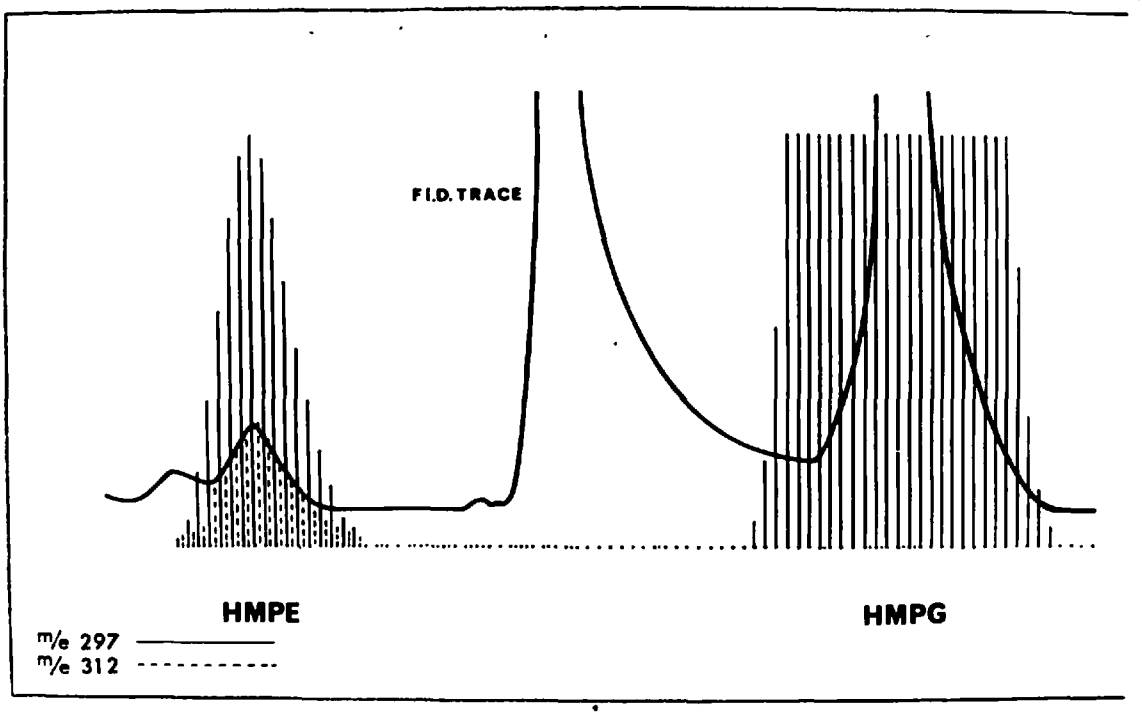


Fig. 8.1. Illustration of how low levels of HMPE in urine as its TE/E could be detected by monitoring ions m/e297 and m/e312 alternatively every 0.5 sec. Here only HMPE derivative have ions at both m/e 297 and m/e 312. HMPG on the other hand has an ion at m/e 312 and so will not give the peak corresponding to ion 297 as shown for HMPE. It will be noticed that the maxima of the traces at m/e 297 and 312 correspond to the FID (Flame ionisation detector) maximum, confirming the identity of the peak as HMPE. The urine was obtained from a patient suffering from neuroblastoma.

TABLE 8.1

Patient	HVA	VMA	Vanillic Acid	P-HPLA	P-HPAA	DOPAC	HMPG	Description
GL	5.8	9.1	<100µg	<500µg	2.48	<500µg	4.3	Preoperative
CNT	2.50	9.4	"	"	10.9	"	4.0	"
BR	4.0	17.2	2.4	"	12.8	"	3.4	"
MR_1	2.3	12.2	<100µg	"	5.8	"	2.8	"
MR_2	3.1	23.4	"	"	14.2	0.74	3.7	"
MR_3	6.7	5.9	3.7	"	28.2	<500µg	1.5	Postoperative
PM_1	29.2	13.3	<100µg	"	9.5	8.5	6.2	Suspected Malignancy
PM_2	32.2	15.7	"	"	13.9	9.45	7.5	"
JD	6.50	52	"	1.38	29.8	<500µg	10.3	Preoperative
SCR	3.2	36	8.5	<500µg	-	"	-	"
DVS_1	1.90	14.6	<100µg	"	7.0	"	6.4	"
DVS_2	1.8	19.1	"	"	7.8	"	4.7	"
DVS_3	2.0	16.4	"	"	4.12	"	7.5	"
RND_1	0.92	29.8	"	8.4	8.6	"	9.5	"
RND_2	1.8	27.6	"	7.3	5.3	"	11.3	"
Mean	6.9	21.0	-	-	10.1	<500µg	6.3	
Range	0.9-32.2	9.1-52	<100µg - 8.5	<500µg - 8.4	2.5-29.8	<500µg - 9	2.8-11.3	

Urinary excretion of phenolic acid and alcohol metabolites (mg/24hr unless stated) in benign phaeochromocytoma.

TABLE 8.2

Patient	HVA	VMA	Vanillic Acid	P-HPLA	P-HPAA	DOPAC	HMPG
RN	12.1	24.2	<100µg	<500µg	27.6	-	2.5
STK	52.6	174	22.0	"	31.4	4.7	20.4
HAR	3.55	11.5	<100µg	8.2	62.5	<500µg	6.2
PHL	28.1	244	"	6.3	7.1	6.3	52.5
Mean	24.1	113	-	-	32.1	3.8	20.4
Range	3.6-52.6	11.5-244	<100µg-22	<500µg-8.2	7.1-62.5	<500µg - 6.3	2.5-52.5

Urinary excretion of phenolic acid and alcohol metabolites (mg/24 hr unless stated) in malignant phaeochromocytoma

TABLE 8.3

Patient	HVA	VMA	Vanillic Acid	P-HPLA	P-HPAA	EOPAC	HMPG	HMPL
BRY	41.5	51.5	5.3	<500/µg	5.3	3.3	21.0	0.50
HRT	79.0	15.8	<100/µg	"	4.3	6.1	7.5	1.85
MAS	19.5	93	22	"	1.1	3.8	41.0	0.75
WHL	211.0	143	100 g	"	1.9	18.7	56.0	9.2
HY_1	43.5	17.9	0.2	"	29.8	6.4	2.4	3.2
HY_2	3.4	2.5	<100/µg	"	8.2	100 g	1.6	<100/µg
HSN_1	56.5	65	3.8	7.2	3.8	5.6	34.4	<100/µg
HSN_2	260	385	2.0	14.6	1.7	40	97	3.8
Mean	68.1	110	-	-	7.0	11.9	51.3	2.43
Range	19.5-260	15.8-385	<100/µg-22	<500/µg - 14.6	1.1-29.8	3.3-40	2.4-97	100 g - 9.2

Urinary excretion of phenolic acid and alcohol metabolite (mg/24 hr unless stated) in patients with neuroblastoma.

Substance	Benign Phaeochromocytoma		Malignant Phaeochromocytoma		Neuroblastoma	
	Range (mg)	Comment	Range (mg)	Comment	Range (mg)	Comment
P-HPPA	< 0.5	Decomposes on storage	<0.5	Decomposes on storage	<0.5	Decomposes on storage
VLA	<1	-	<1	Detected in traces in one patient (about 1mg/24hr)	<1	Detected in large amount in one patient (9.7mg/24hr)
VMA	<1	Decomposes on storage	<1	Detected in traces in one patient (about 1mg/24hr) Decomposes on storage	<1	Detected in large amount in one patient (8.0mg/24 hr). Decomposes on storage
HMPE	<0.1	Detected in one patient (0.8 and 1.4 mg/24 hr) who excreted large amounts of HVA	<0.1 - 2.1	Detected in two out of three patients (0.7 and 1.1 mg/24 hr).	<0.1 - 9.2	Detected in six patients out of eight see table 8.3
DHMA	<2	-	<2	-	<2	Detected in only one patient (14mg/24hr) who had very high excretion values of HVA and VMA
DHPG	<0.1-1	Detected in two specimens from one patient (9.5 and 11.3 mg/24hr)	<0.1-1	Detected in one patient (1.1 mg/24hr)	<0.1-1	Detected in three patients (approx. 0.3 mg, 8 mg and 10.2 mg/24 hr)
Vanilyl Alcohol	<0.1	-	<0.1	-	<0.1	Detected in two patients (2.2 and 0.75 mg/24 hr.)
DHPE	<0.1	-	<0.1	-	<0.1	Detected in one specimen only (2.9mg/24hr.)
P-HPE PHPE	<0.02	-	<0.02	-	<0.02	-

Summary on the excretion pattern of biologically interesting [illegible] and alcohol metabolites in catechaolamine secreting tumours.

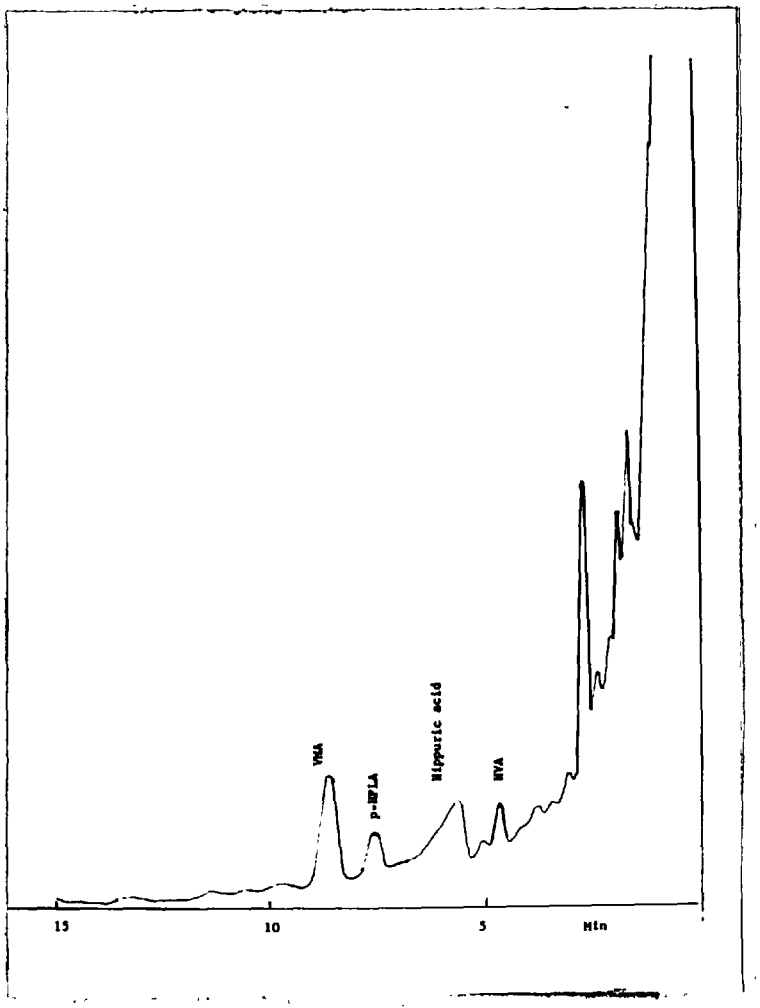


Fig. 8.2. Chromatogram of the ME/TE derivative of an ethylacetate urine extract from a patient with phaeochromocytoma.

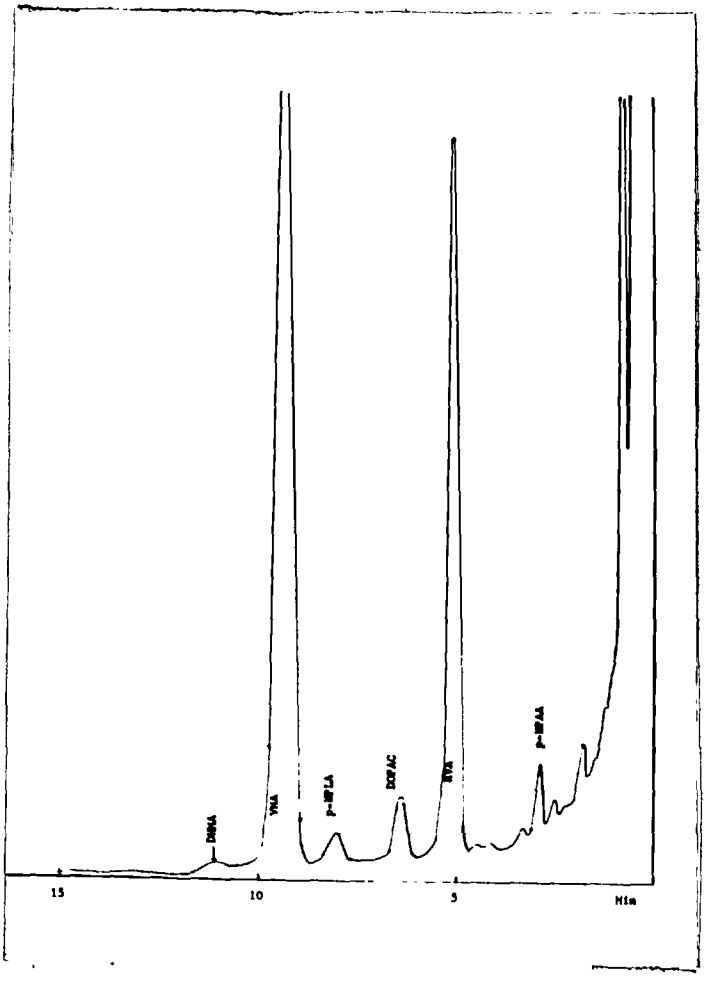


Fig. 8.3. Chromatogram of the ME/TE derivative of an ethyl acetate urine extract from a patient with neuroblastoma.

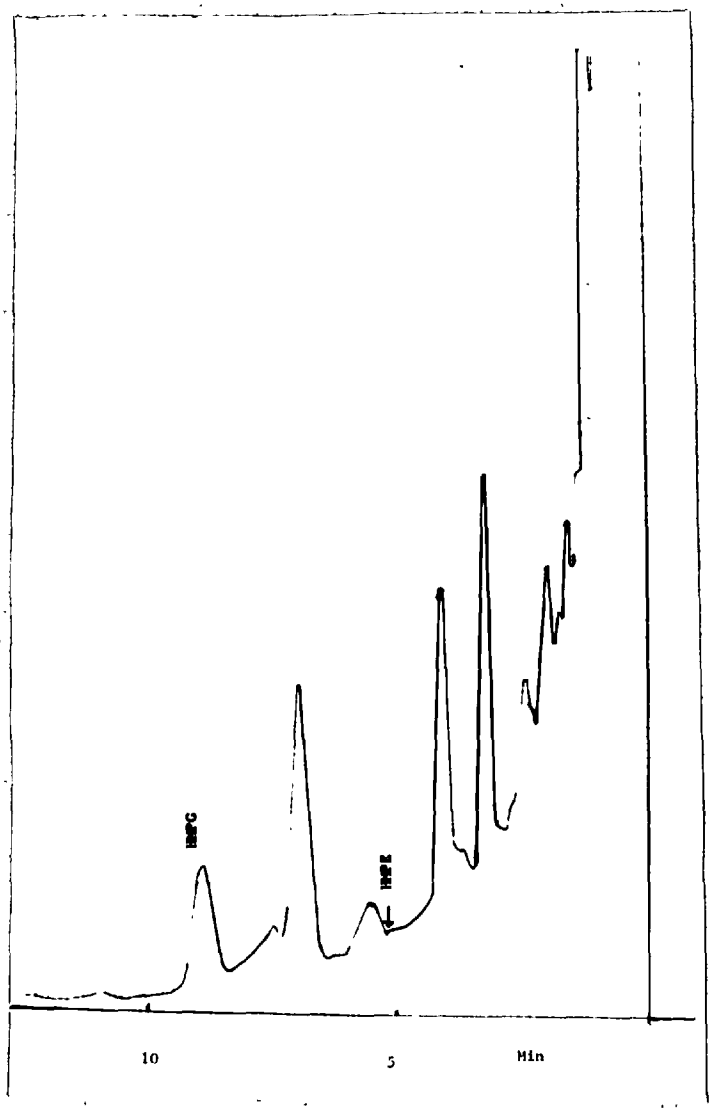


Fig. 8.4. Chromatogram of the TE/E derivative of an ethyl acetate hydrolysed urine extract from a patient with phaeochromocytoma.

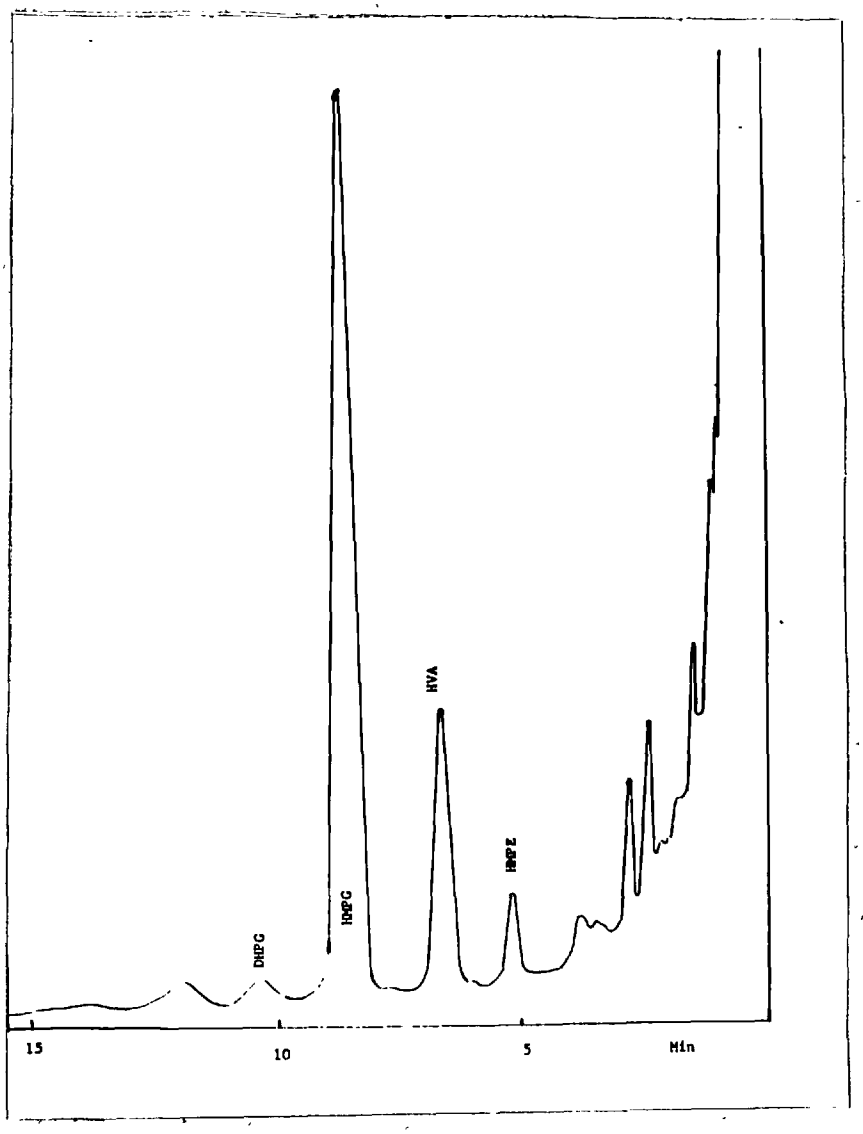


Fig. 8.5. Chromatogram of the TE/E derivative of an ethyl acetate hydrolysed urine extract from a patient with neuroblastoma.

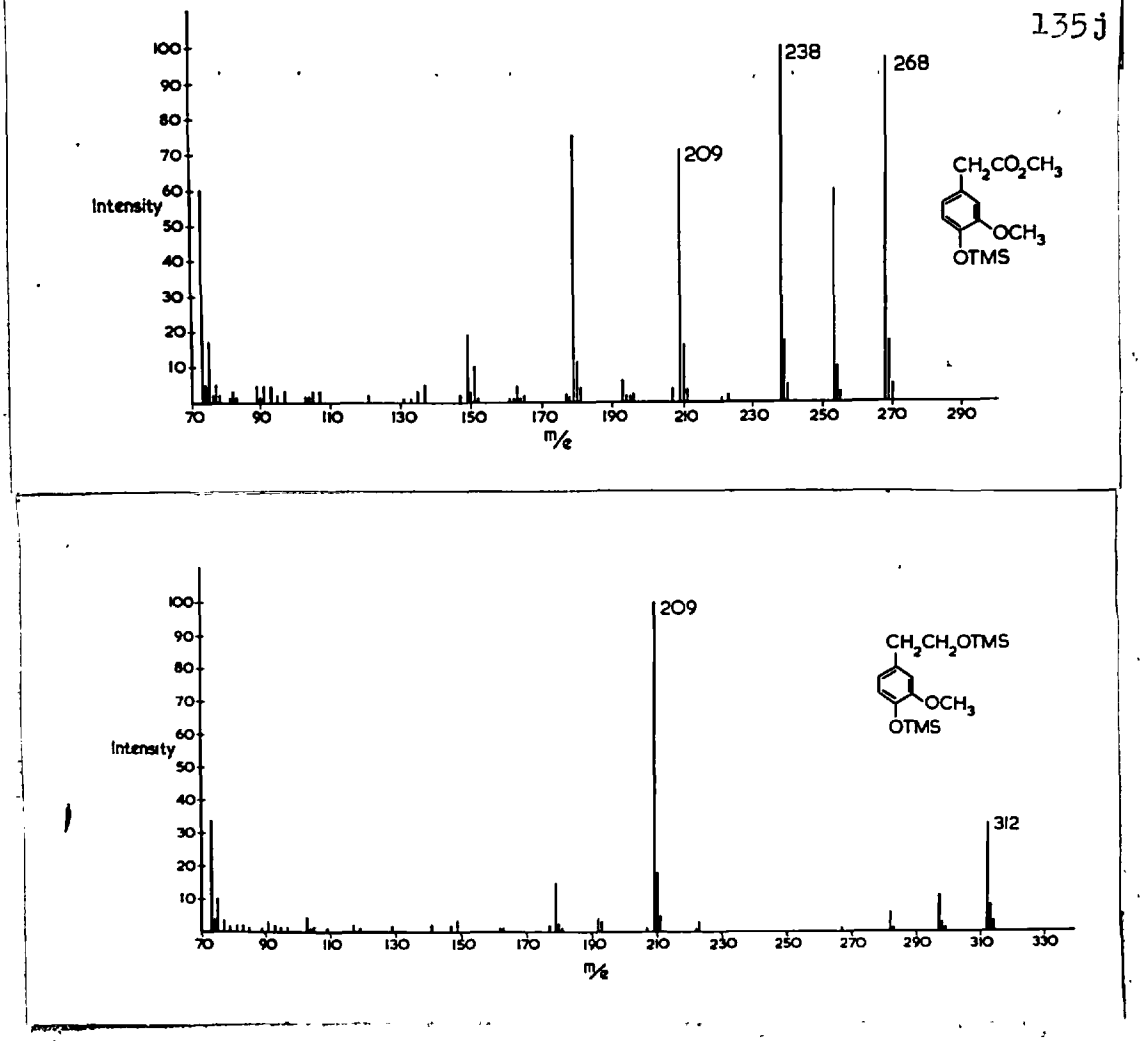


Fig. 8.6a. Mass-spectra of HVA (ME/TE derivative above) and HMPE (TE/E below) from a patient with neuroblastoma. The spectra are identical to those of the corresponding authentic compounds.

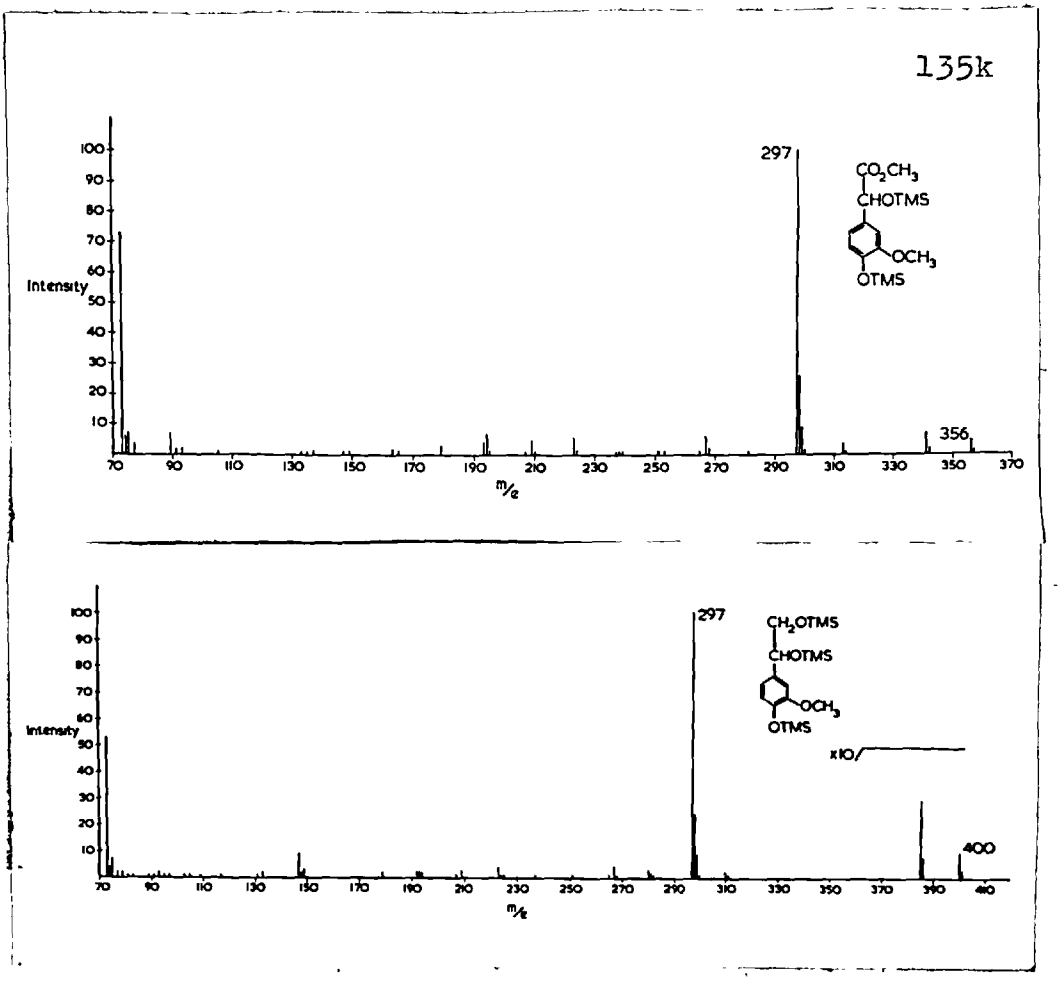


Fig. 8.6b. Mass-spectra of VMA (ME/TE derivative above) and HMPE (TE/E derivative below) from a patient with phaeochromocytoma. The spectra are identical to those of the corresponding authentic compounds.

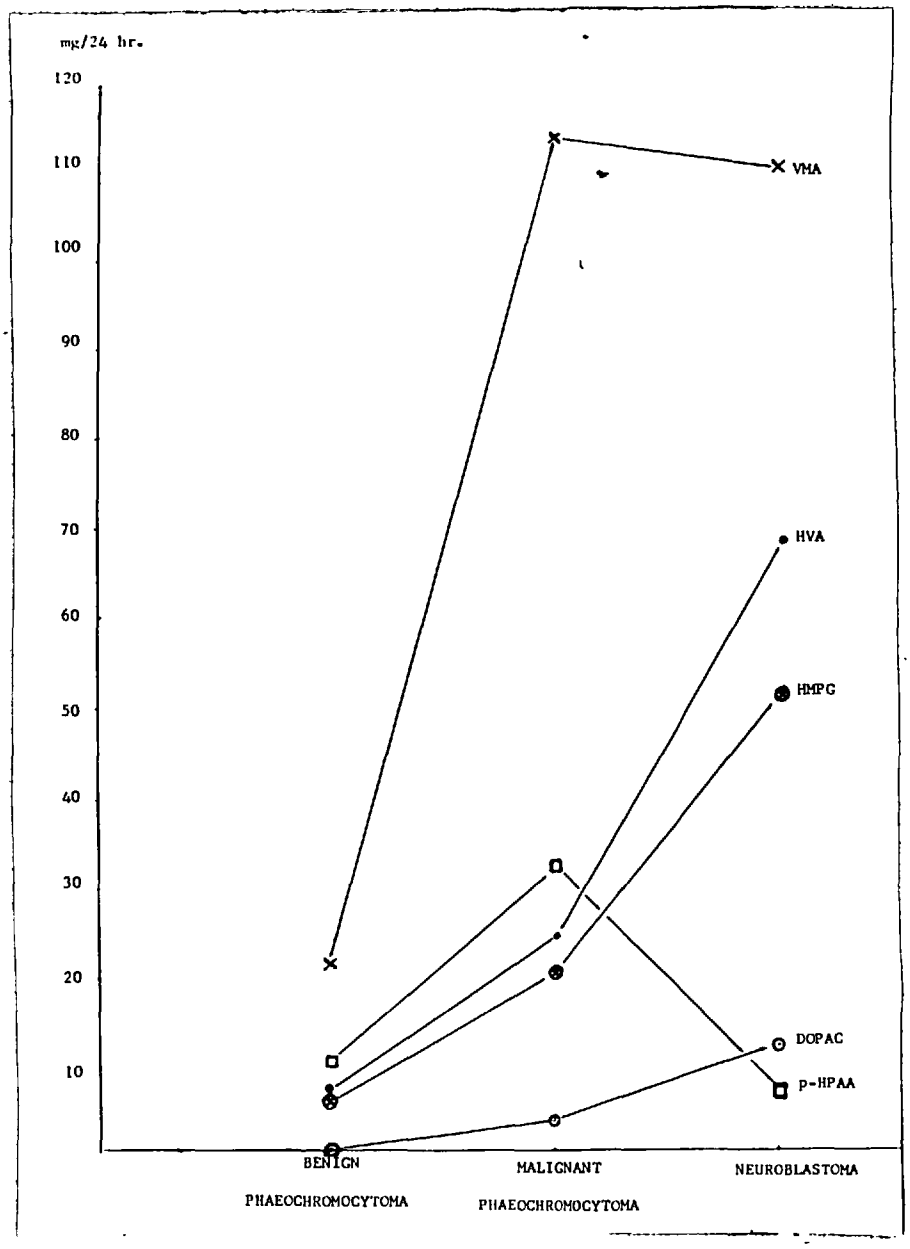


Fig. 8.7. Graphical representation of the mean excretion of five important metabolites in the three major types of catecholamine secreting tumours.

samples one from a benign phaeochromocytoma and the other from a neuroblastoma patient were also confirmed by direct scanning of the appropriate peaks (Fig. 8.6). An unknown compound which was termed substance "x" was detected in substantial quantity in specimens from six benign phaeochromocytoma patients; it was also present in one post-operative neuroblastoma sample. It possessed the same MU value as HMFE. Attempts to work out its structure by GC-MS were unsuccessful due to its weak ionic properties. Certain clues have emerged however: it is likely to be an amine without a hydroxyl group attached to the benzene ring or the side chain; it has a molecular weight of 199.

Discussion

Reports providing a broad coverage of phenolic metabolite assay in catecholamine secreting tumours are few and studies tend to be confined to the major metabolites (von Studnitz, 1966; Crout, 1966; Kaser, 1966). Phaeochromocytoma particularly tends to have been badly served (Robinson et al., 1964). Urinary metabolites in neuroblastoma have been investigated rather more thoroughly (von Studnitz, 1960; Greenberg et al., 1960; Gjessing, 1963; von Studnitz et al., 1963; Gjessing, 1964; Greer et al., 1965; Voorhess, 1968).

The excretory patterns reported here for the major metabolites of DOPA, dopamine NA and A are essentially

similar to those observed by others in phaeochromocytoma (Axelrod, Kopin and Mann, 1959; Gjessing, 1963; Crout, 1966) and neuroblastoma (Gjessing, 1963, 1968; Williams and Greer, 1962).

Excretion values of VMA and HMPG were greater than normal (see chapter 5) in all patients with secreting tumours who were studied and were considerably higher in neuroblastoma than in phaeochromocytoma (Table 8.1, 8.2, 8.3). HVA and free DOPAC excretion values, reflecting DOPA and dopamine production by tumour tissue, were normal in all but one case of benign phaeochromocytoma (see table 8.1); in contrast, output of these metabolites was elevated in all neuroblastoma patients (see table 8.3). The patients with malignant phaeochromocytoma tend to be intermediate within these two extremes, three out of four excreting moderately increased amounts of HVA and free DOPAC. These results tend to support the putative relationship between dopamine excretion and malignancy in phaeochromocytoma (Robinson et al., 1964; Robinson, 1966).

HMPE, first reported in urine from the normal human subject by Goldstein, Friedhoff, Pomerantz and Contrera, (1961) and by La Brosse and Karon (1962) in urine from patients with neuroblastoma, was found to parallel the high output of HVA (see table 8.3). The ratio of HMPE to HVA

however, was higher than that observed after oral L-DOPA administration (see chapter 10), presumably because the reductive pathway of 4-hydroxy-3-methoxyphenyl acetaldehyde is more prominent within the tumour than in normal tissues. The same pattern is observed between HMPG and VMA in neuroblastoma (Gjessing, 1963; La Brosse, 1968); La Brosse (1968) even suggested that HMPG might be the main tumour metabolite which is in part converted elsewhere in the body to VMA (La Brosse, 1969).

No metabolic importance can be attached to the excretion of vanillic acid or its corresponding alcohol, 4-hydroxy-3-methoxy benzyl alcohol (vanilyl alcohol), since variations in their excretion pattern did not appear to correlate with those of the major metabolites (table 8.4) Nevertheless, vanillic acid may arise as a minor product of DOPAC (Alton and Goodall, 1969) and of NA and A (Imaizumi, Yashida and Kita 1958). High excretion of vanillic acid has been reported in malignant phaeochromocytoma (Robinson et al., 1964). Moreover, the high levels of vanillic acid observed during the present work in one case of benign and one of malignant phaeochromocytoma and in a patient with neuroblastoma (tables 8.1, 8.2, 8.3) would seem unlikely to arise solely from dietary sources, in view of the very low values found in other patients.

The inconstant excretion of VLA and VPA (Gjessing, 1963; Smith, 1965; Coward and Smith, 1968) identified in substantial output in only one case of neuroblastoma (table 8.4) and in traces in a malignant phaeochromocytoma appears to be of little value as an aid to differential diagnosis. One suggestion to account for their presence is extreme malignancy (Gjessing, 1966); another possibility is that overproduction of vanilalanine occurs (von Studnitz, 1960) which is not as easily decarboxylated as DOPA (Gjessing, 1965) so that more is shunted down the transamination pathway.

von Studnitz et al., (1963) observed an increased urinary excretion of tyramine in six out of 21 patients with neural crest tumours. Such enhanced production of tyramine might be expected to result in a rise in the excretion of its oxidatively deaminated metabolite, P-hydroxyphenylacetic acid (P-HPAA). Although there is a wide normal range of P-HPAA excretion because of variation in dietary tyramine intake (see chapter 5), values greater than 25 mg/24 hr are likely to be considered high. It is interesting therefore to note that two out of 9 cases of benign and 3 out of 4 of malignant phaeochromocytoma and one out of 6 patients with neuroblastoma excreted more than 25 mg/24hr P-HPAA (tables 8.1 to 8.3). There was no correlation with VLA, VPA of P-HPLA output; in

only one out of four subjects (HAR, table 8.2) was there an increased P-HPLA excretion (over 1 mg/24 hr) and this was accompanied by a P-HPAA. Increased P-HPLA excretion has been observed in a number of pathological conditions including phaeochromocytoma (Robinson et al., 1964; Coward and Smith 1968_a) and neuroblastoma (Coward and Smith, 1968_a). One possible explanation for this phenomenon may lie in an uptake of tyrosine by the tumour in excess of that which can be handled by tyrosine hydroxylase. Thus larger amounts will be available for transamination, perhaps bringing about an accumulation of its product, P-HPPA, which may subsequently act as an inhibitor of its own degradative enzyme, P-HPAA oxidase.

The procedure employed here is not capable of measuring less than 2 mg dihydroxymandelic (DHMA) or 1 mg dihydroxyphenylglycol (DHPG)/24 hr, unless specifically modified. According to De Quattro et al., (1964) normal excretion values of free and conjugated DHMA are between 240 - 575 µg/24 hr and C-264 µg/24 hr respectively, while in phaeochromocytoma and neuroblastoma, marked increases were reported (400 - 9850 µg/24 hr in phaeochromocytoma and 5050 µg/24 hr in a case of neuroblastoma). Dihydroxyphenylglycol (Kopin and Axelrod, 1960) the glycol analogue of DHMA, has not previously been

detected in the urine of patients with catecholamine secreting tumours. Amounts in excess of normal (less than 500 µg/24 hr) were found in one benign (KN) and one malignant (STK) phaeochromocytoma and one adult (MAS) and two children (WHL and HA) with neuroblastoma. A high output of DHPG was accompanied by increased excretion of HMPG rather than DHMA, the excretion of which was lower than 2 mg/24 hr in all but one case. This finding suggests that in tumour tissue, the intermediate aldehyde formed by the action of MAO on NA and A is preferentially reduced to DMPG rather than oxidised to DHMA. The comparatively low incidence of increased DHMA output in catecholamine secreting tumour can be explained by its rapid conversion to HMPG by COMT in tumour tissue. It is also tempting to speculate along the same lines as La Brosse (1968) did about HMPG in tumour tissue: DHPG may be the prominent dihydroxy metabolite in tumour tissue whilst DHMA is formed after DHPG is released into the blood stream. A similar situation is encountered in the brain where radioactive NA is predominantly catabolised to HMPG (Mannarino, Kirshner and Nashold, 1963; Glowinski, Kopin and Axelrod, 1965).

CHAPTER 9

The effect of reserpine on the excretion of catecholamine metabolites in man

Reserpine, an alkaloid extracted from rauwolfia serpentina and related species (Muller, Schlittler and Bein, 1952) exhibits a multiplicity of pharmacological actions (Goodman and Gilman, 1965; Carlsson, 1966) and at least some of them may be explained in terms of the depleting effect of the drug on tissue stores of the biologically active monoamines, 5HT (Pletscher, Shore and Brodie, 1955; Shore, Silver and Brodie, 1955) and the catecholamines (Carlsson and Hillarp, 1956; Holzbauer and Vogt, 1956; Bertler, Carlsson and Rosengren, 1956; Burn and Rand, 1957; Brodie, Olin, Kuntzman and Shore, 1957; Carlsson, Rosengren, Bertler and Nilsson, 1957; Muscholl and Vogt, 1958; Bertler, 1961; Higuchi, 1962). The drug probably exerts its effect directly rather than through an intermediate, since none of the known metabolites are as potent in their action as reserpine itself (Carlsson, 1966). It is almost completely metabolised within a few hours of its administration (Numerof, Gordon and Kelly, 1955) apart from a very small fraction detectable in the brain (Plummer, Sheppard and Schulert, 1957; Sheppard, Tsien, Plummer, Peets, Giletti and Schulert, 1958; Maggiolo and Haley, 1964; Mueller and Shideman, 1968) and other organs (Alpers

and Shore, 1969) long after the drug had disappeared from the circulation.

Whilst the time course of depletion of monoamines by reserpine is relatively short, it depends on anatomical site, drug dosage and route of administration, and varies considerably in different animal species (Carlsson, 1966). The restoration of tissue monoamines to normal values, on the other hand, usually requires periods ranging from one (Shore and Brodie, 1957) to several weeks (Carlsson, Rosengren, Bertler and Nilsson, 1957_a; Haggendal and Lindqvist, 1964).

The mechanism by which reserpine causes amine depletion is not clearly understood, although many hypothesis have been proposed (Costa, Boullin, Hammer, Vogel and Brodie, 1966). In vitro experiments on the immediate effects of the drug (Brodie, Tomich, Kuntzman and Shore, 1957; Hughes, Shore and Brodie, 1958; Hughes and Brodie, 1959) suggest that reserpine acts by blocking an active pumping mechanism responsible for maintaining the high levels of monoamines in storage granules against a concentration gradient (Hughes and Brodie, 1959; Costa et al, 1966). This effect is probably mediated via inhibition of an ATP-Mg dependent mechanism (Kirshner, 1962, 1962_a; Carlsson, Hillarp and Waldeck, 1962, 1963). This mechanism envisages the incorporation of amines, bound with ATP, into a large pool within the granule from which they

are not easily released. Another mechanism, however, not affected by reserpine may be available whereby the amines are taken up into a small but important pool, loosely bound, and hence easily released. Long term, reserpine may exert an effect by interfering with amine synthesis and metabolism. Thus conversion of dopamine to noradrenaline is inhibited (Kirshner, 1962; Rutledge and Weiner, 1967) perhaps by a small residue of drug remaining in the brain and other organs long after the bulk of the drug has been metabolised. This hypothesis was recently supported by the findings of Kopin and Weise (1968); after chronic administration of reserpine to man and rat, there was a marked increase in HVA output whilst that of HMPG was significantly reduced. The total amount of HVA plus HMPG excreted however, was not greatly different from control values, suggesting that tyrosine hydroxylase proceeds at a normal rate but that dopamine conversion to noradrenaline is diminished. Other factors which may contribute to "long-term effect" of the drug include enhenced MAO activity (Izumi, Oka, Yashida and Imaizumi, 1967, 1969; Youdim and Sandler, 1968), inhibition of aldehyde dehydrogenase (Youdim and Sandler, 1968) and increased membrane permeability (Izumi et al., 1967, 1969).

It is now well established that degradation of monoamines by MAO essentially takes place intracellularly. For example

in in vitro experiments with platelets, unchanged 5HT released by reserpine could only be achieved by inhibiting MAO (Carlsson, Shore and Brodie, 1957). Similar findings have directly (Kopin and Gordon, 1962, 1963) and indirectly (Anden and Werdinius, 1963, 1964) been demonstrated for catecholamines. A number of metabolites arising from the degradation of monoamines after release by reserpine have been studied including VMA (McDonald and Weise, 1962; Anton and Greer, 1966), HMPG (Kopin and Weise, 1968; Sandler and Youdim, 1968), HVA (Williams, 1962; Kopin and Weise, 1968) and 5HIAA (Shore, Silver and Brodie, 1955; Erspamer, 1956; Fischer and Lecomte, 1956; Erspamer and Ciceri, 1957; Sano, Kakimoto, Okamoto, Nakajima and Kudo, 1957; Todrick, Dick and Tait, 1958; Valcourt, 1959). Recently reserpine has been found to possess a further effect similar to that of ingested ethanol (Smith, Gitlow, Gall, Martis and Mendlowitz, 1960; Davis, Brown, Huff and Cashaw, 1967_a) on the metabolism of adrenaline and noradrenaline. It causes a shift from a predominently oxidative to a reductive route of further degradation of the intermediate aldehyde (Sandler and Youdim, 1968) so that there is an increase alcohol production at the expense of the acid. To examine the effect of reserpine on catecholamine metabolism as a whole and to determine whether this applies to aldehyde intermediates of other

endogenous or exogenous monoamines other than A and NA a study was carried out on urine samples from a group of volunteers given reserpine intravenously and the urinary metabolites were compared with control samples employing GLC assay techniques.

Subjects, material and methods

Six healthy male volunteers, aged 24-26 years, were given 1.25 mg reserpine intravenously. Urine samples were collected after 1, 3, 6, 9, 12 and 24 hr following the drug, put into plastic containers, preserved with 2-5 ml 6 N HCl and stored at 4°C until analysed. Similar urine collections were made after placebo administration one month before administering reserpine.

GLC analysis was carried out on a 7 ft. 10% SE52 column. 10 ml urine portions from placebo samples and 5 ml from reserpine samples were employed.

For the determination of acidic metabolites (VMA, HVA, p-HPAA and vanillic acid) urine specimens were extracted twice with ethyl acetate at pH2 and ME/TE derivatives were prepared as described. 0.1 mg VMA and 0.05 mg HVA were used as internal standards for each batch of 6 urines. Phenolic acids other than VMA and HVA were quantified by making use of their relative response values with respect to HVA (see

table 3.3).

Phenolic alcohols were determined in urine aliquots by preliminary hydrolysis, extracting twice at pH8 with ethyl acetate and preparing TE/E derivatives. 0.1 mg HMPG was employed as internal standard forevery batch of 6 urines, and used to quantitate other alcoholic metabolites after correcting with an appropriate factor derived from their relative response to HMPG (table 3.3).

The identity of alcoholic metabolites (HMPG, HMPE, P-HPE and HMBA) were checked in randomly selected urines by studying their MU values when TE/E and ME/TE derivatives were prepared. In addition, the structures of HMPE and HMPG were studied and confirmed by direct GC-MS as described in chapter 8.

Results

Excretion rates of the different metabolites after placebo and reserpine administration are summarised in tables 9.1 to 9.8 and shown graphically for the means of HVA, HMPG, HMPE, P-HPE and HMBA in figures 9.1 to 9.3. Mean 24 hr excretion values of acidic and alcoholic metabolites in each group are given in tables 9.9 and 9.10.

VMA, vanillic and P-HPAA output showed no significant difference between groups. ~~Although~~ In the two groups, the mean excretion rate of HVA after reserpine, starting from specimens collected after the 6th hr post injection was significantly

TABLE 9.1

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 hr	60	246	113	224	137	425	200	± 71
3 "	-	203	125	131	83	212	151	± 32
6 "	175	79	192	257	-	196	180	± 40
9 "	253	155	193	182	71	-	171	± 44
12"	263	147	109	204	76	231	172	± 36
24"	292	204	100	128	65	172	160	± 37

PLACEBO

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 hr	225	156	250	225	159	96	185	± 25
3 "	154	150	165	210	45	169	149	± 27
6 "	177	135	127	254	59	285	173	± 37
9 "	258	300	210	194	117	329	235	± 34
12"	402	269	179	417	110	312	282	± 50
24"	288	235	115	379	60	177	209	± 52

RESERPINE

Rate of urinary excretion of HVA (g/hr) after i.v. administration of placebo and reserpine.

N.B. p less than 0.01 after the 9th.hr.

TABLE 9.2

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 hr	112	545	633	633	479	391	256	± 70
3 "	-	318	240	240	206	240	223	± 50
6 "	114	146	230	230	-	352	198	± 58
9 "	363	350	570	240	290	-	283	± 36
12"	387	131	354	145	380	486	280	± 68
24"	354	263	205	205	254	245	251	± 31

PLACEBO

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 hr	237	289	250	187	889	54	214	± 45
3 "	202	373	308	219	260	227	263	± 32
6 "	167	304	317	387	146	354	279	± 46
9 "	219	591	335	225	364	304	339	± 73
12"	300	152	122	364	633	375	324	± 98
24"	171	288	115	158	117	177	171	± 33

RESERPINE

Rate of urinary excretion of VMA (μg/hr) after i.v. administration of placebo and reserpine.

N.B. p values greater than 0.2 for all.

TABLE 9.3

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 Hr	10	10	10	10	137	60	39.5	± 020.5
3 "	10	10	10	10	83	43	27.6	± 11.8
6 "	10	153	38	46	-	39	47.6	± 23.1
9 "	10	249	380	187	127	-	102	± 59.7
12"	10	333	100	97	76	117	122	± 52.2
24"	5	83	212	4	65	8	63	± 32.6

PLACEBO

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 Hr	24	44	26	36	705	15	136	± 112.2
3 "	138	23	14	15	152	13	59.2	± 22.9
6 "	104	45	16	8	146	36	59.1	± 21.9
9 "	10	63	21	15	414	67	98.3	± 65.2
12"	4	350	36	115	387	85	162.8	± 61.8
24"	4	59	53	14	71	20	36.8	± 10.8

RESERPINE

Rate of urinary excretion of vanillic acid (μg/hr) after i.v. administration of placebo and reserpine.

N.B. p values are greater than 0.2 in each case.

TABLE 9.4.

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 hr	-	884	226	307	988	510	583	± 145
3 "	-	584	173	183	640	386	559	± 196
6 "	1390	2316	527	598	510	500	974	± 293
9 "	2332	1343	234	508	546	-	993	± 340
12"	2141	969	133	255	623	649	795	± 334
24"	1731	770	190	231	455	616	473	± 249

PLACEBO

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 hr	720	1137	132	722	-	336	609	± 192
3 "	219	2609	152	562	-	180	744	± 466
6 "	720	459	149	396	-	333	411	± 110
9 "	843	1852	245	415	-	225	716	± 312
12"	294	1134	284	1134	-	370	643	± 163
24"	345	753	257	513	-	237	421	± 99

RESERPINE

Rate of urinary excretion of p-hydroxyphenylacetic acid (/'g/hr) after i.v. administration of placebo and reserpine.

N.B. p values greater than 0.2 for all.

TABLE 9.5.

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 hr	85	102	315	172	109	238	170	± 37
3 "	-	55	210	162	103	238	154	± 35
6 "	79	83	226	124	133	242	148	± 26
9 "	40	93	230	156	129	-	130	± 36
12"	88	83	100	105	137	157	112	± 12
24"	106	130	47	193	78	86	106	± 24

PLACEBO

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 hr	275	354	233	102	76	245	214	± 41
3 "	290	185	240	233	225	175	225	± 19
6 "	651	180	400	231	302	242	334	± 76
9 "	454	252	504	234	209	154	301	± 48
12"	412	314	425	271	185	270	313	± 37
24"	275	175	404	232	73	217	229	± 53

RESERPINE

Rate of urinary excretion of HMFG (/tg/hr) after i.v. administration of placebo and reserpine.

N.B. p values less than 0.001 in each case.

TABLE 9.6

Time	R.C.	M.Y.	M.S.	C.H.	D.H.	P.C.	Mean	Standard Error
1 hr	<10	<10	<10	<10	<10	<10		
3 "	"	"	"	"	"	"		
6 "	"	"	"	"	"	"		
9"	"	"	"	"	"	"		
12"	"	"	"	"	"	"		
24"	"	"	"	"	"	"		

PLACEBO

Time	R.C.	M.Y.	M.S.	C.H.	D.H.	P.C.	Mean	Standard Error
1 hr	18	55	10	<10	30	<10	22	± 7
3 "	16	17	"	85	27	"	28	± 12
6 "	350	<10	"	27	183	"	98	± 55
9 "	350	13	"	<10	264	"	110	± 55
12"	253	<10	"	110	10	20	59	± 39
24"	122	18	"	24	10	72	43	± 16

RESERPINE

Rate of urinary excretion of p-hydroxyphenyl ethanol (p-HPE) (/tg/hr) after i.v. administration of placebo and reserpine.

N.B.pvalues less than 0.001 in each case.

TABLE 9.7.

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 hr	10	10	10	10	10	10	10	-
3 "	"	"	"	"	"	"	"	-
6 "	"	"	"	"	"	"	"	-
9 "	"	"	"	"	"	"	"	-
12"	"	"	"	"	"	"	"	-
24"	"	"	"	"	"	"	"	-

PLACEBO

Time	R.C.	M.Y.	M.S.	C.R.	D.R.	P.C.	Mean	Standard Error
1 hr	36	10	85	43	402	583	193	
3 "	328	"	212	29	198	349	187	
6 "	207	"	103	13	296	388	170	
9 "	403	"	273	27	72	240	171	
12"	72	"	19	46	384	404	156	
24"	88	"	10	12	27	342	82	

RESERPINE

Rate of urinary excretion of HMPE (μg/hr) after i.v. administration of placebo and reserpine.

N.B. p values less than 0.001 in each case.

TABLE 9.9

Subject	HVA		VMA		VANILLIC		p-hydroxyphenyl acetic acid.	
	Res.	Plac.	Res.	Plac.	Res.	Plac.	Res.	Plac.
K.S.	6.495	5.635	4.747	6.944	0.703	100	10.868	38.367
M.Y.	5.300	4.244	7.627	5.908	2.168	4.064	25.717	25.175
M.S.	3.513	3.044	4.569	7.040	0.973	3.090	5.557	5.536
C.R.	7.700	3.955	5.453	5.423	0.649	1.058	13.819	7.514
D.R.	1.828	1.522	6.224	5.942	4.702	1.690	-	11.390
P.C.	5.376	4.199	5.731	6.329	0.839	0.714	6.329	12.121

Excretion of phenolic acids (mg/24hr) after i.v. administration of placebo and reserpine.

TABLE 9.10

Subject	HMPR Res.	HMPR Plac.	HMPG Res.	HMPG Plac.	HMBA Res.	HMBA Plac.	D-HPE Res.	D-HPE Plac.
M.S.	3.799	<0.1	8.030	1.977	2.220	100µg	4.376	0.340
M.Y.	0.1	"	5.064	2.560	0.614	100µg	0.382	0.274
M.S.	1.900	"	9.549	2.966	6.221	0.684	0.020	0.020
C.R.	0.509	"	5.554	3.960	<0.1	0.392µg	0.597	0.020
D.R.	3.377	"	3.485	2.445	1.746	<0.1	1.485	0.179
P.C.	7.800	"	5.194	2.541	3.674	<0.1	0.997	0.020

Excretion of phenolic alcohols (mg/24hr) after i.v. administration of placebo and reserpine.

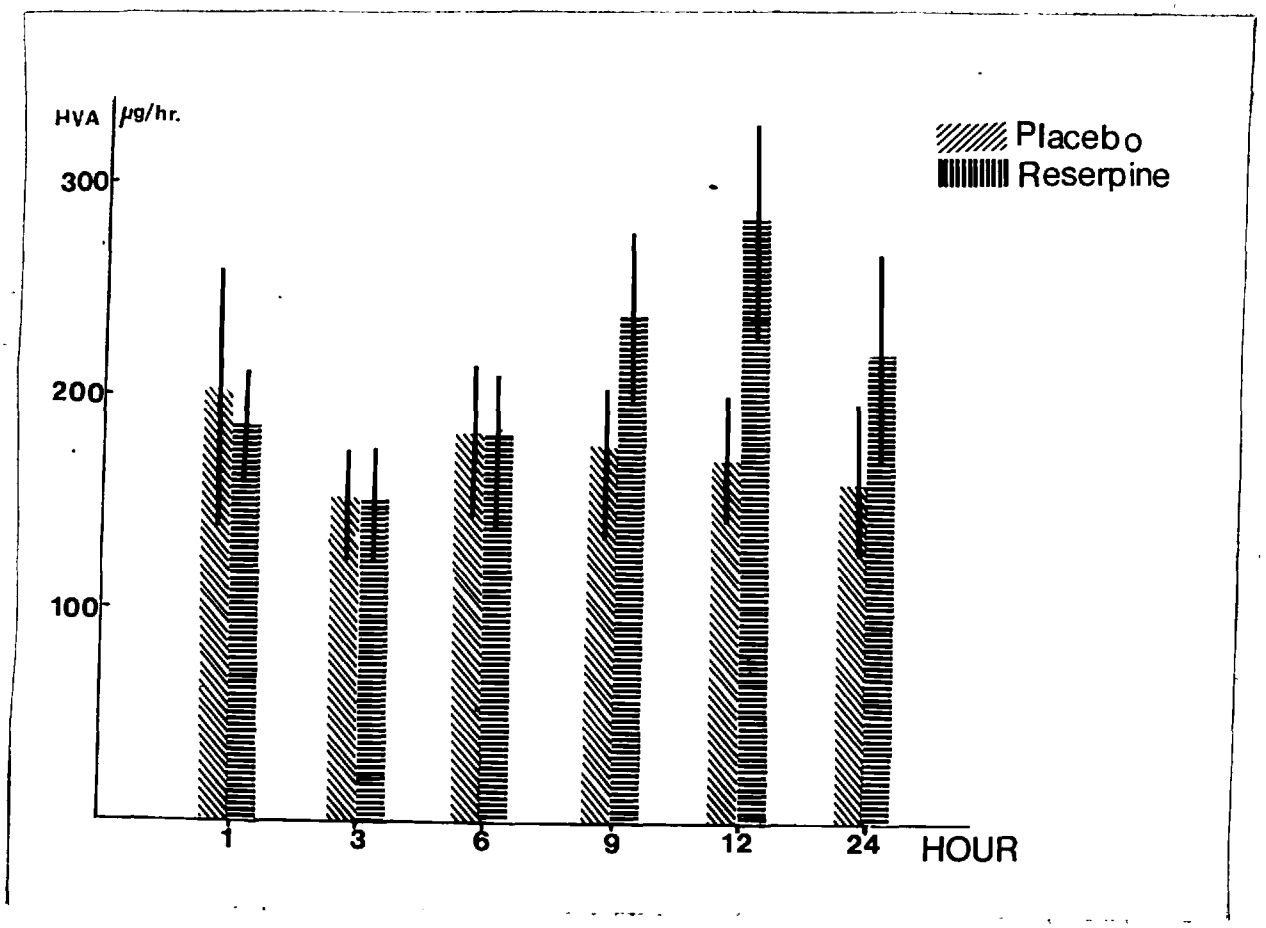


Fig. 8.1. Comparison between the mean rate of HVA excretion in six volunteers before (placebo) and after i.v. reserpine (1.25 mg), $p<0.01$ after the 9th hour.

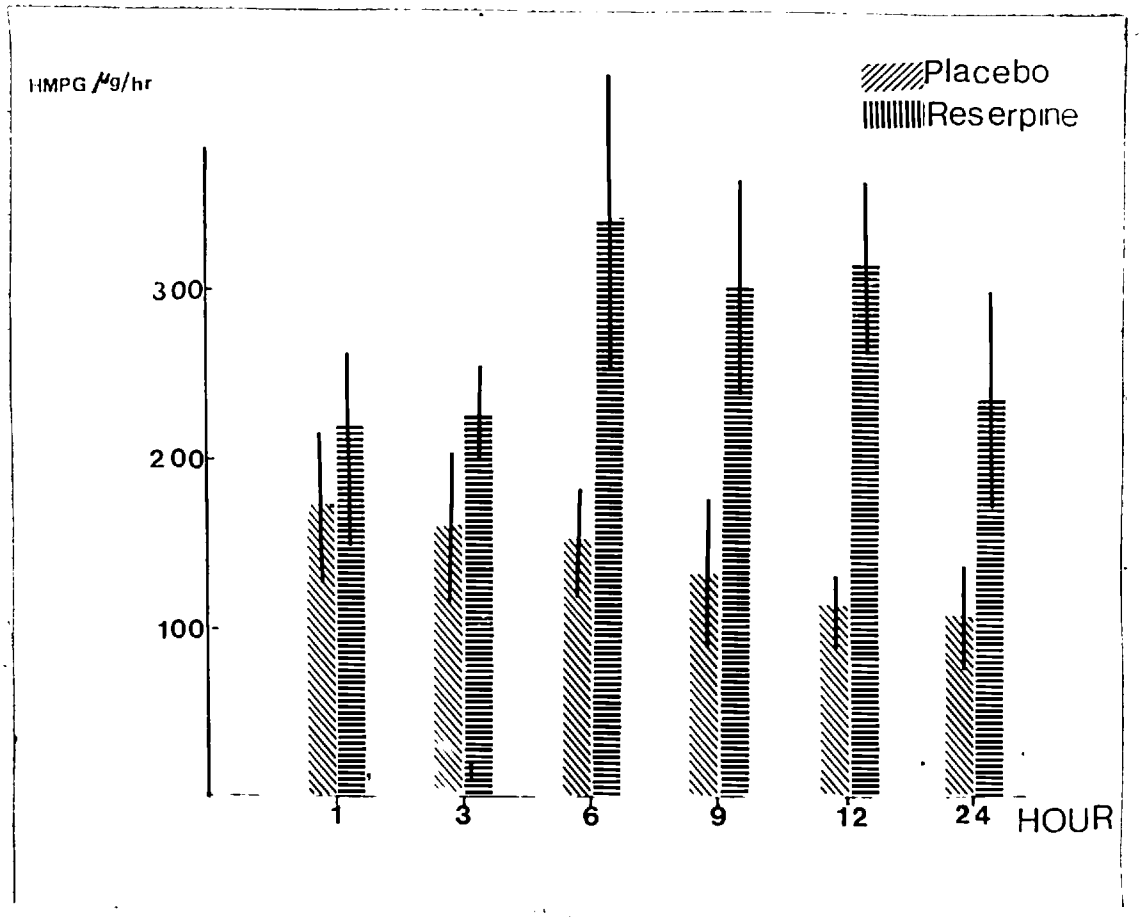


Fig. 8.2. Comparison between the mean rate of HMPG excretion in six volunteers before (placebo) and after i.v. reserpine (1.25 mg), $p < 0.01$ in each case.

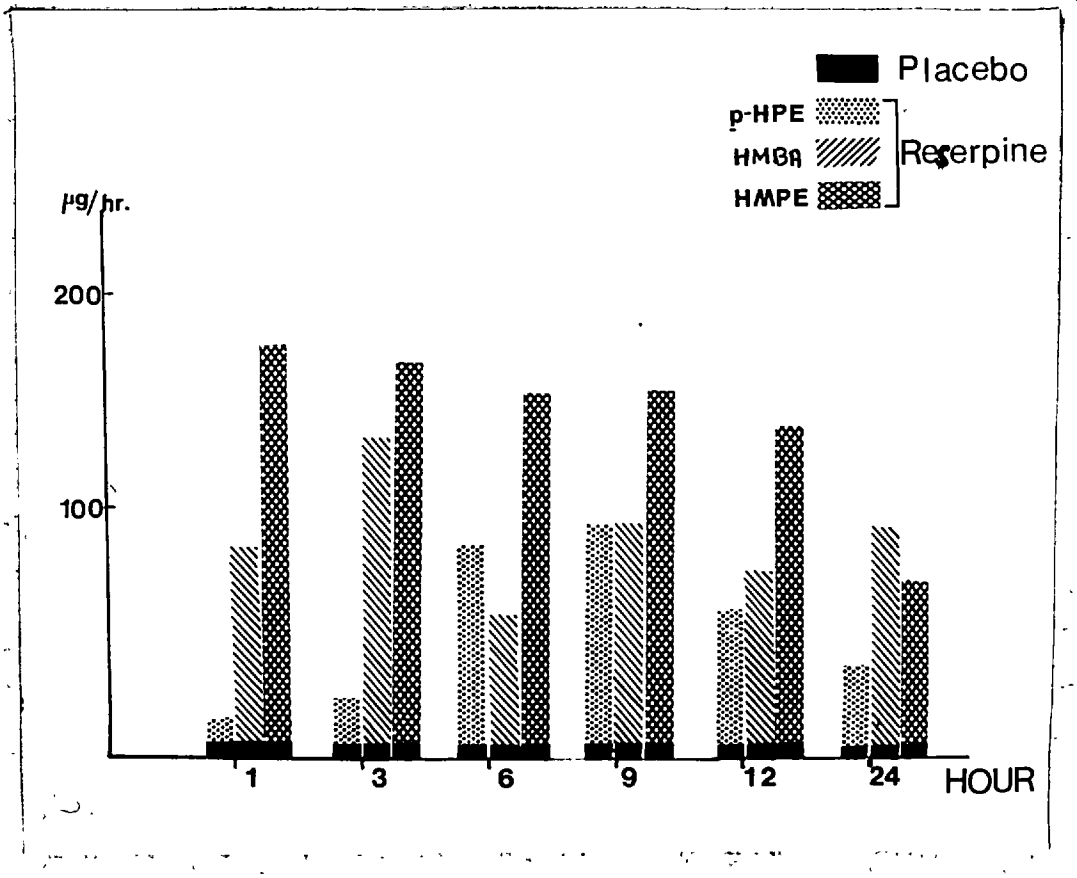


Fig. 6.3. Comparison between the mean excretion of p-HPE, HMBA, HMPE before (placebo, solid columns) and after i.v. reserpine (1.25 mg). $p < 0.001$ in each case.

higher ($p < 0.001$) than the corresponding value during placebo treatment. (figure 9.1). Output of alcoholic metabolites was significantly increased after reserpine compared with placebo dosage (see tables 9.5 to 9.8 and figures 9.2 and 9.3).

The excretion of P-HPAA and vanillic acid in the two groups was variable, reflecting their dietary origin (Tables 9.3 and 9.4) and similar variation was observed for their corresponding alcohols, P-HPE and HMBA (Tables 9.6 and 9.8).

The ratio of acidic to its corresponding alcoholic metabolite showed a significant decrease ($p < 0.01$) after reserpine, when compared with placebo. Thus a shift in the metabolism of the aldehyde intermediate of oxidative deaminated amines from a predominantly oxidative to a reductive pathway was evident.

Discussion

The increased excretion of alcoholic metabolites of a number of monoamines together with the shift from an oxidative to a reductive pathway are in accordance with data of Sandler and Youdim (1968) on the excretion of HMPG after reserpine. The findings are similar to those noted following ethanol ingestion on the metabolism of noradrenaline (Smith et al., 1960; Davis et al., 1967a), adrenaline (Davis, Cashaw, Huff, Brown and Nicholas, 1967_c) and 5HT (Davis, Brown, Huff and Cashaw, 1967b). The absence of significant

change in VMA excretion after reserpine compared with placebo administration conflicts, however, with certain reports that reserpine increases VMA output (McDonald and Weise, 1962; Anton and Greer, 1966; Sandler and Youdim, 1968). The reason for this discrepency is not at present understood. Apart from HVA (Kopin and Weise, 1968) no comparable study of the excretion of other phenolic acids has previously been reported; reserpine appears to cause very little change in their excretion pattern. Although Sandler and Youdim (1968) attributed the metabolic shift observed after reserpine to a possible inhibition of aldehyde dehydrogenase, other alternative explanations which deserve consideration is that reserpine interferes with the conversion of phenolic alcohols to their corresponding acid (as discussed in chapter 8) a view based on preliminary reports by La Brosse (1968, 1969). It is possible, therefore, that the observed increase in urinary excretion of alcoholic metabolites noted after reserpine administration may reflect the actual tissue metabolism of released amine. Studies in a number of isolated tissue indicate that the HMPG at least may be the predominent primary metabolite (Breese, Chase, and Kopin, 1969; Shimizu and La Brosse, 1969). It is also possible that a sudden rise in the production of alcoholic metabolites overwhelms the mechanism responsible for such conversion. A third possibility

worth considering is facilitated conjucation, whereby the alcohol is prevented from being converted to the acid. In any instance aldehyde dehydrogenase need not be inhibited.

Inspection of tables 9.1 to 9.8 reveals a tendency to an inverse relationship between the excretion rate of alcoholic and acidic metabolites after reserpine dosage. This is seen particularly clearly in the case of HVA and HMPE; during the first 6 hr after reserpine (figure 9.1), the excretion rate of HMPE was dramatically increased whilst that of HVA remained unchanged compared with controls. From the 6th hr. onwards, a gradual fall in HMPE excretion was accompanied by a corresponding increase in HVA output.

The significantly increased excretion of HVA after reserpine is compatible with some form of dopamine release from its binding sites whilst the effect on acid; alcohol ratio is becoming attenuated. Thus the two actions of reserpine may well be independent of each other.

5HIAA and 5HTOH were not quantified, as it had not been realised at the time of collection that they were relatively labile in diluted HCl (see chapter 3, preservation of urine samples). Because of the apparent similarity between the actions of reserpine and ethanol on HMPG excretion (Davis et al., 1967a and c; Sandler and Youdim, 1968) and

as 5HTOH output increases after ethanol ingestion (see chapter 7), it is likely that its excretion also increases after reserpine administration in a similar manner to other alcoholic metabolites.

The increased output of HMPG and HMPE is likely to be accounted for by the respective release of noradrenaline and dopamine (Carlsson, 1966); if this conclusion be accepted, it is difficult to find an explanation for the increased excretion of p-HPE and HMBA without postulating the existence of some storage mechanism for their precursor amines. Claims have in fact been made for the presence of tyramine in certain areas of the brain (Spector, Melmon, Lovenberg and Sjoerdsma, 1962) although this has never been convincingly confirmed. The related amine, octopamine has recently been detected in tissue (Malinoff and Axelrod, 1969) although no consistent increase in the output of its corresponding alcohol p-HPG was detected during the course of the present work.

CHAPTER 10

L-DOPA metabolism in Parkinsonism

Parkinson's disease, first described by James Parkinson (1817), is a clinical disorder characterised by dysfunction of the basal ganglia. Although anatomical lesions tend to be scanty, depegmentation of the substantia nigra has been noted (Alvord, 1958) and there is sometimes evidence of neural degeneration (Harriman, 1966). Clinically, the disease presents a disturbance of motor function characterised by slowing and enfeeblement of emotional and voluntary movement, muscular rigidity and tremor (Lord, 1962). Several subdivisions of the disease have been defined. The post-encephalitic variety tend to include the more severely affected patients (Harriman, 1966). This group appears to consist solely of survivors of the 1920-1924 pandemic of encephalitis lethergica who developed the disease at varying intervals after the infection. Apart from a poorly defined group associated with arteriosclerosis and a drug-induced group, the term "parkinson's disease" (paralysisagitans) is usually used for perhaps the most common group of affected subjects whose illness is idiopathic. During the past decade, it has gradually become obvious that parkinson's disease is

associated with a disturbance of dopamine metabolism in the basal ganglia (Hornykiewiez, 1966). Dopamine is now believed to possess a function of its own within the brain, probably acting as a neurotransmitter (Carlsson, Lindqvist, Magnusson and Waldeck, 1958; Holtz, 1959; Barbeau, 1961; Sourkes, 1961; Greenberg and Lind, 1961; Horwitz, Fox and Goldberg, 1962; Hornykiewicz, 1962; Dagirmanjian, Laverty, Mantegazzim, Sharman and Vogt, 1963). The characteristic distribution of dopamine within the brain (Bertler and Rosengren, 1959_a; Carlsson, 1959) especially the basal ganglia (Carlsson, Lindqvist, Magnusson and Waldeck, 1958; Sano, Gamo, Kakimoto, Tanguchi, Takesada and Nishinuma, 1959; Hornykiewicz, 1960; Bertler, 1961; Ehringer and Hornykiewicz, 1960; Hornykiewicz, 1963), together with its pharmacological properties (Gurd, 1937; Curtis and Davis, 1961; Bloom, Costa and Salmoiraghi, 1965; McLennan and York, 1967) strongly support this hypothesis. A key observation was the demonstration of decreased brain dopamine levels (Ehringer and Hornykiewicz, 1960; Bernheimer, Birkmayer, Hornykiewicz, Jellinger and Seitelberger, 1965) particularly in the basal ganglia. To complement this finding, a diminished urinary excretion of dopamine (Barbeau, 1960; Barbeau, Murphy and Sourkes, 1961) has been demonstrated. The cerebrospinal fluid level of HVA is also reduced (Bernheimer, Birkmayer and Hornykiewicz, 1966; Johansson and Roos, 1967; Rinne and

Sonninen, 1968). It appears to be relevant to this argument that reserpine, which depletes brain stores of dopamine (Carlsson et al., 1958), as well as other amines, can produce a parkinsonian syndromes. All these factors firmly point to the involvement of dopamine in parkinsonism. It has been suggested that Parkinson's syndrome might result from an imbalance between colinergic-exitatory and dopaminergic inhibitory control in the direction of cholinergic dominance (McGeer, Boulding, Gibson, Foulkes, 1961; Barbeau, 1962; Arvidsson, Roos and Steg, 1966). It is possible that this imbalance is induced by a variety of causes which directly or indirectly affect the metabolism and storage of dopamine in the basal ganglia (Hornykiewicz, 1966).

As a logical extention of the demonstration of dopamine deficiency in this disease various attempts have been made to correct it by administering DOPA. During the early history of administration of this drug (Birkmayer and Hornykiewicz, 1961) theraputic benefit could not be demonstrated convincingly. While some improvement had been reported (Birkmayer and Hornykiewicz, 1962; Barbeau, 1962; Gestenbrand and Patiesby, 1962; Friedhoff, Hekimian, Alpert and Tobach, 1963; Hirschmann and Mayer, 1964; Umbach and Baumann, 1964), others could not confirm it (McGeer, Boulding, Gibson and Foulkes, 1961; Greer and Williams, 1963; Rinne and Sonninen, 1968). This

discrepancy was probably due to the use of the D-L-isomer and to the administration of low dosage for periods insufficient for the drug to take its effect. Recently, trials with large oral doses of D-L-DOPA (Cotzias, van Woert and Schiffer, 1967) and L-DOPA (Cotzias, Papavasiliou, Gellene, Aronson and Mena, 1969) for sufficiently long periods have shown very encouraging results and have aroused great interest in the therapeutic role of the drug (Calne, Stern, Laurence, Sharkey and Armitage, 1969; Godwin-Austen, Tomlinson, Frears and Kok, 1969; O'Reilly, 1969; Calne, Spiers, Stern, Laurence and Armitage, 1969c; Yahr, Duvoisin, Schear, Burrett and Hoehm, 1969).

The decreased urinary excretion of dopamine (Barbeau, 1960; Barbeau et al., 1961) and its major metabolite (Bernheimer and Hornykiewicz, 1964; Bernheimer et al., 1965) has not been confirmed at other centres, (Greer and Williams, 1963; Westlake and Tew, 1966; Rinne, Laitinen and Sonninen, 1967; Rinne and Sonninen, 1968) so that the case for a generalised defect of amine metabolism is as yet unproven. Whilst decreased brain levels of noradrenaline (Ehringer and Hornykiewicz, 1960; Bernheimer, Birkmayer and Hornykiewicz, 1963) and 5HT (Bernheimer, Birkmayer and Hornykiewicz, 1961) have been noted in the brain of parkinsonian patients, these changes are similarly not reflected by any alteration in the

output of their respective metabolites, VMA (Rinne et al., 1967) or 5HIAA (Rinne and Sonninen, 1968).

Although the clinical effect of L-DOPA has now been studied on a number of occasions, biochemical data are scanty. In this chapter therefore the excretion of phenolic metabolites is assessed in a group of affected subjects before, during and after the administration of oral L-DOPA. These studies were performed with two aims in mind: to clarify the controversy as to whether any alteration existed in excretion of VMA, HVA and perhaps other metabolites in untreated parkinsonian patients; to study the catecholamine metabolite excretion pattern after L-DOPA, and to see if any possible correlation could be detected between the therapeutic effect of the drug and their urinary output.

Patients and methods

Urine specimens (24 hr), preserved with 6N HCl (25 ml), were obtained from forty-long-term hospital patients (18 men and 22 women) with postencephalitic Parkinsonism undergoing a "double-blind" therapeutic trial of oral L-DOPA (dose range 0.5-2.5 g/day; mean 1.3g for 47 days). Full details of this trial are given by Calne et al., (1969).

Urine collections were made at four stages in the trial: (1) either before commencing placebo or L-DOPA regimes

or while control subjects were on placebo ("pre-treatment"); (2) some days after starting oral L-DOPA ("under treatment"); (3) within 24-48 hr of stopping the L-DOPA regime ("end of treatment"); and (4) two weeks later ("follow-up").

Analysis was not carried on every 24 hr urine collection; instead analyses were carried out in two series. In the "homogeneous" series, six female subjects were followed through the four stages enumerated above. In the heterogeneous series, the "pre-treatment" group consisted of three males and eleven females; seven males and five females were "under treatment"; seven females constituted the "end of treatment" and ten females the "follow-up" groups. Some subjects were common to all groups. The full range of metabolites was not measured in every subject in the heterogeneous series; the number of subjects from whom the mean of each metabolite level was calculated is shown in parentheses in Tables 10.7.

Urine samples (24 hr) were also collected for analysis from four patients with idiopathic Parkinsonism who were on 4.25 to 4.75 g oral L-DOPA per day, almost double the maximum dose given to any of the post-encephalitic patients.

Six patients with idiopathic Parkinsonism maintained on different doses of L-DOPA (between 2 and 8g per day) were treated with neomycin (1g/day) as an intestinal antibiotic. Urine samples were collected before and during the third day

of neomycin treatment.

Procedure

Samples from patients in the "pre-treatment" or "follow-up" groups were analysed by extracting urine aliquots twice (10 ml of either unhydrolysed or hydrolysed urines) with ethyl acetate, ME/TE and TE/E derivatives were prepared for acidic and alcoholic metabolites respectively. Internal standards employed were 0.1 mg VMA, 0.05 mg HVA and 0.1 mg HMPG, and other metabolites were determined by making use of their response values relative to any of these three standards (see Table 3.3).

Analysis of specimens from patients in the "under-treatment" and "end of treatment" groups, and before and during neomycin was modified in order to assure satisfactory quantification of the large amounts of HVA and DOPAC present and prevent their interfering with the estimation of metabolites excreted in lesser concentration. HVA and DOPAC were assayed by extracting 0.5 or 1 ml of urine sample diluted to 10 ml with 0.01 HCl twice with ether and preparing the TE/E derivative. HVA and DOPA (0.5 mg) were included as internal standards for every batch of seven specimens.

To eliminate DOPAC, which interferes with measurement of VMA and other phenolic acids at the high concentrations present in patients under treatment, urine (5 ml) was

was adjusted to pH 8.4, and neutral alumina was added and vortex mixed ("Whirlimixer") for 1 min. After centrifuging and adjusting the pH of the supernatant to 2, phenolic acids were extracted with ethyl acetate and the ME/TE prepared. 0.1 mg of VMA, added at the beginning of experiment and passed through all the various stages, was employed as an internal standard for every batch of seven specimens. For phenolic alcohol assay, phenolic acids were first extracted into ether (2 x 25 ml) from urine (10 ml) at pH 1. The separated aqueous phase was heated at 60°C for a few minutes under vacuum to expel traces of solvent and then hydrolysed. Following hydrolysis, the pH was raised to 8.4, alumina added, mixed, centrifuged, and the phenolic alcohols isolated from the supernatant and analysed as before by preparing the TE/E derivative. 0.1 mg HMPG was used as internal standard for every batch of seven specimens, having been added at the beginning of the experiment.

No evidence of free HMPG could be detected in either the untreated or treated urines. This fact was established by careful examination of all chromatograms where free HMPG might have been present (those containing VMA). The similar M.U. value of HMPG (18.37) to that of VMA (18.43) implies that if there had been any free HMPG, its presence in any large quantity would have reduced the VMA retention distance

or it would have appeared as a hump on the ascending arm of the peak; neither was observed. All VMA peaks encountered had exactly the same retention time as that of standard VMA, so that the presence of any substantial amount of free HMPG could be excluded.

Statistical tests

Means and standard errors (S.E.) of means were calculated for the 24 hr urinary excretion values of each metabolite and t-tests were carried out to ascertain the significance of differences between treatment groups as well as their relationship to dose of L-DOPA. Within each group, an examination of the degree of correlation was made for the following variables: selected metabolites, urine volume, dose of L-DOPA in the previous 24 hours and total dose to date of collection of specimen under test.

Each group was regarded as a random sample from its appropriate population and no attempt was made to match patients who appeared in more than one group. It was assumed but not tested that these samples were not subject to selection. The results of statistical tests on the small homogeneous series of 6 patients analysed during all four phases of the investigation support those found in the larger but heterogeneous series. The "between group" analysis in the

heterogeneous series was based on the logarithms of the recorded data as this transformation appeared to create greater similarity of variance within each of the groups. For the analysis of the homogeneous series, the actual readings were used as their range was smaller compared with the unmatched groups. Means and S.E. of means for the measured metabolites are given in tables 10.7 and 10.8. Salient features of the statistical examination are recorded and discussed below.

Results

Individual results of all four groups in the homogeneous and heterogeneous series and of the idiopathic Parkinsonians are shown in tables 10.1 - 10.6.

The results of GLC analysis on the heterogeneous series expressed as mean 24 hr excretion values $\pm$ S.E. of means, together with observed ranges, are tabulated in Table 10.7 and grouped according to the four collection schedules. Values are given for urinary phenolic acids and alcohols and for the ratio of 4-hydroxy-3-methoxymandelic acid (VMA) to 4-hydroxy-3-methylphenylglycol (HMPG). A parallel but extended set of results from the homogeneous series is shown in Table 10.8.

The urinary excretion of metabolites in post-encephalitic Parkinsonian subjects in "pre-treatment" and

TABLE 10.1.

Name	HVA	VMA	DCPAC	p-HPAA	m-HPAA	p-HPLA	HMPG	HMPE	VMA/HMPG	VLA
WL*	1.8	4.80	2.0	7.80	4.00	1.00	1.84	0.1		0.1
HUB*	2.34	5.00	"	3.16	2.18	2.00	1.37	"		"
HLT*	2.54	5.05	"	4.75	0.86	1.00	1.86	"		"
FTS*	3.02	3.50	"	13.9	1.00	"	1.21	"		"
OSB*	3.75	5.25	"	8.20	1.10	"	1.51	"		"
TUR*	2.74	4.20	"	6.40	0.92	"	0.81	"		"
CAR*	1.60	3.20	"	3.00	1.21	"	1.32	"		"
JY	5.20	9.30	"	10.80	4.00	"	3.3	"		"
BL	2.26	4.20	"	2.98	0.85	"	0.8	"		"
BRY	3.30	1.40	"	2.80	0.86	"	3.14	"		"
CMB	3.12	7.85	"	9.10	3.75	"	3.5	"		"
HL	1.87	4.95	"	4.50	1.49	"	2.42	"		"
DV	2.20	5.70	"	6.05	1.14	"	1.42	"		"
BRY	5.95	4.65	"	9.20	1.00	"	-	"		"
MED	3.70	3.90	"	4.80	1.00	"	-	"		"
EV	2.88	1.36	"	7.75	4.20	"	2.26	"		"
MRK	1.86	3.75	"	3.28	1.58	"	1.70	"		"
LLY	3.04	3.80	"	16.50	1.80	"	-	"		"

TABLE 10.1 (Cont.)

Name	HVA	VMA	DOPAC	p-HPAA	m-HPAA	p-HPLA	HMPG	HMPG	VMA/HMPG	VLA
CRP	2.80	3.50	2.0	2.90	1.72	1.00	-	0.1		0.1
CK	3.12	5.45	"	8.30	2.12	"	1.92	"		"
BRA	3.28	2.72	"	2.50	2.90	"	-	"		"

Excretion of urinary phenolic metabolites (mg/24hr) in The "pretreatment" group of post encephalitic Parkinsonian subjects.

x Patients in the homogenous group.

TABLE 10.2

Name	HVA	VMA	DOPAC	p-HPAA	m-HPAA	p-HPLA	HMPG	HMPE	VMA/ HMPG	VLA	Dosage of L-DOPA
WL*	181	5.10	192	3.95	4.05	-	0.57	-	8.9	12.40	2g on prev day
HUB*	350	9.10	230	12.20	10.40	23.0	2.18	-	4.1	108.00	1g
HLT*	252	6.55	200	15.80	7.60	-	-	-	-	8.05	2g
FTS*	135	2.88	151	7.05	2.56	-	0.54	-	5.3	2.00	0.5 on prev. day
OSB*	248	7.90	194	16.7	2.8	-	1.44	1.20	5.5	16.7	1.75g
TUR*	460	7.80	336	2.10	2.9	-	0.86	2.3	9.1	18.3	2.5g
NAS	200	7.65	268	-	-	-	1.34	0.1	5.7	-	1g
IL	52	4.55	110	-	-	-	0.39	1.12	4.1	-	0.5g
PP	230	11.8	140	-	-	-	2.46	0.1	4.8	-	2.25g
HL	190	7.80	298	-	-	-	0.76	0.1	10.2	-	2.25g
ASB	78	9.85	82	-	-	-	3.16	0.1	3.1	-	1.5g
TLY	19.8	5.05	30	-	-	-	2.16	0.1	2.3	-	0.5g
JY	51	2.86	64.5	-	-	-	0.20	0.1	14.0	-	1.25g
MYN	8.05	3.50	123	-	-	-	0.18	0.1	20.0	-	0.5g on prev. day
RD	56.5	1.68	112	-	-	-	0.62	1.26	8.7	-	2.25g
KLY	280	8.40	390	-	-	-	1.14	2.40	7.4	-	1.5g
DYS	37.5	-	26	-	-	-	-	-	-	-	0.5g on prev. day

TABLE 10.2 (cont).

Name	HVA	VMA	DOPAC	p-HPAA	m-HPAA	p-HPLA	HMPG	HMPE	VMA/ HMPG	VLA	Dosage of L-DOPA
GAR	518	16.7	320	-	-	-	0.19	1.12	89	-	1.75g

Excretion of urinary phenolic metabolites (mg/24hr) in the "under treatment" group of post encephalitic Parkinsonian subjects.

* Patients in the homogenous group.

TABLE 10.3.

Name	HVA	VMA	DOPAC	p-HPAA	m-HPAA	p-HPLA	HMPG	HMPL	VMA/HMPG	VLA
JL*	33.20	0.64	13.4	6.90	7.55	-	0.67	0.25		0.5
HUB*	218	5.05	146	9.8	14.30	11.00	1.40	0.1		65.5
HLT*	71.5	3.32	111	2.1	2.1	-	1.42	0.1		0.5
FTS*	57.5	1.33	75.00	8.7	3.9	-	0.4	0.1		1.5
OSB*	84	1.01	141	8.2	2.8	-	1.32	0.5		1.7
TUR*	505	4.3	365	15.6	4.6	-	0.9	0.5		4.3
BER	12.3	-	20.4	-	-	-	0.7	0.1		-

Excretion of urinary phenolic metabolites (mg/24hr) in the "end of treatment" group of post encephalitic Parkinsonian subjects.

* Patients in the homogenous group.

TABLE 10.4.

Name	HVA	VMA	DOPAC	p-HPAA	m-HPAA	p-HPLA	HMPG	HMPE	VMA/HMPG	VLA
WL	2.10	2.36	2.00	3.0	2.10	1.00	1.30	0.1	1.8	0.1
HUB	2.48	4.90	2.00	3.95	2.66	2.6	0.82	0.1	5.9	0.1
HLT	1.73	3.60	"	5.6	1.08	1.00	1.18	"	3.0	"
FTS	1.82	3.8	"	6.35	1.73	"	0.62	"	6.0	"
OSB	5.05	3.85	"	18.5	2.53	"	2.0	"	1.9	"
TUR	3.7	5.1	"	7.7	2.36	"	1.5	"	3.4	"
DYS	1.23	1.70	"	-	-	"	1.96	"	0.9	"
GAR	2.24	3.75	"	-	-	"	1.27	"	3.7	"
BFR	0.96	1.42	"	-	-	"	0.72	"	2.0	"
KLY	2.6	3.22	"	-	-	"	0.93	"	3.4	"

Excretion of urinary phenolic metabolites (mg/24hr) in the "follow up" group of post encephalitic Parkinsonian subjects.

x Patients in the homogenous group.

TABLE 10.5.

Name	HVA	VMA	DOPAC	p-HPAA	m-HPAA	HMPG	HMPE	VLA	p-HPLA	VMA/HMPG
CAR	1220	10.00	2080	5.2	12.50	2.92	0.87	54	3.5	
JNS	2820	10.50	3400	12.0	16.10	3.4	0.42	32.2	1.73	
COP	1290	9.65	1910	11.0	15.00	4.3	3.3	44.5	4.8	
MRG	1500	10.20	1110	12.9	13.50	7.15	1.25	31.8	3.6	
Mean	1707	10.00	2135	10.0	14.00	4.5	1.5	41	2.8	2.5

Excretion of urinary phenolic acids and alcohols (mg/24hr) in the high dosage (4.25-4.75g L-DOPA/day) series of idiopathic Parkinsonian patients.

TABLE 10.6

	HVA		DOPAC		p-HPAA		m-HPAA	
Name	pre-neomycin	post-neomycin	pre-neomycin	post-neomycin	pre-neomycin	post-neomycin	pre-neomycin	post-neomycin
SMT	950	640	895	615	8.0	15.0	16.5	4.0
KNY	393	455	410	385	6.5	25.0	14.5	9.3
FLT	1144	1570	955	1222	6.6	9.0	12.9	6.9
DVS	640	945	515	800	32.8	56.0	17.1	2.8
EDW	840	600	880	685	30.0	19.5	29.0	9.0
PTR	485	415	440	367	9.0	17.8	5.7	1.4
Mean ± S.E	742±121	770±186	683±88	679±138	15.5±4.2	23.7±7.6	15.9±3.7	5.5±1.2

Excretion of urinary phenolic metabolites (mg/24hr) before and during the third day of oral neomycin (1g/day) treatment in six idiopathic Parkinsonian patients under oral therapy with L-DOPA (2g-8g/day).

TABLE 10.7

Metabolite	Pre-treatment Mean±S.E	Pre-treatment Observed Range	Under treatment Mean±S.E	Under treatment Observed Range	End of treatment Mean±S.E	End of treatment Observed Range	Follow-up Mean±S.E	Follow-up Observed Range
HVA	3.1±0.33 (14)	1.6-6.0	201±38 (12)	51-460	140±66 (7)	12-505	2.4±0.41 (10)	1.0-5.1
Free DOPAC	2.0 (14)	-	195±33 (12)	30-390	124±45 (7)	13-365	2.0(10)	-
VMA	4.6±0.54 (14)	1.4-9.3	7.3±0.75 (11)	2.7-11.8	2.6±0.83 (6)	0.6-5.1	3.4±0.37 (10)	1.4-5.1
Total HMPG	2.2±0.32 (9)	0.8-3.5	1.5±0.28 (10)	0.2-3.2	1.0±0.16 (7)	0.7-1.4	1.2±0.14 (10)	0.6-2.0
VMA/HMPG ratio	2.5±0.5 (9)	0.4-4.9	6.6±1.2 (10)	2.3-14.0	2.5±0.6 (6)	0.8-3.6	3.2±0.52 (10)	

Excretion of urinary phenolic acids and alcohols (mg/24hr) in the heterogenous series. Number of patients in parentheses.

TABLE 10.8

	Pre-treatment		Under treatment		End of treatment		Follow-up	
Metabolite	Mean±S.E	Observed Range	Mean±S.E	Observed Range	Mean±S.E	Observed Range	Mean±S.E	Observed Range
HVA	2.7±0.27	1.8-3.8	271±48	135-460	161±73	33-505	2.9±0.53	1.7-5.1
Free DOPAC	2.0	-	217±50	151-336	142±21	13-365	2.0	-
VMA	4.6±0.27	3.5-5.3	6.6±0.92	3.0-9.1	2.6±0.83	0.6-5.1	3.9±0.40	2.4-4.9
Total HMPG	1.4±0.16	0.9-1.9	1.1±0.25	0.5-2.2	1.0±0.18	0.4-1.4	1.2±0.20	0.6-1.5
p-HPAA	7.4±1.52	3.2-13.9	8.8±2.18	2.1-16.7	8.5±1.79	2.1-15.6	7.5±2.30	3.0-18.5
m-HPAA	1.7±0.51	0.9-4.0	5.1±1.32	2.6-10.4	5.8±1.88	2.1-14.3	2.1±0.24	1.1-2.5
VLA	0.1	-	27.5±16.3	2-108	12.3±10.6	0.5-6.6	0.1	-
VMA/HMPG	3.3±0.33	2.7-4.8	6.3±0.85	4.1-9.0	2.5±0.60	0.8-4.5	3.6±0.78	1.8-6.1

Excretion of urinary phenolic acids and alcohols (mg/24hr) in the homogeneous series.

"follow-up" groups (Tables 10.7 and 10.8.) did not differ from the normal range (Karoum et al., 1969, also see chapter 5).

Results on the determination of phenolic acids before and after neomycin administration are recorded in table 10.6.

Compared with "pre-treatment" values, a several-hundredfold rise in HVA excretion was detected in both series of analyses while the patients were "under treatment". At the "end of treatment", these levels fell significantly (heterogeneous series, $P<0.05$; homogeneous series $P<0.025$) to about one-third of the "under treatment" value and had returned to normal in all subjects tested by "follow-up".

There was a very similar high output of free DOPAC "under treatment" which fell considerably at the "end of treatment", returning to normal in the "follow-up" samples. In both the "under treatment" and "end of treatment" groups, there was a strong positive correlation ($P<0.01$) between output of HVA and DOPAC. Total dose of drug up to the time of urine sampling and dose in the 24 hr period preceding urine collection were positively correlated with HVA and DOPAC excretion values in both the "under treatment" and "end of treatment" groups. There was also a positive correlation ($P<0.02$) between urinary HVA or DOPAC output in the "end of treatment" group and urine volume.

Although total 4-hydroxy-3-methoxyphenylethanol (HMPE)

excretion in the "under treatment" or "end of treatment" groups was small compared with output of HVA or DOPAC, it represents a substantial increase over the small quantities (less than 100 μg/24 hr) found in normal urine. In four subjects from the heterogeneous series "under treatment" (Table 10.2), levels of 1.12, 1.12, 1.26 and 2.4 mg HMPE/24 hr were recorded, whilst three others belonging to the "end of treatment" (Table 10.3) group in this series had an output of 0.25, 0.5 and 0.5 mg HMPE/24 hr.

Although the absolute increase in urinary VMA was not as dramatic in patients "under treatment", it was still significantly higher (heterogeneous series $p < 0.02$; homogeneous series $P < 0.05$) than the "pre-treatment" group and remained high ($P < 0.02$) in the "follow-up" group of the homogeneous series.

In the heterogeneous series, total HMPG excretion whilst "under treatment" was significantly ($P < 0.05$) lower than in the "pre-treatment" group. The "end of treatment" output was still lower ($P < 0.01$) than control values and even lower ($P < 0.05$) than the "under treatment" excretion. There was a tendency for the "end of treatment" HMPG output in the homogeneous series to be lower than the "pre-treatment" level but this did not reach significance ($P > 0.1$) in this small series.

There was a positive correlation ($P < 0.02$) between HMPG and VMA output in patients in the heterogeneous series "under treatment", although a similar relationship was not observed in any of the other groups. The mean ratio of the excretion values of VMA to total HMPG in "under treatment" groups in both series was higher than in any of the other groups (Tables 10.7 and 10.8). In the homogeneous series, this rise was significant ($P < 0.02$) for comparisons between "under treatment" and "pre-treatment" or "end of treatment" groups. Inspection of ratios in the heterogeneous series suggested a similar relationship.

4-hydroxy-3-methoxyphenyllactic acid (VLA) was almost undetectable (less than 100 μg/24 hr) in urine from "pre-treatment" and "follow-up" groups but was excreted in large amounts (Table 10.8) by patients "under treatment" ($P < 0.025$) (see Fig. 1,2,3,4). 4-hydroxy-3-methoxyphenylpyruvic acid (VPA) could not be detected in any sample.

Oral L-DOPAC did not affect the excretion of p-hydroxyphenylacetic acid which remained within normal range (see chapter 5) throughout the investigation. In contrast there was a significant increase in output of m-hydroxyphenylacetic acid (Table 10.8) in both "under treatment" and "end of treatment" groups when compared with "pre-treatment" ($P < 0.05$) and "follow-up" ($P < 0.05$) groups.

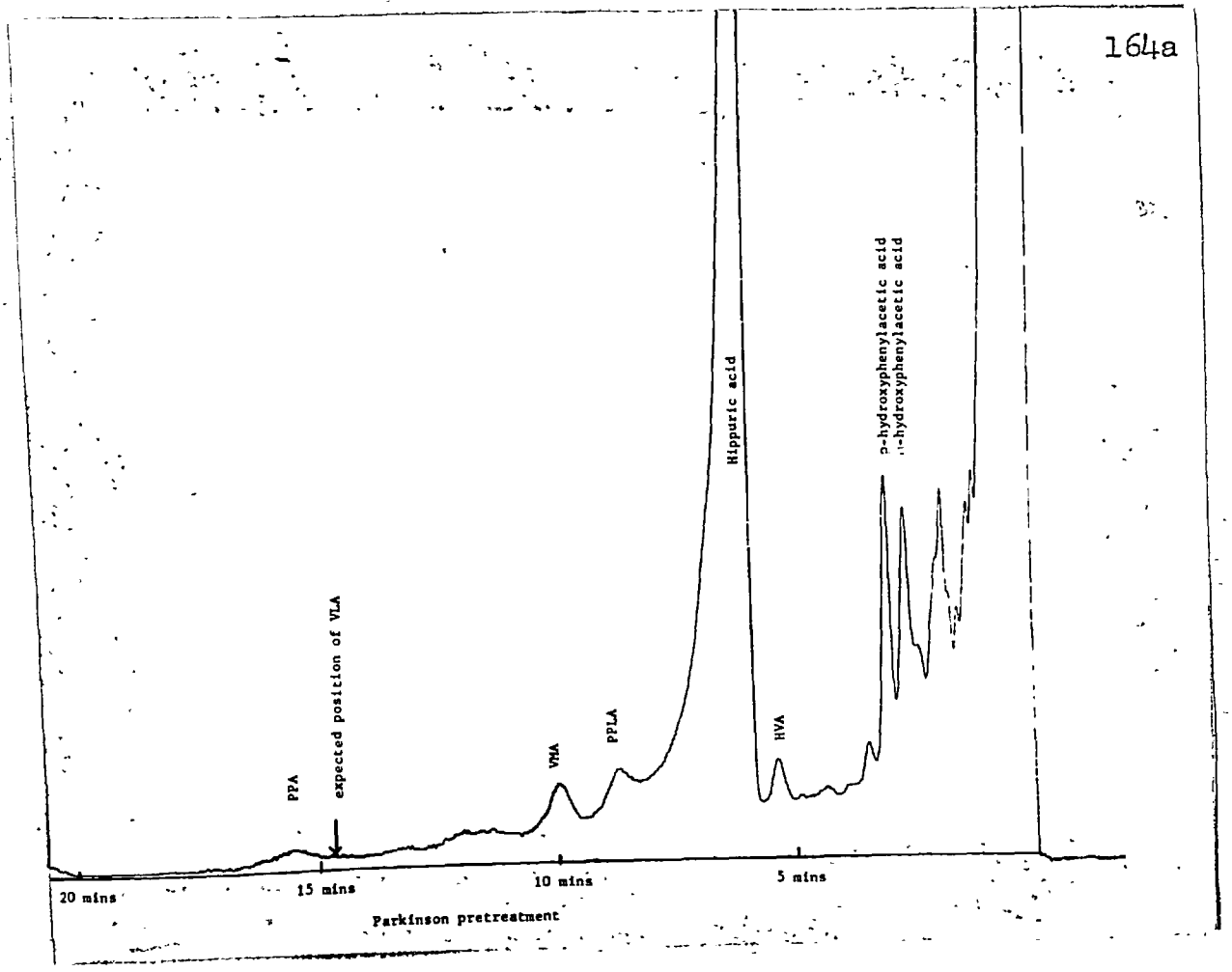


Fig. 10.1. Chromatogram of the ME/TE derivative of an ethyl acetate urine extraction of a post-encephalic Parkinsonian patient before treatment with L-DOPA.

PPLA = p-hydroxyphenllactic acid.
PPA = p-hydroxyphenylpyruvic acid.

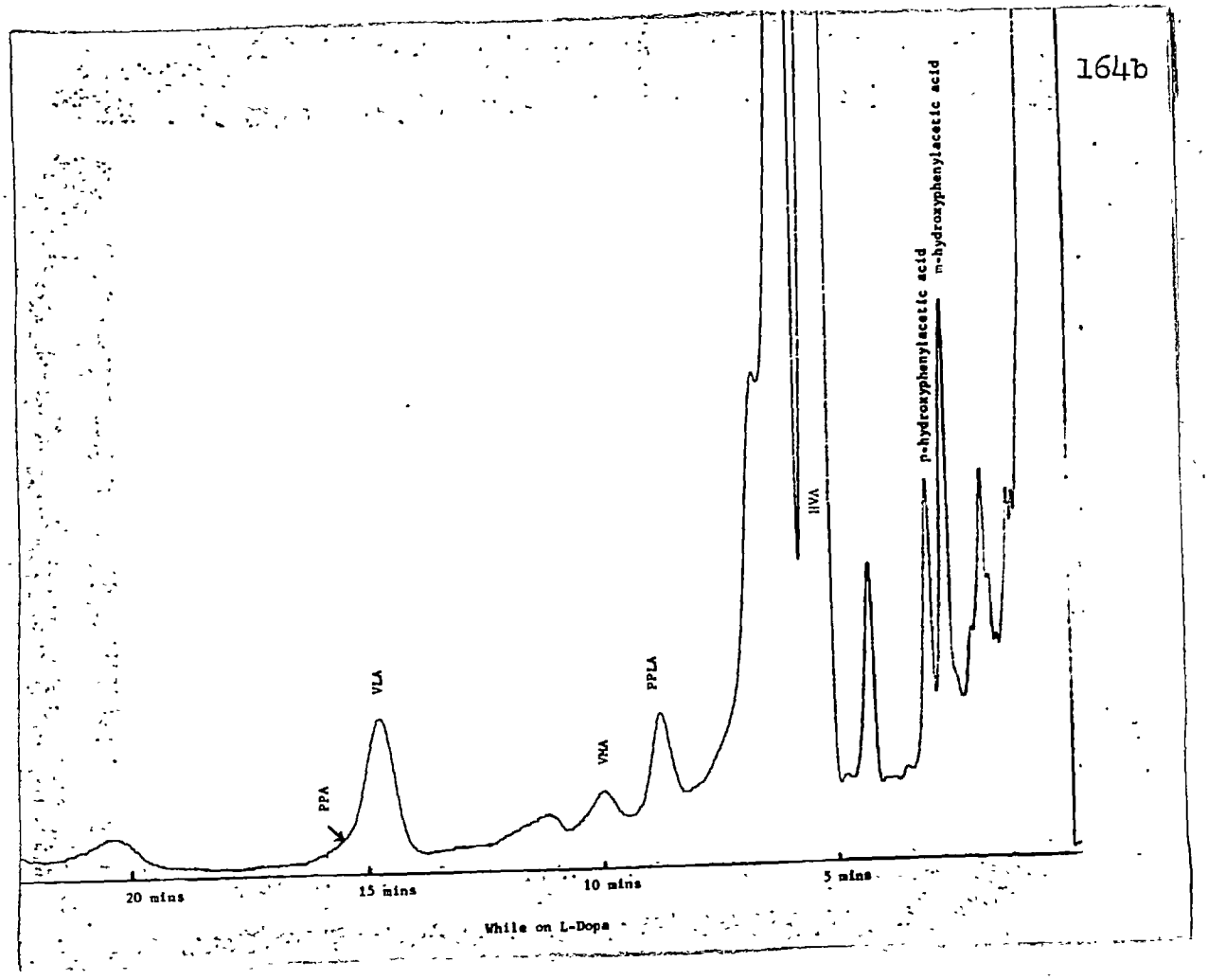


Fig. 10.2. Chromatogram of the ME/TE derivative of an ethyl acetate urine extraction of a post-encephatic Parkinsonian patient while on L-DOPA treatment.

PPLA = p-hydroxyphenyllactic acid.
PPA = p-hydroxyphenylpyruvic acid.

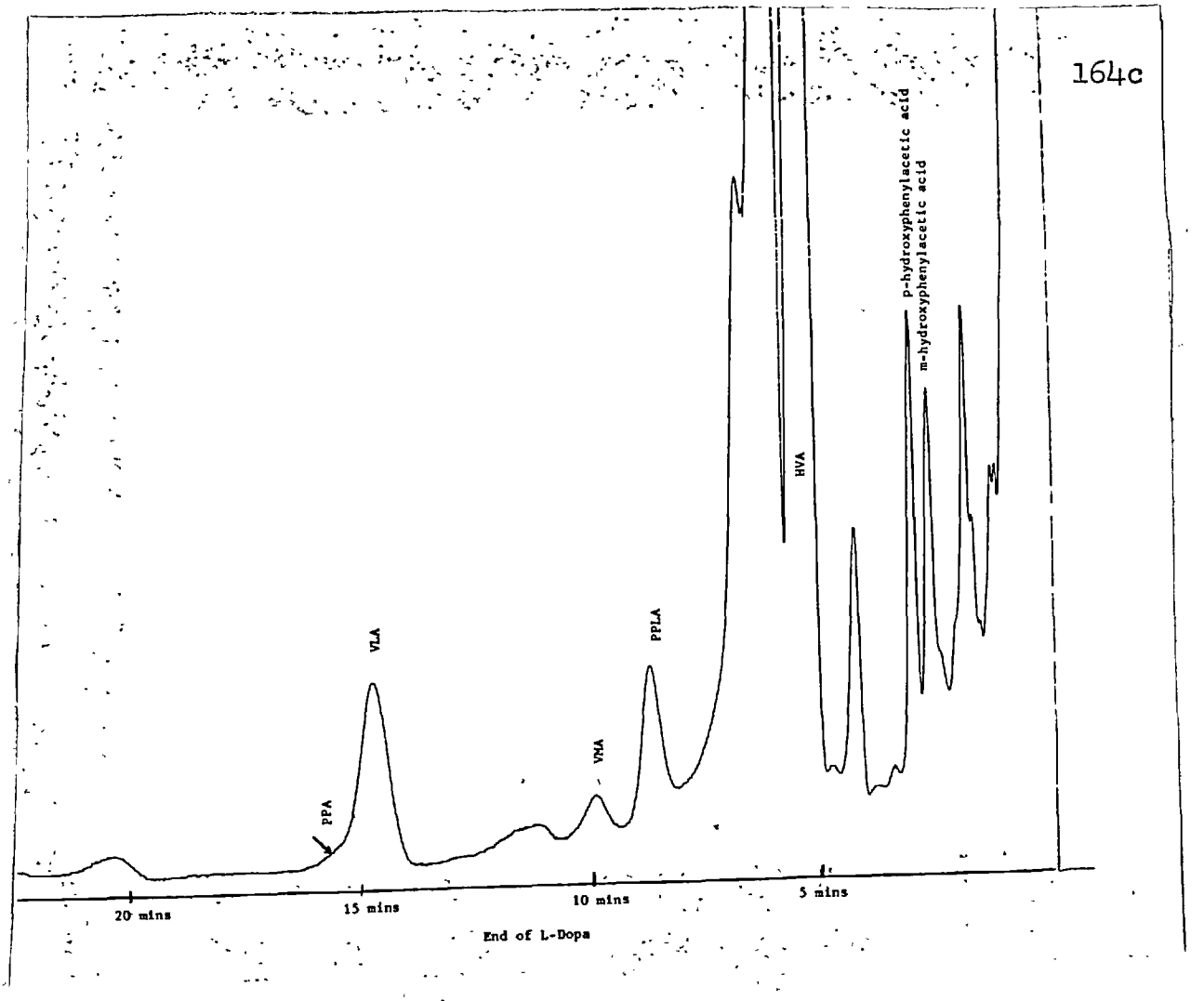


Fig. 10.3. Chromatogram of the ME/TE derivative of an ethyl acetate urine extraction of a post-encephalic Parkinsonian patient 24-48 hr. after completion of L-DOPA treatment.

PPLA = p-hydroxyphenyllactic acid.
PPA = p-hydroxyphenylpyruvic acid.

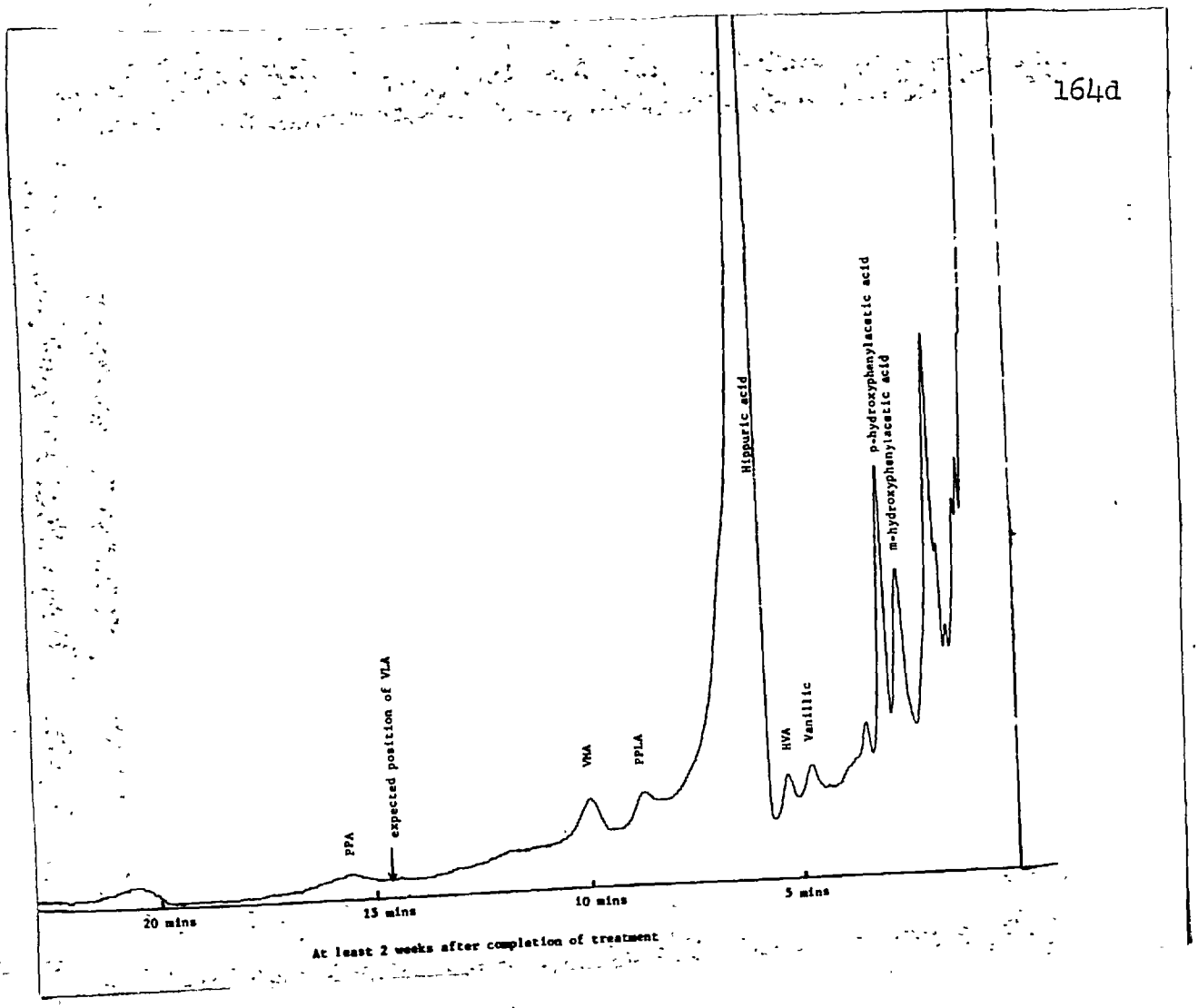


Fig. 10.4. Chromatogram of the ME/TE derivative of an ethyl acetate urine extraction of a post-encephalic Parkinsonian patient at least 2 weeks after completion of L-DOPA treatment.

PPLA = p-hydroxyphenyllactic acid.
PPA = p-hydroxyphenylpyruvic acid.

The range of metabolites measured in the homogeneous series was also estimated in a small group of patients with idiopathic Parkinsonism on a higher dosage of L-DOPA (Table 10.5). Apart from p-hydroxyphenylacetic acid, the value of each was greater than after treatment with the lower dosage. m-Hydroxyphenylacetic acid excretion was so increased as to be greater than that of its p-isomer; its output is normally considerably less (see chapter 5).

Comparing the excretion of phenolic acids before and after neomycin, no significant differences could be observed for HVA, DOPAC, p-HPLA or VLA. m-Hydroxyphenylacetic acid output was significantly reduced ($P < 0.01$) after neomycin (Fig. 10.5). p-Hydroxyphenylacetic acid excretion was increased in five out of six patients after neomycin. Although mean excretion values of p-HPAA before and after neomycin were not significantly different ($0.2 < P > 0.1$) on a two tail variance scale, the difference becomes significant if one were to assume a one tail variance.

Discussion

Since the earliest observations on L-DOPA metabolism in man and rabbit by Guggenheim (1913), its major pathways of degradation have become well established (Sandler and Ruthven, 1969a). The greater proportion is decarboxylated

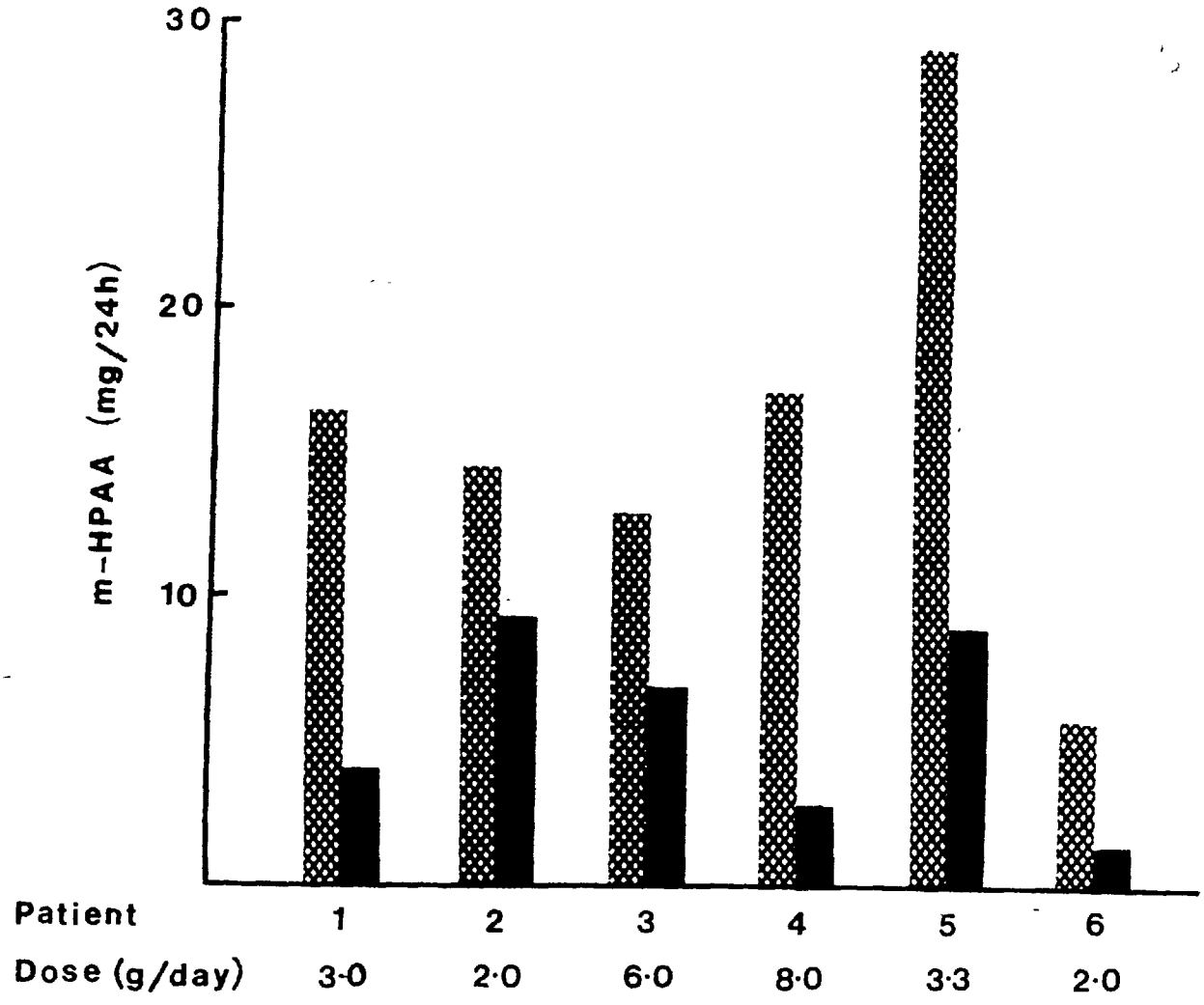


Fig. 10.5. Excretion of m-hydroxyphenylacetic acid (m-HPAA) before (hatched columns) and during (solid columns) the third day of oral neomycin (1g/day) treatment in six Parkinsonian patients under oral therapy with L-DOPA at the dosage shown.

to dopamine which is either β -hydroxylated to noradrenaline or, in common with the other catecholamines, inactivated by two alternative routes, involving the primary action of either catechol O-methyltransferase or monoamine oxidase (MAO) (see Fig. 1.4 in chapter 1). HVA, which results from the action of both enzymes on dopamine, is quantitatively the most important endogenous DOPA metabolite in normal human urine (see chapter 1).

The "pre-treatment" data presented here which do not appear to differ from normal values (see chapter 5), together with the previous finding of a normal HVA output in this disease group (Greer and Williams, 1965; Rinne, Laitinen and Sonninen, 1967; Rinne and Sonninen, 1968) argue against there being a generalised disturbance of dopamine metabolism in Parkinsonism. This is understandable, since dopamine concentration in the brain represents only a small proportion of the total dopamine content in the body (Rinne and Sonninen, 1968).

Sourkes et al., (1965) gave L-DOPA to one postencephalitic Parkinsonian subject and noted some delay in the rise in urinary HVA excretion compared with controls; but, in general, the present findings in patients with this disease during treatment are not too dissimilar from those following L-DOPA administration to normal subjects (Shaw et al., 1957;

Sourkes et al., 1965). There was a very large increase in urinary excretion of HVA which was equalled (Tables 10.2, 10.3) and at high dosage even exceeded (Table 10.5), by DOPAC output. This increase in relative concentration of DOPAC appears to depend to some extent on route of administration (Sourkes et al., 1965).

It is of interest that children with dopamine-secreting tumours may sometimes put out as much HVA as the patients "under treatment" (Table 10.2). DOPAC excretion, on the other hand, although markedly raised, forms a proportionately smaller part of the total metabolite output (von Studnitz, 1960; Sourkes et al., 1963) (see chapter 8). This finding implies that the site of metabolic degradation of this endogenously secreted dopamine differs from that generated from exogenously administered DOPA.

There are other points of difference from dopamine-secreting tumours. The intermediate aldehyde produced by oxidative deamination of dopamine is preferentially oxidised further to HVA rather than reduced to HMPE (Breese et al., 1969); during the present investigation, an even smaller proportion of HMPE was found after L-DOPA treatment than is observed in cases of dopamine-secreting tumour (see chapter 8).

A second difference concerns the degree of β-hydroxy-

lation. Apart from metabolites derived from the direct metabolism of dopamine, patients with dopamine-secreting tumours almost invariably have a large output of the β-hydroxylated noradrenaline series of metabolites with a relatively large contribution from the aldehyde reduction product, HMPG. Oral DOPA treatment however resulted in a comparatively small increase only in VMA and a marginal decrease in HMPG, resulting in an increased VMA/HMPG ratio. In the light of these findings, some re-interpretation of views published from these laboratories concerning the site of β-hydroxylation of dopamine derived from a secreting tumour (Sandler and Ruthven, 1966_a) is probably necessary. Whereas this had been localised at sites peripheral to the tumour, it now seems likely that the metabolic change occurs within the tumour tissue itself.

DeEds et al., (1957) found that DOPA administration to rabbits gave rise to an increased urinary output of m-hydroxyphenylacetic acid, presumably by p-dehydroxylation brought about to a large extent by the gut flora (Scheline, 1968). Dehydroxylation has been demonstrated in vivo for a wide range of substances in the mammalian organism (Scheline, 1968). Because this reaction is attenuated or abolished by gut sterilization with neomycin, it is generally accepted that intestinal micro-organisms are responsible for its occurrence

(Shaw, Gutenstein and Jepson, 1961; Booth and Williams, 1963; Dacre and Williams, 1968; Dacre, Scheline and Williams, 1968; Dayman and Jepson, 1969). Recently a strain of bacterium responsible for this dehydroxylation has been isolated (Perez-Silva, Rodriguez and Perez-Silva, 1966). Although Shaw et al., (1957) were not able to marshal any evidence for the existence of this pathway in man, the present findings show that a small but significant proportion of L-DOPA is degraded by this route (Table 10.5). This view is further supported by the significant reduction of m-hydroxyphenylacetic acid output after gut sterilization (Table 10.6). There was no evidence of m-dehydroxylation, as the output of p-HPAA was unchanged after L-DOPA administration. Nevertheless, this may have occurred since both p- and m-dehydroxylation of catecholic acids have been described in some species (Dacre et al., 1968); but whereas the m-isomers remain intact, p-isomers are readily decarboxylated in the gut (Scheline, 1966 and 1968a). In view of the claims by Boulton et al., (1967) and Smith and Kellow (1969) that patients with Parkinsonism have an increased urinary output of p-tyramine, the p-hydroxyphenylacetic acid precursor, the finding of a normal output of the acid is at first sight puzzling. Both observations may be reconciled however by assuming a decrease of in vivo MAO activity. Tryptamine

out; ut also appears to be increased in Parkinsonism (Kuehl et al., 1968), an observation consistent with this view. Preliminary direct observations in this laboratory (J. Southgate - unpublished) indicate that there may be a decrease in platelet MAO activity in affected subjects. The increased p-hydroxyphenylacetic acid output observed after gut sterilisation of patients with Parkinson's disease during L-DOPA treatment is similarly observed in normal subjects following gut sterilisation (Dayman and Jepson, personal communication); it is therefore likely that L-DOPA does not contribute to this increase. The phenomenon is presumably explained by speculating that p-tyramine the main precursor of this phenolic acid is normally further metabolised in the gut, a degradation prevented by antibiotic administration.

In their classical paper, Shaw et al., (1957) discussed the possible existence of an alternative pathway of DOPA metabolism via transamination, although they were not able to prove it directly. For many years, it has been known that L-DOPA can participate in transamination reactions (Cammarata and Cohen, 1950) and Fonnum, Haavaldsen and Tanger (1964) have characterised several DOPA transaminases in brain. Smith (1967) claimed to find an increased urinary excretion of the unstable VPA but no evidence of its more stable derivative, VLA, after L-DOPA administration to volunteers. Despite the sensitivity

of the methods employed and the considerably higher dosage range of L-DOPA, it was not possible to detect VPA in any sample. There was a considerable increase, however, of its reduction product (Weber and Zannoni, 1966; Zannoni and Weber, 1966), VLA, confirming earlier observations in patients with DOPA-secreting tumours (Gjessing 1963; Smith, 1965).

The major metabolite of tyrosine metabolism is p-hydroxyphenylpyruvic acid (p-HPPA) which is normally further metabolised by p-HPPA-oxidase (La Du, 1966) undergoing the recently described "NIH shift" (Guroff et al., 1967), to 2,5-dihydroxyphenylacetic acid (homogentisic acid). The increased output of the immediate reduction product of p-HPPA, p-HPLA, which tends to correlate with VLA output, suggests that DHPPA or VPA may compete with p-HPPA for p-HPPA-oxidase. If this were so, DHPPA or VPA might itself be expected to be metabolised by a similar "NIH shift" medianism, to 2,4,5-trihydroxyphenylacetic or 2,5-dihydroxy-4-methoxyphenylacetic acid. A recent attempt to detect these substances by GC-MS, has suggested the presence of minute amounts of both in one normal urine sample out of six, two Parkinsonian (pretreatment) urine samples out of 12 and in 2 treated Parkinsonian samples out of 3.

Although no significant correlation has so far been established between excretion of its urinary metabolites and

the therapeutic response of L-DOPA (Calne et al., 1969_a) there is the suggestion that such correlation may exist between HVA levels in the CSF and clinical improvement (Klowans and Weiner, 1969). As CSF, anatomically at least, is more intimately associated with the site of action of the drug, its investigation is more likely to yield result of value (Rinne and Sonninen, 1968), were it not for ethical consideration. The time lag between start of clinical improvement after L-DOPA therapy and the presumed rapid generation of dopamine within the central nervous system (Calne et al., 1969_a) however, suggests other biochemical factors need to be considered. For instance it is possible that the delay is directly related to a build-up of some minor metabolite unconnected with the main routes of DOPA degradation. A full account of this aspect of the problem is given by Sandler (1970).

GENERAL DISCUSSION

Since Sweeley and Williams's first publication in 1961, the application of gas chromatography to the analysis of aromatic acids and alcohols has largely been confined to a study of authentic substances, thus by-passing many of the difficulties encountered during metabolic investigations. In the last three years, however, interest in gas chromatography has broadened as the scope and potentiality of the approach has been appreciated. With the advent of the electron capture detector, the possibility of measuring minute concentrations of endogenous metabolites in body fluids and tissues has been opened up and methods continue to be published with increasing frequency attesting to the adaptability of this analytical tool. Such a sensitive, versatile and quantitative analytical approach makes these procedures unique, and as GLC can be linked to a mass spectrometer, its scope is accordingly broadened. Much structural information can be obtained about unknown metabolites, an aspect of the subject of special relevance to investigations of metabolic disorders and studies of drug metabolism.

As shown during the present investigation, the preparation of the ME/TE derivative for phenolic and indolic acid assay and the TE/E for that of the corresponding

alcohols, together with chromatography on an SE52 column, seems to offer the best arrangement for rapid and accurate analysis of a wide range of metabolites. New phases have recently been reported such as OV_1 and OV_{17} (Horning, Boucher and Moss, 1967), and new methods of preparing the TE/E derivatives (e.g. Chambaz and Horning, 1969; Pierce, 1968) are continually appearing in the literature. A brief survey of these innovations (unpublished) has not revealed any advantage over the procedure described here; accordingly the methods described in this thesis are recommended at least for metabolic investigations of urine in man and rat. One of the main reasons why the technique promises so well is that it is highly flexible and more manoeuvreable than other chromatographic methods. By adjusting a number of different variables, a procedure can be "tailor-made" to suit almost any type of analysis required (see chapters 2 and 3). The specificity of such procedures can be further improved by selective extraction (Karoum et al., 1969) to eliminate interfering substances which might otherwise affect accurate quantification. It is however, important to realise that although the application of GLC is simple and straightforward, initial setting up of methods requires a sound knowledge of the fundamental principles of its operation; such expertise helps considerably in assessing column performance, resolution

and in selecting optimal efficiency with respect to the overall performance and analysis time.

Using the present procedure, excretion values of certain metabolites in human and rat urine were essentially similar to those reported by other techniques (see chapter 5), although differences were observed in the human new-born infant. A rather higher incidence of tyrosyluria than had been expected was observed and this excretory pattern was highly correlated with the incidence of jaundice. Whether enzyme immaturity was responsible for both phenomena, or whether bilirubin inhibits p-HPPA oxidase and is thus responsible for the increased output of p-HPLA in tyrosyluria must be the subject of further work. Further information is also required in the neonate on the excretion of a wider range of metabolites derived from phenylalanine and tyrosine which might be of diagnostic assistance in discriminating between true phenylketonuria and transitory forms of disturbed phenylalanine and tyrosine metabolism.

GLC emerged as being particularly suited to the investigation of amine-secreting tumours (see chapters 7 and 8). In carcinoid syndrome, apart from abnormal excretion values of 5HIAA, a raised output of 5HTOH was observed. Published data are extremely scanty concerning excretion of this metabolite, for no simple assay procedure had previously been available.

During the investigation of catecholamine secreting tumours, a wide range of metabolites was determined; some have already demonstrated their diagnostic usefulness, whilst others will obviously prove of diagnostic value when sufficient experience is obtained and further data accumulate. The methodology is particularly suited to this type of investigation for many more than one metabolite are present on the same chromatogram. Thus HVA as well as VMA can be quantified at the same time, a valuable ancillary test in view of the putative association between dopamine secretion and malignancy in phaeochromocytoma (see chapter 8). During these studies output of a number of compounds which had not been measured before in this condition was noted. Of special interest is an unknown peak, designated as substance x, the excretion of which was found to be increased in about 60% of cases of phaeochromocytoma. Identification of this substance and of certain other commonly observed unknown peaks are urgent tasks for future GC-MS studies.

The shift in metabolism of noradrenaline (Sandler and Youdim, 1968) from a predominently oxidative pathway of the intermediate aldehyde to a reductive one after intravenous reserpine was shown to be a general phenomenon and to apply to the metabolism of other monoamines (see chapter 9). One might well view the conduct of this type of experiment as a

prototype. The approach can obviously be extrapolated to a study of the action of many different categories of drugs on phenolic acid and alcohol metabolism. A further example of the usefulness of this set of techniques is demonstrated by present studies on the metabolism of L-DOPA in Parkinsonism. A number of minor metabolic pathways of L-DOPA metabolism were demonstrated. It is probably of considerable importance to chart them, for the mechanism of action of L-DOPA in Parkinsonism is by no means completely explained. There are some rather puzzling discrepancies in the time course, whilst the beneficial effect may only be observed after a number of days or weeks have elapsed (Calne et al., 1969; O'Reilly, 1969 Godwin-Austen et al., 1969; Calne et al., 1969c; Yaher et al., 1969), dopamine generation is likely to be rapid (Hornykiewicz, 1966). Thus the build-up of some minor metabolite may contribute in greater or lesser measure to the theraputic action of the drug.

So far, techniques have only been developed for the assay of phenolic and indolic acids and alcohols. It is hoped to broaden the scope of this work by devising procedures for bases also. This would undoubtedly entail preparing a halogenated derivative and employing the electron capture detector. It seems likely that such procedures, which have so far been relatively scantily investigated, will contribute as much as their predecessors.

REFERENCES

Airaksinen, M.M. (1961). Biochem. Pharmac. 8, 245

Alpers, H.S. and Shore, P.A. (1969). Biochem. Pharmac. 18, 1363

Alton, H. and Goodall, McC. (1969). Biochem. Pharmac. 18, 1373

Alvord, E.C. (1958). In: Pathogenesis And Treatment of Parkinsonism, ed. W.S. Fields, C.C. Thomas, Springfield, Ill. p. 161.

Ambrose, D., James, A.T., Keulemans, A.I.M., Kovats, E., Rock, H., Rouit, C. and Stross, F.H. (1960). In "Pure and Applied Chemistry", 1, 423.

Anden, N.E., Roos, B.E. and Werdinuis, B. (1963). Life Sci. 2, 319.

Anden, N.E., Roos, B.E. and Werdinuis, B. (1964). Life Sci. 3, 149.

Anton, A.H. and Greer, M. (1966). Clin. Pharmac. Ther. 7, 727.

Anderson, P.J. and D'Ioris, A. (1968). Biochem. Pharmac. 17, 1949.

Anderson, J.A., Ziegler, M.R. and Doeden, D. (1959). Science, N.Y. 127, 236.

Applegarth, D.A., Hardwick, D.F. and Ross, P.M. (1968). Clin. Chim. Acta. 22, 131.

Asatoor, A.M. (1968). Clin. Chim. Acta. 22, 223.

Asatoor, A.M., Chamberlain, M.J., Emerson, S.T., Johnson, J.R., Levi, A.J. and Milne, M.D. (1967). Clin. Sci. 33, 111.

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

Armstrong, M.D. and Shaw, K.N.F. (1955). J. biol. Chem. 213, 805.

Armstrong, M.D., Shaw, K.N.F., Gortatowski, M.J. and Singer, H. (1958). J. biol. Chem. 232, 17.

Armstrong, M.D., Shaw, K.N.F. and Robinson, K.S. (1955). J. biol. Chem. 213, 797.

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

Arvidsson, J., Roos, B.E., Steg, G. (1966). Acta Physiol. Scand. 67, 399.

Avery, M.E., Clow, C.L., Menkes, J.H., Romas, A., Scriver, C.R., Stern, L. and Wasserman, B.P. (1967). Pediatrics, Springfield, 39, 378.

Awapara, J., Perry, T.L., Hanly, C. and Peck, E. (1964). Clin. Chim. Acta. 10, 286.

Awapara, T., Sandman, R.P. and Hanly, C. (1962). Archo. Biochem. Biophys. 98, 520.

Axelrod, J. (1959). Physiol. Rev. 39, 791.

Axelrod, J. (1960). Biochim. biophys. Acta. 45, 614.

Axelrod, J. (1960_a). In: Ciba symposium on Adrenergic Mechanisms, ed. G.E.W. Wolstenholme and M. O'Connor, Churchill, Lond. p. 28.

Axelrod, J. (1961). Science, N.Y. 134, 343.

Axelrod, J. (1962_a). J. biol. Chem. 237, 1657.

Axelrod, J. (1962_b). J. Pharmac. exp. Ther. 138, 28.

Axelrod, J. (1963). In: Clinical Chemistry of Monoamines, ed. H. Varley and A.H. Gowenlock, Elsevier, Amsterdam 2, p.5.

Axelrod, J. (1966). Pharmac. Rev. 18, 95.

Axelrod, J. and Tomchick, R. (1958). J. biol. Chem. 233, 702.

Axelrod, J. and Szara, S. (1958_a). Biochim. biophys. Acta. 30, 188.

Axelrod, J. and Weissbach A. (1960). Science, N.Y. 131, 1312.

Axelrod, J. and Weissbach, A. (1961). J. biol. Chem, 236, 1281.

Axelrod, J., Kopin, I.J. and Mann, J. (1959). Biochim. biophys. Acta. 36, 576.

Axelrod, J., Albers, W. and Clemente, C.D. (1959_a). J. Neurochem. 5, 68.

Ayd, F.J., (1961). J. Amr. med. Ass. 175, 1054.

Bagchi, S.P. and McGeer, P.L. (1964). Life Sci. 3, 1195.

Barbeau, A. (1960). Neurology Minneap., 10, 442.

Barbeau, A. (1961). Archs. Neurol., Paris, 4, 97.

Barbeau, A. (1962). Canad. Med. Ass. J. 87, 802.

Barbeau, A., Murphy, C.F. and Sourkes, T.L. (1961). Science, N.Y. 133, 1706.

Barbato, L. and Abood, L.G. (1963). Biochim. biophys. Acta 67, 531.

Baron, D.N., Dent, C.E., Harris, H., Hart, E.W. and Jepson, J.B. (1956). Lancet. 2, 421.

Baudhin, P., Beaufay, H., Rahman, Li. Y., Sellinger, O.Z., Wattiaux, R., Jacques, P and de Duve, C. (1964) Biochem. J. 92, 179.

Bentley, R., Sweeley, C.C., Makita, M. and Wells, W.W. (1963) Biochem. biophys. Res. Commun. 11, 14.

Berlyne, G.M. (1965), Brit. Heart J. 70, 143.

Bernheimer, H., Birkmayer, W and Hornykiewicz, O. (1961). Klin. Wschr. 39, 1056.

Bernheimer, H., Birkmayer, W. and Hornykiewicz, O. (1963). Klin. Wschr. 41, 465.

Bernheimer, H., Birkmayer, W and Hornykiewicz, O. (1966). Wien. Klin. Wschr. 78, 417.

Bernheimer, H., Birkmayer, W., Hornykiewicz, O., Jellinger, K. and Seitelberger, F. (1965). Proc. 8th Intern. Cong. Neurol. Vienna, Wiener Medizinische Akademie, 145.

Bernheimer, H. and Hornykiewicz, O. (1964). Archs. exp. Path. Pharmak. 247, 305.

Bernheimer, H. and Hornykiewicz, O. (1965), Klin. Wsch. 43, 711.

Bernheimer, H. and Hornykiewicz, O. (1964). Arch. exp. Path. Pharmakol. 247, 305.

Bertler, A., Carlsson, A. and Rosengren, E. (1956). Naturwissenschaften, 22, 521.

Bertler, A and Rosengren, E. (1959_a). Experientia, 15, 10.

Bertler, A. (1961). Acta. Physiol. Scand. 51, 75.

Bertler, A. and Rosengren, E. (1959). Experientia, 15, 382.

Birkmayer, W. and Hornykiewicz, O. (1961). Wien. Klin. Wschr. 73, 787.

Birkmayer, W. and Hornykiewicz, O. (1962). Arch. Psychiat. Nervkrankh. 203, 560.

Blakley, R.R. (1966). Anal. Biochem. 15, 350.

Blaschko, H. (1939). J. Physiol, Lond. 96, 50p.

Blaschko, H. (1952). Pharmac, Rev. 4, 415.

Blaschko, H. (1952_a). Biochem. J. 52, 10p.

Blaschko, H. (1957). Experientia, 13, 9.

Blaschko, H. (1963). In: The Enzymes ed. by P.D. Boyer, H.A. Lardy and K. Myrback, 8, 337. Academic Press. Inc. N.Y.

Blaschko, H. and Holton, P. (1950). J. Physiol. Lond., 110, 484.

Blaschko, H. and Levine, W.G. (1966). In: Handbook of Experimental Pharmacology, Vol. 19, ed. by O. Eichler and A. Farah. Springer-Verlag. Berlin. p. 212.

Blaschko, H. and Sloane-Stanley, G.H. (1948). Biochem. J. 42, iii

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

Bloom, F.E., Costa, E. and Salmoiraghi, G.C. (1965). J. Pharmac. exp. Ther. 151, 244.

Bloxam, H.R., Day, M.G., Gibbs, N.K. and Woolf, L.I. (1960). Biochem. J. 77, 320.

Boadle, M.C. and Bloom, F.E. (1969). J. Histochem. Cytochem. 17, 331.

Bodian, M. (1959). Pediat. Clin. N. Amer. 6, 449.

Boehm, J.J. and O'Brien, D. (1963). Pediatrics. Springfield, 31, 861.

Bohemen, J., Langer, S.H., Perrett, R.H. and Purnell, J.H. (1960). J. Chem. Soc. 2444.

Bohuon, C. and Guerinot, F. (1966). Clin. Chem. Acta. 14, 414.

Bondurant, R.E., Greer, M. and Williams, C.M. (1966). Anal. Biochem. 15, 364.

Booth, A.N., Emerson, O.H. Jones, F.J. and De Eds, F. (1957). J. biol. Chem. 229, 51.

Booth, A.N. and Williams, R.T. (1963). Biochem. J. 88, 66p.

Booth, A.N. and Williams, R.T. (1963). Nature, Lond. 198, 684.

Booth, A.N., Masri, M.S., Robbins, D.J., Emerson, O.H., Jones, F.T., and De Eds, F. (1960). J. biol. Chem. 235, 2649.

Breese, G.R., Chase, T.N. and Kopin, I.J. (1968). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 27, 399.

Breese, G.R., Chase, T.N., and Kopin, I.J. (1969). J. Pharmac. exp. Ther. 165, 9.

Bremer, W., Jaeniche, V. and Leupold, D. (1969). Clin. chim. Acta. 23, 244.

Brenda, C.E. and Cobb, S. (1942). Medicine, Baltimore, 21, 95.

Brenneman, A.R. and Kaufman, S. (1964). Biochem. biopys. Res. Commun. 17, 177.

Brett, E.M., Oppé, T.E., Ruthven, C.R.J. and Sandler, M. (1964). Archs. Dis. Childh. 39, 403.

Bridgers, W.F., and Kaufman, S. (1962). J. biol. Chem. 237, 526.

Brodie, B.B., Tomich, E.G., Kuntzman, R. and Shore, P.A. (1957). J. Pharmac. exp. Ther. 119, 461.

Brodie, B.B., Olin, J.S., Kuntzman, R. and Shore, P.A. (1957). Science, N.Y. 125, 1293.

Brook, J.L., Biggs, R.H., St.John, P.A. and Anthony, D.S. (1967). Analyt. Biochem. 18, 453.

Brown, K.A., and Zuelzer, W.W. (1958). J. Clin. Invest. 37, 332.

Bumpus, F.M. and Page, I.H. (1955). J. Biol. Chem. 212, 111.

Burger, M. and Langemann, H. (1956). Klin. Wschr. 34, 941.

Burn, J.H. and Rand. M.J. (1957). Lancet, ii, 1097.

Calne, D.B., Karoum, F., Ruthven, C.R.J. and Sandler, M. (1969_a). Br. J. Pharmac. Chemother. 37, 57.

Calne, D.B., Stern, G.M., Laurence, D.R., Sharkey, J. and Armitage, P. (1969_b). Lancet. i, 744.

Calne, D.B., Spiers, A.S.D., Stern, G.M., Laurence, D.R. and Armitage, D. (1969_c). Lancet. ii, 973.

Cammarata, P.S. and Cohen, P.P. (1950). J. biol. Chem. 187, 439.

Campbell, A.C.P., Gowenlock, A.H., Platt, D.S., and Snow, P.J.D. (1963). Gut. 4, 61.

Cavellakis, Z.N. and Cohen, P.P. (1956). J. biol. Chem. 222, 53.

Carlsson, A. (1959). Pharmac. Rev. 11, 490.

Carlsson, A. (1966). In: Handbook of Experimental Pharmacology, ed. O. Eichler and A. Farah, XIX, 529, Springer-Verlag, Berlin.

Carlsson, A. and Hillarp, N-A. (1956). K. Fysiogr. Sallk. Lund. Forh, 26. No. 8.

Carlsson, A. and Hillarp, N-A. (1962). Acta Physiol. Scand. 52, 95.

Carlsson, A. Hillarp, N-A. and Hokfelt, B. (1957). J. biol. Chem. 227, 243.

Carlsson, A., Hillarp, N-A. and Waldeck, B. (1962). Med. exp. (Basel), 6, 47.

Carlsson, A., Hillarp, N-A. and Waldeck, B. (1963). Acta. Physiol. Scand. 59, Suppl. 215, 1.

Carlsson, A. Lindqvist, Magnusson, T. and Waldeck, B. (1958). Science, N.Y., 127, 471.

Carlsson, A., Lindqvist and Magnusson, T. (1960). In. Ciba symposium on adrenergic mechanisms, ed. G.E.W. Wolstenholme and M. O'Connor, 434, Churchill, Lond.

Carlsson, A., Rosengren, E., Bertler, A. and Nilsson, J. (1957). In: Psychotropic Drugs, ed. S. Garattini and V. Ghetti, 363, Elsevier, Amsterdam.

Carlsson, A., Shore, P.A. and Brodie, B.B. (1957b). J. Pharmac. exp. Ther. 120, 334.

Catroux, G. and Blachere, H. (1966). Compt. Rend., Serie, D. 262, 1345.

Ceasar, P.M., Ruthven, C.R.J. and Sandler, M. (1969). Br. J. Pharmac. Chemother. 36, 70.

Chadwick, B.T. and Wilkinson, J.H. (1960). Biochem. J. 70, 102.

Chambaz, E.M. and Horning, E.C. (1969). Anal. Biochem. 30, 7.

Chidsey, C.A., Kaiser, G.H. and Braundwald, E. (1963). Science, 139, 828.

Clark, W.G. (1963). In: Metabolic Inhibitors. ed. by R.M. Hochster and J.H. Quastel, 1, 315, Academic Press, Inc. Lond. N.Y.

Clark, W.G. and Pogrund, R.S. (1961). Circulation Res. 9, 721.

Clark, C.T., Weissbach, H. and Udenfriend, S. (1954). J. biol. Chem. 210, 139.

Collins, G.G.S., Youdim, M.B.H., and Sandler, M. (1958). FEBS Letters, 1, 215.

Contractor, S.F. (1966). Biochem. Pharmac. 15, 1701.

Contractor, S.F., Sandler, M. and Wragg, J. (1964). Life. Sci. 3, 999.

Cooper, J.R. and Melcer, I. (1961). J. Pharmac. exp. Ther. 132, 265.

Coq, M.H. and Baron, C. (1964). Experientia, 20, 374.

Costa, A., Boullin, D.J., Hammer, W., Vogel, W. and Brodie, B.B. (1966). Pharmac. Rev. 18, 577.

Cotzias, G.C., Papavasiliou, P.S. and Gellene, R. (1969). New Engl. J. Med. 280, 337.

Cotzias, G.C., Papavasiliou, P.S., Gellene, R., Aronson, R.B. and Mena, I, (1969). In: Third symposium on Parkinson's Disease, p.178. E and S Livingston Ltd. Edinburgh and Lond.

Cotzias, G.C., van Woert, M.H. and Schiffer, L.M. (1967). N. Engl. J. Med. 276, 374.

Coward, R.F. and Smith, P. (1968). Biochem. Med. 2, 216.

Coward, R.F. and Smith, P. (1968_a). J. Chromat. 37, 111.

Coward, R.F. and Smith, P. (1968_b). Biochem. Med. 2, 216.

Coward, R.F., Smith, P. and Middleton, J.E. (1967). Nature, Lond. 213, 520.

Cramer, K., Cramer, H and Selander, S. (1967). Clin. chim. Acta. 15,331.

Creveling, C.R., Daly, J.W., Witkop, B. and Udenfriend,S.(1962). Biochim. biophys, Acta, 64, 125.

Creveling, C.R., Van Der Schot, J.B. and Udenfriend, S. (1962_a). Biochem. biophys. Res. Commun. 8, 215.

Crout, J.R. (1966). Pharmac. Rev. 18, 651.

Crout, J.R. (1968). Anesthesiology, 29, 661.

Crout, J.R. and Sjoerdsma, A. (1964). J. Clin. Invest. 43, 94.

Crout, J.R., Pisano, J.J. and Sjoerdsma, A. (1961). Am. Heart J. 61, 375.

Curzon, G. (1957). Archs. Biochem. biophys. 66, 497.

Dacre, J.C. and Williams, R.T. (1968). J. Pharm. Pharmac. 20, 610.

Dacre, J.C., Scheline, R.R. and Williams, R.T. (1968). J. Pharm. Pharmac. 20, 619.

Dagirmanjian, R., Laverty, R., Mantegazzini, P., Sharman, D.F. and Vogt. M. (1963). J. Neurochem. 10, 177.

Dalgliesh, C.E. (1955_a). Adv. Protein Chem. 10, 31.

Dalgliesh, C.E. (1955_b). Adv. Protein Chem. 10, 79.

Dalgliesh, C.E. (1955_c). Adv. Protein Chem. 10, 110.

Dalgliesh, C.E. (1958). In: Advances in Clinical Chemistry. 1, 193. Academic Press. Inc. N.Y.

Dalgliesh, C.E., Horning, E.C., Horning, M.G., Knox, K.L. and Yarger, K. (1966). Biochem. J. 101, 792.

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

Dargeon, H.W. (1962). J. Pediat. 61, 456.

Davis, R.B. and Rosenberg, J.C. (1961). Am. J. Med. 30, 167.

Davis, V.E., Brown, H., Huff, J.A. and Cashaw, J.L. (1967_a). J. Lab. clin. Med. 69, 132.

Davis, V.E., Brown, H., Huff, J.A. and Cashaw, J.L. (1967_b). J. Lab. clin. Med. 69, 787.

Davis, V.E., Cashaw, J.L., Huff, J.A., Brown, H. and Nicholas, N.L. (1967_c). Proc. Soc. exp. Biol. Med. 125, 1140.

Davis, V.E., Cashaw, J.L., Huff, J.A. and Brown, H. (1966). Proc. Soc. exp. Biol. Med. 122, 890.

Davis, P., Peart, W.S. and van't Hoff, W. (1955). Lancet. ii, 274.

Dayman, J. and Jepson, J.B. (1969). Biochem. J. 113, 11p.

de Champlain, J., Axelrod, J., Krakoff, L.P. and Mueller, R.A. (1968). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 27, 399.

De Eds, F., Booth, A.N., and Jones, F.T. (1957). J. biol. Chem. 225, 615.

De Laey, P., Hooft, C., Timmermans, J. and Snoek, J. (1964). Annals. Pediat. 202, 145.

De Potter, W.P., Bacq. Z.M., Criel, G., De Schaepdryver, A.F. and Renson, J. (1963). Biochem. Pharmac. 12, 661.

Dengler, H.J., Michaelson, I.A., Spiegel, H.E. and Titus, E.O. (1962). Int. J. Neuropharmac. 1, 23.

DeQuattro, V.L. and Sjoerdsma, A. (1967). Clin. chim. Acts, 16, 227.

DeQuattro, V.L., Nybenga, D., von Studnitz, W. and Brunjes, S. (1964). J. Lab. clin. Med. 63, 864.

Dew, C.M. and McNesky, J.R. (1965). In: Vapour Phase Chromatography. ed. D.H. Desty, p. 213, Butterworths, London.

Dicke, W.K., Kingma, B.E., Wadman, S.K., de Jongh, S.E. and Nooch, E.L. (1959). Presented at the IXth Intern. Cong. of Pediatrics, Montreal.

Dirscherl, W. and Wirtzfeld, A. (1964). Z. Physiol. Chem. 336, 81.

Doft, F.S. (1965). Paper presented to NATO Advanced Study Institute, in Hartley Wintney, Hampshire. England, August. 1965.

Donaldson, R.M., Jr., Gray, S.J., and Letsou, V.C. (1959). Lancet, ii, 1002.

Donath, A., Kaser, H., Roos, B., Ziegler, W. Oetliker, O., Colombo, J.P. and Bettex, M. (1965). Heh. Pediat. Acta. 20, 1.

Drujan, B.D., Alvarez, N. and Borges, J.M. (1966). Analyt. Biochem. 15, 8.

Duchoň, J., Matouš, B and Procházková, B. (1967). Clin. chim. Acta. 18, 487.

Eccleston, D., Moir, A.T.B., Reading, H.W., and Ritchie, I.M. (1966). Br. J. Pharmac. Chemother. 28, 367.

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

Engelman, K. and Sjoerdsma, A. (1964). J. Am. med. Asso. 189, 81.

Engelman, K., Lovenberg, W. and Sjoerdsma, A. (1967). New Engl. J. Med. 277, 1103.

Erspamer, V. (1954). Pharmac. Rev. 6, 425.

Erspamer, V. (1954$_a$) in Ciba Foundation symposium on Hypertension, London: Churchill, ed. G.E.W. Wolstenholme and M.P. Camerson, p. 78.

Erspamer, V. (1955). J. Physiol., Lond. 127, 118.

Erspamer, V. (1956). Experientia, 12, 63.

Erspamer, V. (1961). Fortschr. Arzneimittlforsch. 3, 151.

Erspamer, V. (1966). In: Handbook of Experimental Pharmacology. ed. O. Eichler and A. Farah. Springer, Berlin. 19, 113.

Erspamer, V. and Asero, B. (1952). Nature Lond. 169, 800.

Erspamer, V. and Bertaccini, G. (1962). Archs. int. Pharmacodyn. 137, 6.

Erspamer, V. and Ciceri, C. (1957). Experientia, 13, 87.

Erspamer, V. and Glässer, A. (1960). Ateneo parmense, 31, 511.

Erspamer, V., Glässer, A. and Nobili, M.B. (1961). Archs. Biochem. 93, 673.

Erspamer, V., Glässer, A., Pasini, C. and Soppani, G. (1961). Nature Lond. 187, 483.

Erwin, V.G. and Hellerman, L. (1967). J. biol. Chem. 242, 4230.

Erwin, V.G. and Deitrich, A. (1966). J. biol. Chem. 241, 3533.

Ewin, A.I. and Laidlaw, P.P. (1910). J. Physiol., Lond. 41, 78.

Euler, V.S. von, (1956). "Noradrenaline" Springfield, Illinois.

Evans, R.S. (1963). Chimia, 17, 137.

Everett, S.D. (1964). J. Pharm. Pharmac. 16, 767.

Falck, B., Häggendal, J. and Owman, C. (1963). Q. Jl exp. Physiol. 48, 253.

Falck, B., Owman, C. and Sjostrand, N-O. (1965). Experientia, 21, 98.

Feldstein, A. and Williams, O. (1968). In: Advances In Pharmacology. ed by S. Garattini, P.A. Shore, E. Costa, and M. Sandler, 6, Part A. 91.

Feldstein, A. and Wong, K.K.K. (1965_a). Life Sci. 4, 183.

Feldstein, A. and Wong, K.K.K. (1965_b). Analyt. Biochem. 11, 467.

Fellman, J.H. (1959). Enzymologia, 20, 366.

Fellman, J.H., Severson, L.J., Robinson, E.H. and Fujita, T.S. (1962). Am. J. clin. Path. 38, 651.

Fischer, P. and Lecomte, J. (1956). C.r. Séanc. Soc. Biol. 150, 1026.

Fonnum, F., Haavaldsen, R. and Tengen, O. (1964). J. Neurochem. 11, 109.

Fowlis, A., Maggs, R.J. and Scott, R.P.W. (1964). J. Chromat. 15, 471.

Freedman, R.W. and Charlier, G.O. (1964). Analyt. Chem. 36, 1880.

Freedland, R.A., Wadzinski, H.A. and Waisman, H.A. (1961). Biochem. biophys. Res. Commun. 5, 94.

Friedhoff, A.J., Hekimian, L., Alpert, M. and Tobach, E. (1963). J. Amer. med. Ass. 184, 285.

Friedman, S. and Kaufman, S. (1965). J. biol. Chem. 240, PC552.

Friedman, S. and Kaufman, S. (1965_a). J. biol. Chem. 240, 4763.

Friedhoff, A.J. and Furiya, K. (1967). Nature, Lond. 214, 1127.

Freyburger, W.A., Graham, B.E., Rapport, M.M., Seay, P.A., Govier, W.M., Swoap, O.E. and Vander Brook, M.J. (1952). J. Pharmac. exp. Ther. 105, 80.

Fuller, R.W. and Hunt, J.M. (1967). Life Sci. 6, 1107.

Gaddum, J.H. (1953_a). J. Physiol., Lond. 121, 15p.

Gaddum, J.H. (1954_a). Ciba Foundation on Hypertension, ed. G.E.W. Wolstenholme and M.P. Camerson, p.75.

Gaddum, J.H. and Giarman, N.J. (1956). Br. J. Pharmac. Chemother. 11, 88.

Gal, E.M. (1965). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 24, 580.

Gal, E.M. Armstrong, J.C. and Ginsberg, B. (1966). J. Neurochem. 13, 643.

Garattini, S. and Valzelli, L. (1965). Serotonin. Elsevier, Amsterdam.

Garattini, S., Shore, P.A., Costa, E. and Sandler, M. (1968). Advances in Pharmacology. 6, Part A. Academic Press, N.Y.

Georges, R.J. (1964). Clin. chim. Acta. 10, 583.

Gerstenbrand, F. and Pateisby K. (1962). Wien. Z. Nervenheilk, 20, 90.

Gessner, P.K., Khairallah, P.A., McIssac, W.M. and Page, I.H. (1960). J. Pharmac. exp. Ther. 130, 126.

Gey, K.F. (1968). Abstract. Fifth meeting. Federation of European Biochemical Societies Praha. No. 472, 118.

Gey, K.F. and Pletscher, A. (1964). Biochem. J. 92, 30C.

Giachetti, A. and Shore, P.A. (1966). Life Sci. 5, 1373.

Gibbs, N.K. and Woolf , L.I. (1959). Br. Med. J. ii, 532.

Gitlow, S.E., Mendlowitz, M., Kruk, E. and Khassis, S. (1961). Circulation Res. 9, 746.

Gitlow, S.E., Mendlowitz, M., Wilk, E.K., Wilk, S., Wolf, R.L. and Bertani, L.M. (1968). J. Lab. clin. Med. 72, 612.

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

Gjessing, L.R. (1963_a). Scand. J. clin. Lab. Invest. 15, 649.

Gjessing, L.R. (1964). Scand. J. clin. Lab. Invest. 16, 661.

Gjessing, L.R. (1966). In: Recent Results In Cancer Research. Neuroblastoma Biochemical Studies. ed. C. Bohuon, p. 26, Springer-Verlag, Berlin, Heidelberg, N.Y.

Gjessing, L.R. (1966_a). Scand. J. clin. Lab. Invest. 18, 540.

Gjessing, L.R. (1968). In: Advances In Clinical Chemistry. ed. O. Bodansky and C.P. Stewart, 11, Academic Press, Inc. N.Y.

Gjessing, L.R. and Borud, O. (1964_a). Lancet, ii, 818.

Gjessing, L.R. and Borud, O. (1965). Scand. J. clin. Lab. Invest. 17, 80.

Glasstone, S. (1960). Textbook of Physical Chemistry, MacMillan, London 2nd. ed.

Glowinski, J. and Baldessarini, R.J. (1966). Pharmac. Rev. 18, 1201.

Glowinski, J. Kopin, I.J. and Axelrod, J. (1965). J. Neurochem. 12, 25.

Goldstein, M. (1962). Experientia, 18, 334.

Goldstein, M. and Contrera, J.F. (1961). Experientia, 17, 447.

Goldstein, M., Gang, H. and Joh, T.H. (1969). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 28, 287.

Goldstein, M., Lauber, E. and McKereghan, M.R. (1965). J. biol. Chem. 240, 2066.

Goldstein, M., Prochoroff, N.N. and Sirlin, S. (1965_a). Experientia, 21, 592.

Goldstein, M., Lauber, E., Blumberg, W.E. and Peisach, J. (1965_b). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 24, 604.

Goldstein, M., McKereghan, M.R., and Lauber, E. (1963). Biochem. biophys. Acta. 77, 161.

Goldstein, L.B., Friedhoff, A.J., Simmons, C. and Prochoroff, N. (1960). Proc. Soc. exp. Biol. Med. 103, 137.

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

Goldstein, M., Friedhoff, A.J., Pomerantz, S. and Simmons, C. (1960_a). Biochem. biophys. Acta. 39, 189.

Goldstein, M., Musacchio, J.M., Kenin, M.C., Contrera, J.F., and Rice, M.D. (1962_a). Biochem. Pharmac. 11, 809.

Goodman, L.S. and Gilman, A. (1965). In: The Pharmacological Basis of Theraputics, MacMillan, N.Y.

Goodall, McC. and Kirshner, N. (1957). J. biol. Chem. 226, 213.

Goodall, McC. and Kirshner, N. (1958). Circulation, 17, 366.

Goodall, McC. and Alton, H. (1965). J. Pharmac. exp. Ther. 14, 1595.

Goodwin-Austen, R.B. Tomlinson, E.R., Fears, C.C. and Kok, H.K.L. (1969). Lancet. ii, 165.

Gorkin, V.Z. (1963). Nature Lond 200, 77.

Gorkin, V.Z. (1966). Pharmac. Rev. 18, 115.

Gorkin, V.Z. and Romanova, L.A. (1959). Biokhimiya, 24, 826.

Graham, J.B. (1951). Surgery Gynec. Obstet. 92, 105.

Grahame-Smith, D.G. (1964_a). Biochim. biophys. Acta, 86, 176.

Grahame-Smith, D.G. (1964_b). Biochem. biophys. Res. Commun. 16, 586.

Grahame-Smith, D.G. (1964_c). Biochem. J. 92, 52p.

Grahame-Smith, D.G. (1967). Biochem, J. 105, 351.

Grahame-Smith, D.G. and Moloney, L. (1965). Biochem. J. 96, 66p.

Green, A.L. (1964). Biochim. biophys. Acta. 81, 396.

Green, H. (1968). In: Advances In Pharmacology. ed. S. Garattini, P.A. Shore, A. Costa and M. Sandler. 6, Part A, 18.

Green, H and Sawyer, J.L. (1965). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 24, 604.

Green, H. and Sawyer, J.L. (1966). Analyt. Biochem. 15, 53.

Greenberg, R.E. and Gardner, L.I. (1959). Pediatrics, Springfield, 24, 683.

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

Greenberg, R.E. and Lind. J. (1961). Pediatrics, Springfield, 27, 904.

Greenfield, J.G. (1955). In: Neuropathology, 529, Edwards, Arnold, Lond.

Greenstein, J.P. and Winitz, M. (1961). In: Chemistry of Amino Acids. 311, John Wiley, N.Y.

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

Greer, M. and Williams, C.M. (1967). Analyt. Biochem. 19, 40.

Greer, M., Anton, A.H., Williams, C.M. and Echevarria, R.A. (1965). Archs. Neurol., Chicago, 13, 139.

Gross, R.E., Farber, S. and Martin, L.W. (1959). Pediatrics. Springfield, 23, 1179.

Grunwald, C., Vendrell, M. and Stowe, B.B. (1967). Analyt. Biochem. 20, 484.

Guggenheim, M. (1913). Hoppeseyler's Z. Physiol. Chem. 88, 276.

Guha, S.R. and Kirshna Murti, M. (1965). Biochem. biophys. Res. Commun. 18, 350.

Gurd. M.R. (1937). Quart. Jl. Pharm. Pharmac. 10, 188.

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

Guroff, G., Daly, J.W., Jerina, D.M., Renson, J., Witkop, B. and Udenfriend, S. (1967). Science, N.Y. 157, 1524.

Haavaldsen, R. (1964). Biochem. J. 92, 23p.

Haavaldsen, R. and Norseth, T. (1965). In: Symposium on Tyrosinosis, ed. L.R. Gjessing, Universitetsforlaget, Oslo, 50.

Hagen, P. (1956). J. Pharmac. exp. Ther. 116, 26.

Hagen, P. (1962). Br. J. Pharmac. Chemother. 18, 175.

Hagen, P.B. and Cohen, L.H. (1966). In: Handbook of Experimental Pharmacology. ed. C. Eichler and A. Farah. 19, 182, Springer-Verlag. Berlin. Heidelberg. N.Y.

Hagen, P. and Lee, F.L. (1958). J. Physiol., Lond. 143, 7 B.

Hagen, P., Ono, S. and Lee, F.L. (1960). J. Pharmac. exp. Ther. 130, 9.

Hager, S.E., Gregerman, R.I. and Knox, W.E. (1957). J. biol. Chem. 225, 935.

Haggendal, J. and Lindqvist, M. (1961). Acta Pharmac. (Kbh). 18, 278.

Halvorsen, K. and Halvorsen, S. (1963). Pediatrics, Springfield, 31, 29.

Hanson, A. (1966). In: Handbook of Experimental Pharmacology. ed. O. Eichler and A. Farah, Vol. 19, p.66.

Happold, F.C. (1950). Adv. Enzymol. 10, 51.

Harriman, D.G.F. (1966). Hospital Medicine, Oct. 17.

Hartmann, W.J. and Clark, W.G. (1953). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 12, 62.

Hartmann, W.J., Akawie, R.W., and Clark, W.C. (1955). J. biol. Chem. 216, 507.

Hassler, R. (1938). J. Physiol. Neurol., Leipzig, 48, 387.

Hedgley, E.J. and Overend, W.G. (1960). Chem. Ind. 378.

Heath, J.W. (1947). Archs.Neurol. Psychiat., Chicago, 58, 484.

Heilmeyer, L and Clotten, V.R. (1958). Dt. med. Wschr. 83, 617.

Herrlich, P. and Sekeris, C.E. (1963). Biochim. biophys. Acta, 78, 750.

Hess, S., Redfield, B.G. and Udenfriend, S. (1959). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 18, 402.

Higuchi, H. (1962). Jap. J. Pharmac. 12, 34.

Hinterberger, H. and Bartholomew, R.J. (1969). Clin. chim. Acta. 23, 169.

Hirschmann, J. and Mayer, K. (1964). Dt. med. Wschr. 89, 187.

Holtz, P. (1939). Naturwissenschaften, 27, 724.

Holtz, P. (1959). Pharmac. Rev. 11, 317.

Holtz, P. and Credner, K. (1942). Archs. exp. Path. Pharmak. 200, 356.

Holtz, P., Heise, R. and Ludtke, K. (1938). Archs. exp. Path. Pharmak. 191, 87.

Holzbauer, M. and Vogt, M. (1956). J. Neurochem. 1, 8.

Hopkins, F.G. and Cole, S.W. (1902). J. Physiol. Lond. 27, 418.

Horii, Z., Makita, M., Takeda, I., Tamura, Y. and Chnishi, Y. (1965). Chem. Pharmac. Bull. 13, 636.

Horita, A. (1967). Nature, Lond. 215, 411.

Horning, M.G., Boucher, E.A. and Moss A.M. (1967). J. Gas Chromatog. 5, 297.

Horning, E.C., Horning, M.G., Vanden Heuvel, W.J.A., Knox, K.L., Holmstedt, B. and Brooks, C.J.W. (1964). Analyt. Chem. 36, 1546.

Horning, M.G., Knox, K.L., Dalgliesh, C.E. and Horning, E.C. (1966). Analyt. Biochem. 17, 244.

Horning, E.C., Sweeley, C.C., Dalgliesh, C.E., and Kelly, W. (1959). Biochim. biophys. Acta. 32, 566.

Hornykiewicz, O. (1962). Germ. med. Mon. 10, 344.

Hornykiewicz, O. (1963). Wein, Klin. Wschr. 75, 309.

Hornykiewicz, O. (1966). Pharmac. Rev. 18, 925.

Hormitz, D., Fox, S.M., Goldberg, L.I. (1962). Circulation Res. 10, 237.

Hosoda, S. and Glick, D. (1966). J. biol. Chem. 241, 192.

Howard, W.F., McDevitt, P.I. and Stander, H.W. (1964). Am. J. Obstet. Gynec. 89, 615.

Hughes, F.B. and Brodie, B.B. (1959). Experientia, 14, 178.

Hughes, F.B., Shore, P.A. and Brodie, B.B. (1958). Experientia, 14, 178.

Ichiyama, A., Nakamura, S., Nishizuka, Y and Hayaishi, O. (1968). In: Advances In Pharmacology, ed. S. Garattini., P.A. Shore, E. Costa, and M. Sandler, 6, Part A.5,Academic Press. p.5.

Iisola, E. and Castren, O. (1967). Annls. Med. exp. Biol. Fenn. 45, 253.

Ikeda, M., Levitt, M. and Udenfriend, S. (1965). Biochem. biophys. Commun. 18, 482.

Ikeda, M., Levitt, M. and Udenfriend, S. (1967). Archs. Biochem. Biophys. 120, 420.

Imaizumi, R., Yoshida, H. and Kita, T. (1958). Jap. J. Pharmac. 8, 9.

Inscoe, J.K. Daley, J. and Axelrod, J. (1965). Biochem. Pharmac. 14, 1257.

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

Iverson, L.L. (1967). The Uptake And Storage of Noradrenaline In Sympathetic Nerves. Cambridge University Press.

Iverson, L.L. (1967_a). Nature, Lond. 214, 8.

Iverson, L.L. (1969). J. Physiol. Lond. 201, 1 p.

Izumi, F., Oka, M., Yoshida, H. and Imaizumi, R. (1967). Life Sci. 6, 2333.

Izumi, K., Oka, M., Yoshida, H. and Imaizumi, R. (1969). Biochem. Pharmac. 18, 1738.

Jacoby, G.A. and LaDu, B. (1962). Biochem. biophys. Res. Commun. 8, 352.

Jaenicke, J. and Bremer, H.J. (1966). Z. Kinderheilk, 97, 281.

Jere, R.S. (1950). Proc. Soc. exp. Biol. Med. 74, 148.

Jepson, J.B. (1955). Lancet, ii, 1009.

Jepson, J.B. (1966). In: The Metabolic Basis of Inherited disease, ed. J.B. Stanbury, J.S. Wyngaarden and D.S. Fredrickson, 2nd edn. 1283, McGraw-Hill. N.Y.

Jepson, J.B. (1969). In: Chromatographic and Electrophoretic Techniques. ed. I. Smith, i, 243, 3rd. edn, Heinemann, London.

Jepson, J.B., Udenfriend, S. and Zaltzman, P. (1959). Fedn. Proc. Am. Socs. Exp. Biol. 18, 254.

Jepson, J.B., Zaltzman, P. and Udenfriend, S. (1962). Biochim. biophys. Acta, 62, 91.

Jequier, E., Robinson, D.S., Lovenberg, W. and Sjoerdsma, A. (1969). Biochem. Pharmac. 18, 1071.

Johansson, B. and Roos, B.E. (1967). Life.Sci. 6, 1449.

Kähane, Z., Mowat, J.H. and Vestergaard, P. (1969). Clin. chim. Acta. 26, 307.

Kahler, H.J. and Heilmeyer, L. (1961). Ergebn. Inn. Med. Kinderheilk, 16, 292.

Karoum, F., Anah, C.C., Ruthven, C.R.J. and Sandler, M. (1969). Clin. chim. Acta. 24, 341.

Karoum, F., Ruthven, C.R.J. and Sandler, M. (1968). Clin. chim. Acta. 20, 427.

Karoum, F. and Sandler, M. (1968). Proc. Assoc. Clin. Biochem. 5, 123.

Karlson, P., Sekeris, C.E. and Herrlich, P. (1963). Dt. Med. Wschr. 39, 1873.

Käser, H. (1966). Pharmac. Rev. 18, 659.

Käser, H., Bettex, M and von Studnitz, W. (1964). Archs. Dis. Childh. 39, 168.

Käser, H., Schweisguth, O., Sellei, K. and Spengler, G.A. (1963). Helv. chim. Acta. 30, 628.

Kaufman, S. (1957). J. biol. Chem. 226, 511.

Kaufman, S. (1958). J. biol. Chem. 230, 931.

Kaufman, S. (1959). J. biol. Chem. 234, 2677.

Kaufman, S. (1963_a). Proc. Natn. Acad. Sci. U.S.A. 50, 1085.

Kaufman, S. (1963_b). In: Oxygenases. ed. O. Hayaishi, 129, Academic Press. N.Y.

Kaufman, S. (1964). Trans. N.Y. Acad. Sci., 26, 977.

Kaufman, S. (1966). Pharmac. Rev. 18, 61.

Kaufman, S., Bridgers, W.P., Eisenberg. F. and Friedman, S. (1962). Biochem. biophys. Res. Commun. 9, 497.

Kaufman, S. and Friedman, S. (1965). Pharmac. Rev. 17, 71.

Kaufman, S. and Lovenberg, B. (1959). J. biol. Chem. 234, 2683.

Keglevic, D., Kveder, S. and Iskric, S. (1968). In: Advances In Pharmacology, ed. S. Garattini, P.A. Shore, E. Costa, and M. Sandler, 6, Part A, 79.

Kenney, F.T. (1959). J. biol. Chem. 234. 2707.

Kennedy, J.S., Synnington, T. and Woodger, B.A. (1961). J. Path. Bact. 81, 409.

King, R.L., Storaasli, J.P. and Bolande, R.P. (1961). Am. J. Roentg. 85, 733.

Kirberger, E. (1962). Dt. Med. Wchsr. 87, 929.

Kirshner, N. (1957). J. biol. Chem. 226, 821.

Kirshner, N. (1959). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 18, 261.

Kirshner, N. (1962). J. biol. Chem. 237, 2311.

Kirshner, N. (1962_a). Science, N.Y. 135, 107.

Kiselev, A.V. (1958). In: The Structure And Properties of Porous Materials, ed. D.H. Everett and F.S. Stone X, 257, Butterworth, Lond.

Klinkenberg A. and Sjeniter, F. (1956). Chem. Eng. Sci. 5, 258.

Kitabchi, A.E. and Williams, R.H. (1969). Biochim. biophys. Acta. 171, 181.

Klawans, M.L. and Weiner, W. (1969). 2nd int. meeting, Int. Soc. Neurochem. p. 245.

Knox, W.E. (1955). In: Symposium On Amino Acid Metabolism. ed. W.D. McElroy and H.B. Glass, Johns Hopkins, Baltimore.

Knox, W.E. (1966). In: The Metabolic Basis Of Inherited Disease. ed. J.B. Stanbury, J.S. Wyngaarden and D.S. Frederickson, 2nd. edn. 258, McGraw-Hill. N.Y.

Kogl, M., Haagen-Smit, A.J. and Erxleben, H. (1933). Z. physiol. Chem. 214, 241.

Koler, R.D., Vanbellinghen, P.J., Fellman, J.H., Jones, R.T. and Behrman, R.E. (1969). Science, N.Y., 163, 1348.

Kopin, I.J. (1966). In: Symposium On Mechanisms Of Release Of Biogenic Amines. ed. U.S. von Euler, S. Rosell and B. Uvnas, 229, Pergamon, Oxford.

Kopin, I.J. (1968). Anesthesiology, 29, 654.

Kopin, I.J. and Axelrod, J. (1960). Archs. Biochem. Biophys. 89, 148.

Kopin, I.J., Axelrod, J. and Gordon, E. (1961). J. biol. Chem. 236, 2109.

Kopin I.J., Fischer, J.E., Musacchio, J.M., Horst, W.D. and Weise, V.K. (1965). J. Pharmac. exp. Ther. 147, 186.

Kopin, I.J. and Gordon, E.K. (1962). J. Pharmac. expt. Ther. 138, 351.

Kopin, I.J. and Gordon, E.K. (1963). J. Pharmac. exp. Ther. 140, 207.

Kopin, I.J. and Weise, U.K. (1968). Biochem. Pharmac. 17, 1461.

Kretchmer, N., Levine, S.Z., McNamara, H. and Barnett, H.L. (1956). J. clin. Invest. 35, 236.

Kretchmer, N. andMcNamara, H. (1956). J. clin. Invest. 35, 1089.

Kuntzman, R., Shore, P.A., Bogdanski, D. and Brodie, B.B. (1961). J. Neurochem. 6, 226.

Kvale, W.F., Roth, G.M., Manger, W.M. and Priestley, J.T. (1956). Circulation, 14, 622.

Kveder, S., Iskrić, S. and Keglevic, D. (1962). Biochem. J. 85, 447.

Kveder, S. and Iskrić, S. (1965). Biochem. J. 94, 509.

La Brosse, E.H. (1968). J. pediat. Surg. 3, 148

La Brosse, E.H. (1969). VIIth. International congress of clinical chemistry, Geneve/Evian, 458.

La Brosse, E.H., Axelrod, J. Kopin, I.J. and Kety, S.S. (1961). J. clin. Invest. 40, 253.

La Brosse, E.H. and Karon, M. (1962). Excerpta Medica. Intern. Con. Series No. 48.

La Brosse, E.H. and Karon, M. (1962_a). Nature, Lond. 196, 1222.

La Du, B.N. (1966_a). In: The Metabolic Basis of Inherited Disease, ed. J.B. Stanbury, J.S. Wyngaarden and D.S. Frederickson 2nd edn. 295, McGraw-Hill, N.Y.

La Du, B.N. (1966_b). In: The Metabolic Basis of Inherited Disease, ed. J.B. Stanbury, J.S. Wyngaarden and D.S. Frederickson 2nd edn. 303, McGraw-Hill, N.Y.

Laduron, P. and Belpaire, F. (1968). Biochem. Pharmac. 17, 1127.

Langemann, H., Boner, A. and Müller, P.B. (1962). Schweiz. med. Wschr. 92, 1621.

Lagunoff, D., Lam, K.B., Roeper, E. and Benditt, E.P. (1957). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 16, 363.

Lancet (1969). i, 1133.

Langer, S.H., Pantages, P. and Wender, I. (1958). Chem. Ind. Lond. 1664.

Lapidus, L. and Amiundson, N.R. (1952). J. physiol. Chem. 56, 984.

Lathe, G.H. and Walker, M. (1958). Biochem. J. 70, 705.

Lathe, G.H. and Ruthven, C.R.J. (1958). clin. Path. 11, 155.

Leeper, L.C. and Udenfriend, S. (1956). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 15, 298.

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

Lembeck, F. (1953). Nature, Lond. 172, 910.

Lembeck, F. (1956). Schweiz. med. Wschr. 86, 943.

Levin, E.Y., Levenberg, B. and Kaufman, S. (1959). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 18, 272.

Levin, E.Y., Levenberg, B. and Kaufman, S. (1960). J. biol. Chem. 235, 2080.

Levin, E.Y. and Kaufman, S. (1961). J. biol. Chem. 236, 2043.

Levine, S.Z., Dann, M. and Marple, E. (1943). J. clin. Invest. 22, 551.

Levine, S.Z., Gordon, H.H. and Marples, E. (1941_a). J. clin. Invest. 20, 209.

Levine, S.Z., Marples, E. and Gordon, H.H. (1941_b). J. clin. Invest. 20, 199.

Levine, S.Z., Marples, E. and Gordon, H.H. (1939). Science, N.Y. 90, 620.

Levitt, M., Spector, S., Sjoerdsma, A. and Udenfriend, S. (1965). J. Pharmac. exp. Ther. 148, 1.

Lin, E.C.C. and Knox, W.E. (1957). Biochim. biophys. Acta. 26,86.

Liberti, A. (1958). In: Gas Chromatography, ed. D.H. Desty, 214, Butterworth, Lond.

Loewi, O. (1921). Arch. ges. Physiol. 189, 239.

Lord, B. (1962). In: Diseases of The Nervous System, Oxford Univ. Press, Lond.

Lovelock, J.E. (1958). J. Chromatog. 1, 35.

Lovelock, J.E. (1958_a). Nature, Lond. 182, 1663.

Lovelock, J.E. (1960). In: Gas Chromatography, ed. R.P.W. Scott, 26, Butterworth, Lond.

Lovenberg, W., Barchas, J., Weissbach, H. and Udenfriend, S. (1963). Archs. Biochem. Biophys. 103, 9.

Lovenberg, W., Jequier, E. and Sjoerdsma, A. (1967). Science, N.Y., 155, 217.

Lovenberg, W., Jequier, E. and Sjoerdsma, A. (1968). In: Advances In Pharmacology, ed. S. Garattini, P.A. Shore, E. Costa and M. Sandler, 6, Part A, 21, Academic Press, N.Y.

Lovenberg, W., Weissbach, H. and Udenfriend, S. (1962). J. biol. Chem. 237, 89.

Maggiolo, C. and Haley, T.J. (1964). Proc. Soc. exp. Biol. Med. 115, 149.

Malinoff, P. and Axelrod, J. (1969). Science, 164, 428.

Mandell, A.J. (1965). Nature, Lond. 207, 977.

Mannarino, E., Kirshner, N. and Nashold, B.S. (1963). J. Neurochem. 10, 373.

Marki, F., Axelrod, J. and Witkop, B. (1962). Biochim. biophys. Acta. 58, 367.

Martin, G.U., Brendel, R. and Beiler, J.M. (1950). Exp. Med. Surg. 8, 5.

Martin, A.J.P. and Synge, R.L.M. (1941). Biochem. J. 35, 1358.

Mathieu, P.P. and Revol, L. (1968). Ann. Biol. clin. 26, 383.

Mathews, J. and Partington, M.W. (1964). Archs Dis. Childh. 39, 371.

Maynert, E.W. and Isaac, L. (1968). In: Advances In Pharmacology, ed. S. Garattini, P.A. Shore, E. Costa and M. Sandler, 6, Part A 113, Academic Press, N.Y.

McDonald, R.K. and Weise, U.K. (1962). J. Pharmac. exp. Ther. 136, 26.

McGeer, P.L. and McGeer, E.G. (1964). Biochem. biophys. Res. Commun. 17, 502.

McGeer, P.L., Boulding, J.E., Gibson, W.C. and Foulkes, R.G. (1961). J. Am. med. Ass. 177, 665.

McIsaac, W.M. and Page, I.H. (1958). Science, N.Y. 128, 537.

McLennan, H. and York, D.H. (1967). J. physiol. 189, 393.

Meister, A. (1957). In: Biochemistry Of The Amino Acids, 1st. edn. 110, Academic Press, Inc. N.Y.

Meister, A. (1965). In: Biochemistry Of The Amino Acids. 2nd. edn. 841, Academic Press, Inc.

Mendez, J. and Stevenson, F.J. (1966). J. Gas Chromatog. 4, 483.

Menkes, J.H. and Avery, M.E. (1963). Bull Johns Hopkins Hosp. 113, 301.

Mitoma, C., Weissbach, H. and Udenfriend, S. (1955). Nature, Lond., 175, 994.

Mitoma, C., Weissbach, H. and Udenfriend, S. (1956). Archs. Biochem. Biophys. 63, 122.

Miyaka, H., Yashida, H. and Imaizumi, R. (1962). Jap. J. Pharmac. 163, 12.

Moorhead, E.L., Caldwell, J.R., Kelly, A.R. and Morales, A.R. (1966). J. Am. med. Ass. 196, 1107.

Mueller, R.A. and Shideman, F.E. (1963). J. Pharmac. exp. Ther. 163, 91.

Musacchio, J.M. and Goldstein, M. (1963). Biochem. Pharmac. 12, 1055.

Muscholl, E. and Vogt. M. (1958). J. physiol. Lond. 141, 132.

Müller, J.M., Schlittler, E. and Bein, H.J. (1952). Experientia 8, 338.

Nagatsu, T., Levitt, M. and Udenfriend, S. (1964). J. biol. Chem. 239, 2910.

Nagatsu, T., Lyman, A.R. and De Quattro, V. (1969). Biochem. Pharmac. 18, 1441.

Nakamura, S., Ichiyama, A. and Hayaishi, O. (1965). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 24, 604.

Nakao, A. and Ball, M. (1960). J. nerv. ment. Dis. 130, 417.

Nara, S. and Yasunobo, K.T. (1966). Pharmac. Rev. 18, 144.

Neff, N.H. and Costa, E. (1966). Life Sci. 5, 951.

Neri, R., Hayano, M., Stone, D., Dorfman, R.I. and Elmadjian, F. (1956). Archs. Biochem. Biophys. 60, 297.

Nicolopoulos, A., Agathopoulos, A., Danelatou-Athanassiadou, C. and Bafataki, M. (1968). Pediatrics, Springfield, 41, 777.

Noble, N.R. (1961). Med. J. Aust. ii, 589.

Novick, W.J. (1961). Endocrinology, 69, 55.

Numerof, P., Gordon, M. and Kelly, J.M. (1955). J. Pharmac. exp. Ther. 115, 427.

Oates, J.A. (1961). In: Methods of Medical Research. 9, 169.

Oates, J.A. and Sjoerdsma, A. (1962). Am. J. med. 32, 333.

Oates, J.A., Gillespie, L., Udenfriend, S. and Sjoerdsma, A. (1960). Science, N.Y. 131, 1890.

Oates, J.A., Melmon, K., Sjoerdsma, A., Gillespie, L. and Mason, D.T. (1964). Lancet, i, 514.

Oka, M., Kajikawa, K., Ohuchi, T., Yoshida, H. and Imaizumi, R. (1967). Life Sci. 6, 461.

Oliver, G. and Schäfer, E.A. (1894). J. Physiol. (Proc.) 18,

O'Reilly, S. (1969). Lancet i, 984.

Oswald, E.O. and Strittmater, C.F. (1963). Proc. Soc. exp. Biol. Med. 114, 668.

Owman, C. and Sjöstrand, N.O. (1965). Z. Zellforschi 66, 300.

Page, I.H. (1954). Physiol. Rev. 34, 563.

Page, I.H. (1958). Physiol. Rev. 38, 277.

Page, I.H., Corcoran, A.C., Udenfriend, S., Sjoerdsma, A. and Weissbach, H. (1955). Lancet, i, 198.

Partington, M.W. (1968). Biol. Neonat. 12, 316.

Pernow, B. and Waldenström, J. (1957). Am. J. Med. 23, 16.

Perry, T.L., Hansen, S. and MacDougall, L. (1966). Can. Med. Ass. J. 95, 89.

Perez-Silva, G., Rodriguez, D. and Perez-Silva, J. (1966). Nature, Lond. 212, 303.

Perry, T.L., Hestrin, M., McDougall, L. and Hansen, S. (1966). Clin. chim. Acta. 14, 116.

Petrack, B., Sheppy, F. and Fetzer, V. (1968). J. biol. Chem. 243, 743.

Pisano, J.J., Crout, J.R. and Abraham, D. (1962). Clin. chim. Acta, 7, 285.

Pisano, J.J., Creveling, C.R. and Udenfriend, S. (1960). Biochim. biophys. Acta. 43, 566.

Pierce, A.E. (1968). Silylation of Organic Compounds, Pierce Chemical Company, Rockford, Illinois.

Pletscher, A. (1966). Pharmac. Rev. 18, 121.

Pletscher, A., Shore, P.A. and Brodie, B.B. (1955). Science, N.Y. 122, 374.

Pletscher, A., Bartholini, G. and Tissot, R. (1967). Brain Res. 4, 106.

Plummer, A.J., Sheppard, H. and Schulert, A.R. (1957). In: Psychotropic Drugs, ed. S. Garattini and V. Ghetti, 350, Elsevier, Amsterdam.

Pohorecky, L.A., Zigmond, M., Karten, H. and Wurtman, R.J. (1969). J. Pharmac. exp. Ther. 165, 190.

Potter, L.T. and Axelrod, J. (1963). J. Pharmac. exp. Ther. 140, 199.

Potter, L.T. and Axelrod, J. (1963). J. Pharmac. exp. Ther. 142, 299.

Potter, L.T. and Parrish, J.M. (1942). Am. J. Phath. 18, 141.

Posner, H.S., Mitoma, C. and Udenfriend, S. (1961). Archs. Biochem. Biophys. 94, 269.

Powell, L.E. (1964). Plant. Physiol., 39, 836.

Prange, A.J., White, Jr. J.E., Lipton, M.A. and Kinkead, A.M. (1967). Life Sci. 6, 581.

Purnell, H. (1962). In: Gas Chromatography, John Wiley. N.Y.

Pscheidt, G.R., Berlet, H.H., Spaide, J. and Himwich, H.E. (1966). Clin. chim. Acta. 13, 228.

Quay, W.B. (1968). In: Advances In Pharmacology, ed. S. Garattini, P.A. Shore, E. Costa and M. Sandler, 6, 283.

Ranadive, N.S. and Sehon, A.H. (1967_a). Canad. J. Biochem. 45, 1681.

Ranadive, N.S. and Sehon, A.H. (1967_b). Canad. J. Biochem. 45, 1689.

Ranadive, N.S. and Sehon, S.H. (1967_c). Canad. J. Biochem. 45, 1701.

Rand, M. and Reid, G. (1951). Nature, Lond. 168, 385.

Renson, J., Goodwin, F., Weissbach, H. and Udenfriend, S. (1961). Biochem. biophys. Res. Commun. 6, 20.

Renson, J., Weissbach, H. and Udenfriend, S. (1962). J. biol. Chem. 237, 2261.

Rinne, U.K. and Sonninen, V. (1968). Ann. Med. Int. Fenn. 57, 105.

Rinne, U.K., Laitinen, L. and Sonninen, V. (1967). Neurology, Minneap. 153, 345.

Robinson, R. (1966). In: Recent Results In Cancer Research, Neuroblastoma Biochemical Studies, ed. C. Bohuon, 37.

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

Robinson, R., Smith, P. and Whittaker, S.R.F. (1964). Br. med. J. i, 1422.

Rodnight, R. (1956). Biochem. J. 64, 621.

Rodnight, R. (1961). Int. Rev. Neurobiol. 3, 251.

Rodnight, R. and McIlwain, H. (1956). Br. med. J. i, 108.

Roos, B.E. and Werdinius, B. (1964). Life. Sci. 3, 351.

Rosengren, E. (1960). Acta physiol. Scand. 49, 370.

Rosenstein, B.J. and Engelman, K. (1963). J. Pediat. 63, 217.

Rowland, M. and Riegelman, S. (1967). Abalyt. Biochem. 20, 463.

Ruthven, C.R.J. (1963). In: The Clinical Chemistry Of Monoamines, ed. H. Varley and A.H. Gowenlock 39, Elsevier, Amsterdam.

Ruthven, C.R.J. (1965). Ph. D. Thesis University of London.

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

Ruthven, C.R.J. and Sandler, M. (1964). Analyt. Biochem. 8, 282.

Ruthven, C.R.J. and Sandler, M. (1965). Clin. chim. Acta. 12, 318.

Ruthven, C.R.J. and Sandler, M. (1966). Clin. chim. Acta. 14, 511

Rutledge, C.O. and Johnason, J. (1967). J. Pharmac. exp. Ther. 157, 493.

Rutledge, C.O. and Weiner, N. (1967). J. Pharmac. exp. Ther. 157, 290.

Sokamoto, Y., Uchida, M. and Ichihara, K. (1953). Med. J. Osaka Univ. 3, 477.

Sano, I., Gamo, T., Kakimoto, Y., Taniguchi, K., Takesada, M. and Nishinuma, K. (1959). Biochim. biophys. Acta. 32, 586.

Sandler, M. (1963). In: The Clinical Chemistry of Monoamines, ed. H. Varley and A.H. Gowenlock, 116, Elsevier, Amsterdam.

Sandler, M. (1967). Proc. R. Soc. Med. 60, 1.

Sandler, M. (1968). Annls. Med. exp. Biol. Fenn. 46, 479.

Sandler, M. (1968_a). In: Advances In Pharmacology, ed. S. Garattini, P.A. Shore, E. Costa and M. Sandler, 6, Part B. 127, Academic Press, Inc. p. 127.

Sandler, M. Karim, S.M.M. and Williams, E.D. (1968). Lancet, ii, 1053.

Sandler, M. and Ruthven, C.R.J. (1959_a). Lancet, ii, 114.

Sandler, M. and Ruthven, C.R.J. (1959_b). Lancet, ii, 1034.

Sandler, M. and Ruthven, C.R.J. (1961). Biochem. J. 80, 78.

Sandler, M. and Ruthven, M. (1961_a). Proc. Fourth int. Congr. clin. Chem. ed. C.P. Stewart, P.C. Jocelyn and S.C. Frazer, 188, Livingstone, Edinburgh.

Sandler, M. and Ruthven, C.R.J. (1963). Association of Clinical Pathologist, Broadsheet, No. 44.

Sandler, M. and Ruthven, C.R.J. (1966). Pharmac. Rev. 18, 343.

Sandler, M. and Ruthven, C.R.J. (1966_a). In: Recent Results in Cancer Research. II Neuroblastomas. Biochemical Studies. ed. Bohuon, C. 55, Springer, Berlin.

Sandler, M. and Ruthven, C.R.J. (1969). In: Chromatographic And Electrophoretic Techniques, ed. I. Smith, 3rd edn. 1, 714. William Heinemann, London.

Sandler, M. and Ruthven, C.R.J. (1969_a). In: Progress In Medical Chemistry, ed. G.P. Ellis and G.B. West, 6, 200. Butterworth, Lond.

Sandler, M., Ruthven, C.R.J. and Contractor, S.F. (1963). Biochem. Pharmac. 12, Suppl. p. 203.

Sandler, M., Ruthven, C.R.J. and Wood, C. (1964_a). Int. J. Neuropharmacol. 3, 123.

Sandler, M. and Snow, P.J.D. (1958). Lancet, i, 137.

Sandler, M., Spector, R.C., Ruthven, C.R.J. and Davison, A.N. (1960). Biochem. J. 74, 42P.

Sandler, M. and Youdim, M.B. (1967). Biochemical Aspects of Phenylamine, Capri, ed. F. Cedrangols, 14, 157.

Sandler, M. and Youdim, M.B.H. (1968). Nature, Lond. 217, 771.

Sandler, M. (1970). Proc. Laurentian Conference on DOPA in Parkinsonism in Press.

Sankoff, I. and Sourkes, T.L. (1963). Can. J. Biochem. Physiol. 41, 1381.

Sano, I.Y., Kakimoto, T., Okamoto, T., Nakajima, H. and Kudo, Y. (1957). Schweiz. Med. Wschr. 87, 214.

Sato, T.L. (1965). J. Lab. clin. Med. 66, 517.

Sato, T.L. and Sjoerdsma, A. (1965). Br. med. J. ii, 1472.

Sato, T.L., Jequier, E., Lovenberg, W. and Sjoerdsma, A. (1967). Eur. J. Pharmac. 1, 18.

Schayer, R.W. (1953). Proc. Soc. exp. Biol. Med. 84, 60.

Scheline, R.R. (1967). Experientia, 23, 493.

Scheline, R.R. (1966). J. Pharm. Pharmac. 18, 664.

Scheline, R.R. (1968_a). J. pharm. Sci. 57, 2021.

Scheline, R.R. (1968_b). Acta pharmac. tox. 26, 189.

Scheline, R.R., Williams, R.T. and Wit, J.G. (1960). Nature, Lond. 188, 849.

Schepartz, B. and Gurin, S. (1949). J. biol. Chem. 180, 663.

Schnaitman, C., Erwin, V.G. and Greenawalt, J.W. (1967). J. Cell. Biol. 32, 719.

Second International Symposium on Catecholamines (1966). pharmac. Rev. 18, No. 1.

Sen, N.P. (1969). J. Food. Sci. 34, 22.

Sen, N.P. and McGeer, P.L. (1963). Biochem, biophys. Res. Commun. 13, 390.

Senoh, S., Daly, J., Axelrod, J. and Witkop, B. (1959. J. Am. chem. Soc. 81, 6240.

Sereni, F., Kenney, F.T. and Kretchmer, N. (1959). J. biol. Chem. 234, 609.

Sharman, D.F. (1963). Br. J. Pharmac. Chemotherp. 20, 204.

Shaw, K.N.F., Gutenstein, M. and Jepson, J.B. (1961). Proc. 5th. int. Cong. Biochem. Moscow, 9, 427. Pergamon Press Oxford.

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

Shaw, K.N.F., Redlich, D., Wright, S.W. and Jepson, J.B. (1960). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 19, 194.

Shimizu, H. and La Brosse, E.H. (1969). Biochem. Pharmac. 18, 1643.

Sjoerdsma, A., Smith T.E., Stevenson, T.D. and Udenfriend, S. (1955). Proc. Soc. exp. Biol. Med. 89, 36.

Sheppard, H., Tsien, W.H., Plummer, A.J., Peets, E.A., Giletti, R.J. and Schulert, A.R. (1958). Proc. Soc. exp. Biol. Med. 97, 717

Shore, P.A. and Brodie, B.B. (1957). In: Psychotropic Drugs, ed. S. Garattini and V. Ghetti, 423. Elsevier, Amsterdam.

Shore, P.A., Silver, S.L. and Brodie, B.B. (1955). Science N.Y. 14, 178.

Shulgin, A.T., Sargent, T. and Naranjo, C. (1969). Nature, Lond. 221, 537.

Sjoerdsma, A. Weissbach, H. and Udenfriend, S. (1955_a). J. Am. med. Ass. 159, 397.

Sjoerdsma, A., Weissbach, H. and Udenfriend, S. (1956). Am. J. Med. 20, 520.

Sjostrand, N.O. (1955). Acta, physiol. Scand. 65, suppl. 257.

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

Smith, C.A. (1959). In: The Physiology of The Newborn Infant. 3rd edn. Blackwell, Oxford.

Smith, P. (1963). In: The Clinical Chemistry of Monoamines, ed. H. Varley and A.H. Gowenlock, 31, Elsevier, Amsterdam.

Smith, P. (1965). Nature, Lond., 205, 1236.

Smith, P. (1967). Nature, Lond., 213, 802.

Smith, I. (1969). Chromatographic And Electrophoretic Techniques 1, 3rd edn. Heinemann, London.

Smith, B. and Carlson, O. (1963). Acta. chem. Scand. 17, 455.

Smith, A., Gitlow, S., Gall, E., Wortis, S.B. and Mendlowitz, M. (1960). Clin. Res. 8, 367.

Smith, A.N., Nyhus, L.M., Dalgliesh, C.E., Dutton, R.W., Lennox, B. and McFarlane, P.S. (1957). Scott. med. J. 2, 24.

Snow, P.J.D., Lennard-Jones, J.E., Curzon, J.E. and Stacey, R.S. (1955). Lancet, ii, 1004.

Spector, S.C., Kuntzman, R., Shore, P.A. and Brodie, B.B. (1960). J. Pharmac. exp. Ther. 130, 256.

Spector, S., Melmon, K., Lovenberg, W. and Sjoerdsma, A. (1963). J. Pharmac. exp. Ther. 140, 229.

Sourkes, T.L. (1955). Br. med. Bull. 21, 66.

Sourkes, T.L. (1961). Rev. Can. Biol. 20, 187.

Sourkes, T.L. (1966). Pharmac. Rev. 18, 53.

Sourkes, T.L., Denton, G.F., Murphy, G.F., Chavez, B. and St. Cyr. S. (1963). Pediatrics, Springfield, 31, 660.

Sourkes, T.L., Pivnicki, D., Brown, W.T., Wiseman-Distler, M.H., Murphy, G.F., Sankoff, I. and Saint Cyr. S. (1965). Psychiat. Neurol. 149, 7.

Spencer, R.P. and Zamcheck, N. (1960). Biochem. biophys. Res. Commun. 3, 386.

Sprinkle, T.J., Porter, A.H., Greer, M. and Williams, C.M. (1969). Clin. chim. Acta. 25, 409.

Stern, L., Greenberg, R.E. and Lind, J. (1961). Acta. paediat., Stockh. 50, 497.

St.John, P.A., Brook, J.L. and Biggs, R.H. (1967). Analyt. Biochem. 18, 459.

Stoll, W.A., (1947). Schweiz, Arch. Neurol. Psychiat. 68, 279.

Stowe, B.B. and Shilke, J.F. (1964). In: Regulateurs Naturels dela Croissance vegelate, Colloq. intern. Centre Notl. Rech. Sci. (Paris). 123, 409.

Stacey, R.S. (1966). In: Handbook of Experimental Pharmacology ed. O. Eichler and A. Farah, 19, 744, Springer, Berling,

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

Sunderman, F.W., Jr., Cleveland, P.D., Low, N.C. and Sunderman, F.W. (1960). Am. J. clin. Path. 34, 293.

Tangen, O., Founum, F and Haavaldsen, R. (1965). Biochim. biophys. Acta. 96, 82.

Todrick, H., Dick, M. and Tait, A.C. (1958). Br. med. J. i, 496.

Tozer, T.N., Neff, N.H. and Brodie, B.B. (1966). J. Pharmac. exp. Ther. 152, 177.

Udenfriend, S. (1958). In: 5-hydroxytryptamine, ed. G.P. Lewis, 44, Pergamon, Lond.

Udenfriend, S. (1962). Fluorescence Assay In Biology and Medicine, Academic Press, Lond.

Udenfriend, S. (1966). Pharmac. Rev. 18, 43.

Udenfriend, S. and Bessman, S. (1953). J. biol. Chem. 203, 961.

Udenfriend, S., Clark, C.T. and Titus, E. (1953). J. Am. chem. Soc. 75, 501.

Udenfriend, S. and Cooper, J.R. (1952). J. biol. Chem. 194, 503.

Udenfriend, S. and Creveling, C.R. (1959). J. Neurochem. 4, 350.

Udenfriend, S., Creveling, C.R., Posner, H., Redfield, B.G., Daly, J. and Witkop, B. (1959). Archs. Biochem. biophys. 83, 501.

Udenfriend, S., Lovenberg, W.M. and Sjoerdsma, A. (1959_a). Archs. Biochem. Biophys. 85, 487.

Udenfriend, S., Lovenberg, W.M. and Weissbach, H. (1960). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 19, 7.

Udenfriend, S., Titus, E and Weissbach, H. (1955_a). J. biol. Chem. 216, 499.

Udenfriend, S., Weissbach, H. and Sjoerdsma, A. (1956_a). Science, N.Y. 123, 669.

Udenfriend, S., Titus, E., Weissbach, H. and Peterson, R.E. (1956_b). J. biol. Chem. 219, 335.

Udenfriend, S. and Weissbach, H. (1958_a). Proc. Soc. exp. Biol. Med. 97, 748.

Udenfriend, S., Weissbach, H. and Bogdanski, D.F. (1957). J. biol. Chem. 224, 803.

Udenfriend, S. and Wyngarden, J.B. (1956). Biochim. biophys. Acta. 20, 48.

Udenfriend, S., Zaltman-Nirenberg, P., Gordon, R. and Spector, S. (1966_a). Mol. Pharmac. 2, 95.

Umbach, W. and Baumann, D. (1964). Arch. Psychiat. Ztchr. Neurol. 205, 281.

Valcourt, A.J. (1959). Archs. Neurol. Psychiat. Chicago. 81, 292.

Van Deemter, J.J., Zuiderweg. F.J. and Klinkenberg, A. (1956). Chem. Eng. Sci. 5, 27.

Van Der Schoot, J.B., Creveling, C.R., Nagatsu, T. and Udenfriend, S. (1963). J. Pharmac. exp. Ther. 141, 74.

Vane, J.R. (1964). Br. J. Pharmac. Chemother. 23, 360.

Vandenheuvel, W.J.A. (1966). J. Chromatog. 25, 29.

Verley, W.G., Koch, G. and Hunebelle, G. (1962). Archs. int. Physiol. 70, 624.

Vogel, A.I. (1961). A Textbook of Practical Organic Chemistry Including Quantitative Organic Analysis. 3rd. edn. 971. Longmans Green, Lond.

Vestergaard, P. and Leverett, R. (1958). J. Lab. clin. Med. 51, 211.

von Euler, V.S. (1958). Recent Prog. Horm. Res. 14, 451.

von Euler, V.S. (1967). Circulation Res. Suppl. III, 10, 63.

von Studnitz, W. (1960). Scand. J. clin. Lab. Invest. 12, Suppl. 48, 58.

von Studnitz, W. (1960_a). Scand. J. clin. Lab. Invest. 12, Suppl. 48, 1.

von Studnitz, W. (1960_b). Scand. J. clin. Lab. Invest. 12, Suppl. 48, 27.

von Studnitz, W. (1960_c). Scand. J. clin. Lab. Invest. 12, Suppl. 48, 1.

von Studnitz, M. (1961). Clin. chim. Acta. 6, 525.

von Studnitz, W. (1962). Klin. Wschr. 40, 163.

von Studnitz, W. (1966). Pharmac. Rev. 18, 651.

von Studnitz, W. (1967). Klin. Wschr. 45, 307.

von Studnitz, W., Käser, H. and Sjoerdsma, A. (1963). New Engl. J. Med. 269, 232.

Voorhess, M.L. (1967). Pediatrics. Springfield, 39, 252.

Voorhess, M.L. (1968). J. pediat. surg., 30, 147.

Voorhess, M.L. and Gardner, L.I. (1962). J. Clin. Endocr. 22, 126.

Voorhess, M.L., Pickett, L.K. and Gardner, L.I. (1963). Am. J. Surg. 106, 33.

Warren, R.J., Eisdorfer, I.B., Thompson, W.E. and Zarembo, J.E. (1965). J. pharm. Sci. 54, 1806.

Weber, W.W. and Zannoni, V.G. (1966). J. biol. Chem. 241, 1345.

Weissbach, H., King, W., Sjoerdsma, A. and Udenfriend, S. (1959). J. biol. Chem. 234, 81.

Weissbach, H., Lovenberg, W. and Udenfriend, S. (1960). Biochem. biophys. Res. Commun. 3, 225.

Weissbach, H., Redfield, B.G. and Axelrod, J. (1960_a). Biochim. biophys. Acta. 43, 352.

Weissbach, H., Redfield, B.G. and Udenfriend, S. (1957). J. biol. Chem. 229, 953.

Weissbach, H., Redfield, B.G. and Udenfriend, S. (1958). Fedn. Proc. Fedn. Am. Socs. exp. Biol. 17, 418.

Wells, H.G. (1940). Archs. Path. 30, 535.

Werle, E. and Aures, D. (1959). Hoppe-Seyler's Z. physiol chem. 316, 45.

Westermann, E., Balzer, H. and Knell, J. (1958). Archs. exp. Path. Pharmak. 234, 194.

Westlake, R.J. and Tew, J.M. (1966). Neurology, 16, 619.

Wilk, S., Gitlow, S.E., Clarke, D.D. and Paley, D.H. (1967). Clin. chim. Acta. 16, 403.

Wilk, S., Gitlow, S.E., Mendlowitz, M., Franklin, M.J., Carr, H.E. and Clarke, D.D. (1965). Analyt. Biochem, 13, 544.

Williams, R.T. (1959). Detoxication Mechanisms, 2nd. edn. Chapman and Hall, Lond.

Williams, C.M. (1962). Analyt. Biochem. 4, 423.

Williams, C.M. (1965). Analyt. Biochem. 11, 224.

Williams, C.M., Dorin, G.E. and Greer, M. (1963_a). Anal. Biochem. 6, 468.

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

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

Williams, C.M. and Greer, M. (1965). Clin. chim. Acta. 11, 495.

Williams, C.M. and Leonard, R.H. (1963). Analyt. Biochem. 5, 362

Williams, C.M. and Sweeley, C.C. (1961). J. clin. Endocr. 21, 1500.

Williams, C.M. and Sweeley, C.C. (1964). In: Biochemical Application of Gas Chromatography, ed. H. Szymanski, 225, Plenum Press, N.Y.

Williams, E.D. and Sandler, M. (1963). Lancet, i, 238.

Wilcox, M. (1966). Analyt. Biochem. 16, 253.

Wilcox, M. (1967). J. Pharm. Sci. 56, 642.

Wiseman-Distler, M.H. and Sourkes, T.L. (1963). Biochem. Physiol. 41, 57.

Woolf, L.I. and Edmunds, M.E. (1950). Biochem. J. 47, 630.

Woolf, L.I. (1965). In: Symposium on Tyrosinosis, ed. L.A. Gjessing, Scandinavian University Books, p.24.

Woolley, D.W. and Shaw, E. (1954). Proc. Natn. Acad. Sci. U.S.A. 40, 228.

Wong, P.W.K., Lambert, A.M. and Kamrower, G.M. (1967). Develop. Med. Child. Neurol. 9, 551.

Wurtman, R.J., Axelrod, J. and Douglas, E.K. (1968). The Pineal Gland, Academic Press, N.Y. Lond.

Wurtman, R.J., Axelrod, J. and Potter, L.T. (1964). J. Pharmac. exp. Ther. 144, 150.

Yahr, M.D., Duvoisin, R.C., Schear, M.J., Burrett, R.E. and Hoehn, M.M. (1969). Arch. Neurol. 21, 343.

Youdim, M.B.H, Collins, G.G.S. and Sandler, M. (1968). FEBS Letters, 1, 215.

Youdim, M.B.H, Collins, G.G.S. and Sandler, M. (1969). Nature, Lond. 223, 626.

Youdim, M.B.H and Sandler, M. (1967). Biochem. J. 105, 43P

Youdim, M.B.H and Sandler, M. (1968). European J. Pharmac. 4, 105.

Youdim, M.B.H. and Sourkes, T.L. (1966). Can. J. Biochem. Physiol. 44, 1397.

Yuwiler, A., Geller, E. and Eiduson, S. (1959). Archs. Biochem. Biophys. 80, 162.

Yuwiler, A., Geller, E. and Eiduson, S. (1960). Archs. Biochem. Biophys. 89, 143.

Zannoni, V.G. and La Du, B.N. (1959). J. biol. Chem. 234, 2925.

Zannoni, V.G. and Weber, W.W. (1966). J. biol. Chem. 241, 1340.

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

Zeisel, H. and Kuschke, H.J. (1959). Klin. Wschr. 37, 1168.

Reprinted from
Clinica Chimica Acta
Elsevier Publishing Company,
Amsterdam
Printed in The Netherlands

GAS CHROMATOGRAPHIC MEASUREMENT OF PHENOLIC ACIDS AND ALCOHOLS IN HUMAN URINE

F. KAROUM, C. R. J. RUTHVEN AND M. SANDLER
Bernhard Baron Memorial Research Laboratories and Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London, W.6 (U.K.)

(Received January 31, 1968)

SUMMARY

Methylene unit values of trimethyl silyl ether/ester and of methyl ester/trimethyl silyl ether derivatives of some biologically important phenolic acids were determined gas chromatographically under isothermal conditions. The former were chromatographed on 4 non-polar stationary phases, methyl-, methyl phenyl-, methyl phenyl vinyl-silicone gums and silicone oil, and the latter on methyl phenyl silicone gum and silicone oil. Methyl ester/trimethyl silyl ether derivatives of a number of phenolic alcohols were also run on methyl phenyl silicone gum.

From a detailed evaluation of these investigations using both authentic compounds and urine extracts, suitable conditions were defined for the quantitative analysis of a large number of urinary phenolic acids and alcohols, with particular reference to 4-hydroxy-3-methoxymandelic and homovanillic acids; the most satisfactory quantitative system involved chromatography of the methyl ester/trimethyl silyl ether derivative on a 7 ft. 10% methyl phenyl silicone gum column.

The application of these procedures to the investigation of abnormal phenolic acid excretion in phenylketonuria, alkaptonuria, tyrosyluria of the newborn and catecholamine-secreting tumours is discussed.

INTRODUCTION

The first systematic assessment of the phenolic acid pattern of human urine stemmed from the paper chromatographic studies of Armstrong *et al.*[1]. More recently however, Williams and his colleagues[2–11], and later others[12–20] have applied gas chromatographic analysis to the separation of the large number of aromatic acids present in urine. At its best, this versatile technique is quick, sensitive, specific and quantitative, although the methods originally employed by Williams and associates variously suffered from certain drawbacks such as poor resolution and tailing[10] and multiple derivative formation from a single compound[5,6,8].

A wide variety of volatile derivatives has been used for gas–liquid chromatography (GLC) of urinary aromatic acids[10,12,19]. It now seems clear that some form of

trimethyl silyl (TMS) derivative, usually the TMS ether/ester, possesses the properties necessary for satisfactory chromatograms[13–15, 17–20]. The TMS ether/ester is simple to prepare in high and reproducible yield, and gives rise to few undesirable side reactions. After some early doubts[10], methyl ester/TMS ether derivatives have also been shown to possess many desirable features[17,18].

A number of different stationary phases has been employed for the separation of these TMS derivatives[9], but many of the available data have been derived from experiments which made use of simple mixtures of authentic compounds rather than urine. Thus many of the problems which arise when urine extracts are studied have been avoided. The object of the present work was to define the most suitable general-purpose system or systems for the analysis of urinary phenolic acids. In the event, the system chosen also proved to be highly satisfactory for the analysis of urinary phenolic alcohols, which have so far received scant attention in the gas chromatographic literature[17].

EXPERIMENTAL

Materials and equipment

Siliconised acid-washed celites 85/100 and 100/120 mesh, methyl silicone gum (SE30), methyl phenyl silicone gum (SE52), methyl phenyl vinyl silicone gum (SE54) and silicone oil (F-60) were obtained from W. G. Pye, Ltd., Cambridge, England. *n*-Alkane standards, dioxane (dry), hexamethyldisilazane (HMDS) and trimethylchlorosilane (TMCS) were obtained from British Drug Houses Ltd., Poole, Dorset, England.

Authentic aromatic and phenolic acids in the purest grade available, were purchased from commercial sources, with the exception of vanillactic (VLA) and vanil-pyruvic (VPA) acids which were gifts from Dr. P. Smith and 3,4-dihydroxyphenyl-glycol from Dr. J. Axelrod.

Ethereal diazomethane (approx. 100 mg/ml) was prepared by the reaction of *p*-tolylsulphonylmethylnitrosamide with an ethanolic solution of KOH[21]. The preparation is stable for at least one month if stored at 0°.

All other reagents were of analytical grade.

GLC analysis was carried out with a Pye Panchromatograph fitted with an argon ionisation detector. The detector temperature was maintained at 250° with an ionising potential of 1250 V. The sensitivity was varied between 10^{-8} to $3 \cdot 10^{-9}$ A full scale deflection. Columns were not connected to the detector until it had reached the temperature of operation.

Preparation of columns. Glass U-tube columns 5 ft. or 7 ft. × 5 mm (internal diameter) were siliconised before packing by allowing them to stand overnight with a mixture of petroleum ether (B.P. 40/60°)–TMCS–HMDS (100:4:2) before emptying and drying in a stream of argon at 200°.

Columns were packed evenly with the appropriate coated siliconised celite. Freshly packed columns were preheated for 48 h at 250° in a stream of argon. This procedure was repeated for at least 6 h whenever a fresh preheated column was used.

Urine extraction. Neonatal urine (15 ml if the 24-h volume was greater than 100 ml or 10 ml if less), normal adult urine (10 ml) or urine from patients with a catecholamine-secreting tumour (5 ml) was adjusted to pH 1 with 6 *N* HCl, saturated with NaCl and extracted twice with ethyl acetate (20 ml). Urine (10 ml) previously hy-

drolysed with a sulphatase–glucuronidase preparation[22] prior to further treatment was used for the detection and estimation of phenolic alcohols. Aliquots (15 ml, 20 ml) of each ethyl acetate extract were combined and evaporated to dryness under vacuum ("Rotary Evapo-Mix") at 40°. An internal standard equivalent to 0.05 mg homovanillic acid (HVA) and 0.1 mg 4-hydroxy-3-methoxymandelic acid (VMA) was added to a duplicate specimen and included with each batch of analyses when quantitation was required. This additional step was not usually necessary when screening for the presence or absence of a particular disease pattern.

TMS ether/ester derivatives were prepared by dissolving the authentic acid (0.5 mg), or the residue obtained from the extracted urine, in dioxane (0.2 ml); HMDS (0.2 ml) and TMCS (0.1 ml) were added to the tube and the contents mixed thoroughly. The preparation was then transferred to a 2-ml test tube, which was tightly stoppered and left for 30 min at room temperature. After centrifugation, 0.5 μl of the clear supernatant fluid was injected into the column with a Hamilton syringe. It was usually convenient to carry out the chromatographic analysis on the same day as the preparation of the derivative although derivatives are stable for at least 3 weeks if stored in a desiccator.

Methyl ester/TMS ether derivatives were prepared by esterifying the authentic acid (0.5 mg), or urine residue, with methanol (0.2 ml) and ethereal diazomethane (2 ml) for 1 min. After evaporation under a stream of nitrogen, the dry residue was silylated as described in the preceding paragraph.

Characterisation of peaks. Peaks were characterised from their methylene unit value (MU) by the procedure described by Dalgliesh *et al.*[17]. The chromatogram was calibrated by introducing even numbered straight chain hydrocarbons (C_{16} to C_{22}) into the column, termed *n*-alkane reference standards. The MU value for a particular peak on a chromatogram was calculated from the formula: $MU = n+2\,y/x$, where y is the distance (mm) from the peak to that of a neighbouring reference standard C_n and x is the distance (mm) between the peak of this standard and that of a second hydrocarbon of chain length C_{n+2}.

RESULTS AND DISCUSSION

MU values of the TMS ether/ester derivatives of a number of biologically important aromatic acids were determined under isothermal temperature conditions on four different non-polar stationary phases (Table I). The methyl ester/TMS ether derivatives of these compounds were also examined and their MU values on two non-polar phases are given in Table I.

From studies of the height equivalent to the theoretical plate (HETP) at different temperature and inlet pressures, optimum conditions for TMS ether/ester derivatives were established at an argon inlet pressure of 1500 mm Hg (flow 100–150 ml/min) and at column temperatures of 190° for SE phases and 195° for the F-60 phase. At these temperatures stationary phases of less than 3% are not suitable. Variations of column temperature by $\pm$ 5° from the optimum and of stationary phases by $\pm$ 5% from a 10% concentration produce a change of MU value of less than 0.04, which is within the overall experimental error of the method. Similar conditions apply to the methyl ester/TMS ether derivatives except that the optimum temperatures were 190° for SE phases and also for F-60. The distances between 3

TABLE I

METHYLENE UNIT VALUES FOR DERIVATIVES OF PHENOLIC ACIDS AND ALCOHOLS ON DIFFERENT STATIONARY PHASES

	Methyl ester/TMS ether derivatives		*TMS ether/ester derivatives*			
	10% F-60	*10% SE52*	*10% SE30*	*10% SE52*	*10% SE54*	*10% F-60*
1 Phenylacetic acid	13.92	13.05	13.35	13.46	13.43	15.00
2 Salicylic acid	14.82	14.28	14.90	15.20	—	—
3 *p*-Hydroxybenzaldehyde	—	14.22	—	—	—	—
4 *m*-Hydroxybenzoic acid	15.25	14.48	15.72	15.68	15.67	16.15
5 *p*-Hydroxybenzoic acid	15.87	14.96	16.16	16.29	16.30	16.66
6 *o*-Hydroxyphenylacetic acid	15.50	14.79	15.70	15.84	15.80	16.23
7 *m*-Hydroxyphenylacetic acid	15.95	15.16	—	—	—	—
8 *p*-Hydroxyphenylacetic acid	16.23	15.48	16.22	16.39	16.38	16.86
9 3,4-Dimethoxybenzoic acid	17.25	16.07	16.69	17.11	17.09	18.19
10 3,4-Dimethoxymandelic acid	—	18.16	—	—	—	—
11 3,4-Dihydroxy-5-methoxyphenylacetic acid	—	18.82	—	—	—	—
12 3,4-Dimethoxyphenylacetic acid	17.62	16.29	16.72	17.18	17.09	18.25
13 Mandelic acid	—	14.11	14.62	14.72	—	15.60
14 *p*-Hydroxymandelic acid	18.10	17.27	17.69	17.92	17.80	18.25
15 *β*-Phenyllactic acid	15.63	14.85	15.94	16.04	16.00	16.33
16 Vanillic acid	17.48	16.53	17.37	17.63	17.60	18.28
17 Homovanillic acid (HVA)	17.94	16.81	17.43	17.78	17.78	18.40
18 3,4-Dihydroxybenzoic acid	18.00	17.21	18.15	18.24	18.33	18.25
19 3,4-Dihydroxyphenylacetic acid (DOPAC)	18.16	17.41	18.24	18.35	18.33	18.71
20 Gentisic acid	17.66	16.96	17.68	17.85	17.85	18.15
21 Homogentisic acid	18.29	17.66	18.24	18.38	18.36	18.72
22 4-Hydroxy-3-methoxymandelic acid (VMA)	19.16	18.43	18.59	18.77	18.78	19.42

27 Dihydroferulic acid	—	—	17.43	17.70	18.90	18.24
28 *p*-Hydroxycinnamic acid	18.77	18.03	19.14	19.37	19.35	20.09
29 3,4-Dihydroxycinnamic acid – main peak	—	21.47	—	—	—	—
– subsidiary peak	—	19.41	—	—	—	—
30 Phenylpyruvic acid*	17.50	17.27	17.25	17.26	17.27	17.50
31 *p*-Hydroxyphenylpyruvic acid*	20.29	19.57	20.43	20.51	21.26	20.85
32 Vanillactic acid (VLA)	—	19.41	20.18	20.31	20.60	20.73
33 Vanilpyruvic acid (VPA)** main peak	—	20.28	—	—	—	21.49
34 *p*-Hydroxyphenyllactic acid	18.68	18.18	18.85	19.05	19.02	19.38
35 Hippuric acid	18.56	17.45	—	—	18.39	19.67
36 Indolylacetic acid	—	18.55	—	—	—	—
37 Indolylacetaldehyde***	—	18.17	—	—	—	—
38 Indolylpyruvic acid	—	22.92	—	—	—	—
39 5-Hydroxyindolylacetic acid (5HIAA)	22.33	21.31	>22	>22	>22	>22
40 5-Hydroxy-6-methoxyindolyl-2-carboxylic acid	—	22.11	—	—	—	—
41 5,6-Dihydroxyindolyl-2-carboxylic acid	—	22.51	—	—	—	—
42 Tryptophol	—	18.42	—	18.42	—	—
43 5-Hydroxytryptophol	—	20.67	—	20.67	—	—
44 4-Hydroxy-3-methoxyphenylglycol (HMPG)	—	18.37	—	18.37	—	—
45 3,4-Dihydroxyphenylglycol	—	18.82	—	18.82	—	—
46 2-(3,4-Dimethoxyphenylethanol)	—	16.54	—	16.54	—	—
47 3-Methoxy-4-hydroxyphenylethanol	—	17.10	—	17.10	—	—
48 3,4-Dihydroxyphenylethanol	—	17.79	—	17.79	—	—

7 ft. columns, inlet pressure of 1500 mm Hg, 85/100 mesh siliconised celite.
SE columns were maintained at 190° and the F-60 at 195° for running TMS ether/ester derivatives and at 190° for methyl ester/TMS ether derivatives.

* TMS ether/ester gave a subsidiary peak.
** Forms many derivatives other than the main one shown in the Table. These may derive from impurities.
*** Forms a subsidiary peak corresponding to tryptophol.

successive reference standards have been found to be related by the following formula

$$\frac{C_{n+4}-C_{n+2}}{C_{n+2}-C_n} = k \text{ (constant)} \pm 0.02$$

This formula serves as a useful check on whether the column has reached a stable temperature or not, and assists in determining the estimated position of a reference standard if it is not represented on the chromatogram.

Resolution was complete when the MU values of two peaks differed by 0.3 or more if between 16 and 18, by 0.2 if between 18 and 20 and by 0.15 when in the 20 to 22 range. Peaks differing in MU values by half or less of these minimum differences were usually inadequately separated.

A comparison was made between peak height of detector response and integrator reading (Technicon Integrator Calculator) of the area under the trace. Using varying concentrations of a C_{18} reference standard applied to the column in a range of 0.05 μg to 0.5 μg, results were similar at two applied voltages (1000 and 1250 V). A comparison of the concentration of a number of phenolic acids in urine samples from 10 normal adults, as determined by peak height and area, showed satisfactory agreement between the respective values; those obtained from peak height tended to be up to 5% higher than those based on area. When increasing amounts of TMS ether/ester derivatives of authentic phenolic acids (HVA, dihydroxyphenylacetic acid (DOPAC), VMA, dihydroxymandelic acid) were run in a concentration range (0.1–1.5 μg of parent acid) similar to that likely to be encountered in clinical practice, a linear detector response was obtained at 800 V and 1000 V, as measured by peak height. For detection of small quantities, 1250 V is more suitable, although the upper limit for linearity is reduced to 0.5 μg.

Horning and her colleagues[18] found it necessary to treat urine samples with three successive volumes of both ethyl acetate and ether to achieve adequate extraction of aromatic acids. Such a sequence will provide information on a wide range of compounds, including indolic acids, some of which have a comparatively low solubility in ethyl acetate although a satisfactory assay of indolic acids can be obtained using ether alone (Fig. 1). With some important exceptions, such as *p*-hydroxymandelic acid[9], most phenolic acids are relatively soluble in ethyl acetate however, so that the procedure described above was suitable for their assay. Recoveries of *p*-hydroxyphenylacetic acid, HVA, VMA and DOPAC from urine were all greater than 90% in a series of three experiments.

Assessed from peak height measurements, the overall recovery of VMA was similar, whether extracted from water or urine; however, HVA recoveries were considerably greater from urine. Because of such variations, it is more accurate to add internal standard to a urine sample on each occasion rather than employing a standard curve, especially one prepared by extracting from aqueous solution.

The resolution of TMS ether/ester derivatives is in general better on F-60 than on any of the SE phases; but neither stationary phase proved useful for the quantitative analysis of this derivative of VMA. Hippuric acid, which is present in large amounts in most adult urine samples, tended to mask normal levels of VMA on F-60. This column also failed to separate VMA from *p*-hydroxyphenyllactic acid which is present in large quantities in most neonatal urine specimens, especially those from premature infants. F-60, however, may be used for screening urine samples for cate-

cholamine-secreting tumours, since high levels of VMA are not masked by the presence of hippuric acid (Fig. 2a).

Methyl ester/TMS ether derivatives are much more suitable for quantitative analysis of urine specimens, whether from adult or infant (compare Figs. 2a and 2b) since they show better resolution than the TMS ether/ester derivatives on both F-60 and SE phases; the SE phases, particularly SE52 (Fig. 3), proved superior to F-60 in their resolving power.

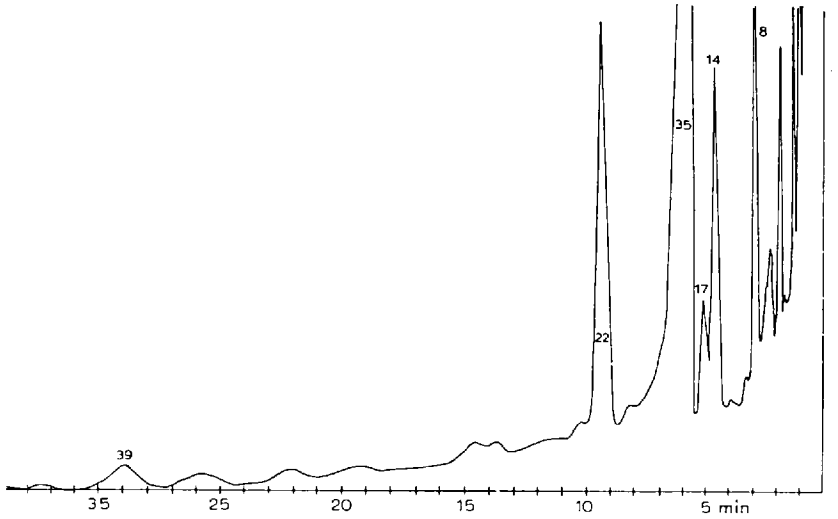


Fig. 1. Chromatogram of methyl ester/TMS ether derivatives of phenolic acids prepared from an ether extract of 15 ml urine from a patient with phaeochromocytoma, and run on a 7 ft. 10% SE52 column. The peak numbers correspond with those used in Table I.

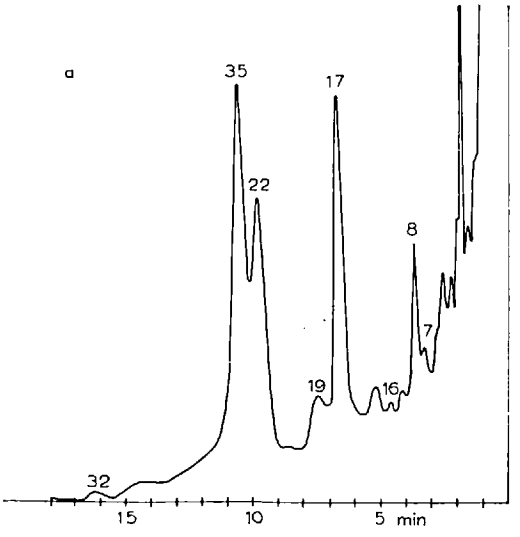


Fig. 2. (a) Chromatogram of TMS ether/ester derivatives of phenolic acids prepared from an ethyl acetate extract of 5 ml urine from a patient with neuroblastoma, and run on a 7 ft., 10% F-60 column. The peak numbers correspond with those used in Table I.

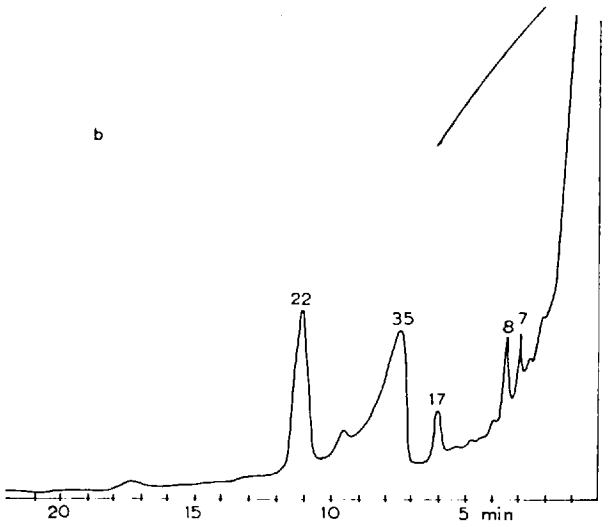


Fig. 2 (b) Chromatogram of methyl ester/TMS ether derivatives of phenolic acids prepared from an ethyl acetate extract of 5 ml urine from a patient with malignant phaeochromocytoma, and run on a 7 ft. 10% SE52 column. The peak numbers correspond with those used in Table I.

As the phenolic alcohols contain no carboxyl group, they do not form a methyl ester with diazomethane under the experimental conditions used here; consequently, their TMS ether/ester and "methyl ester/TMS ether" derivatives are identical.

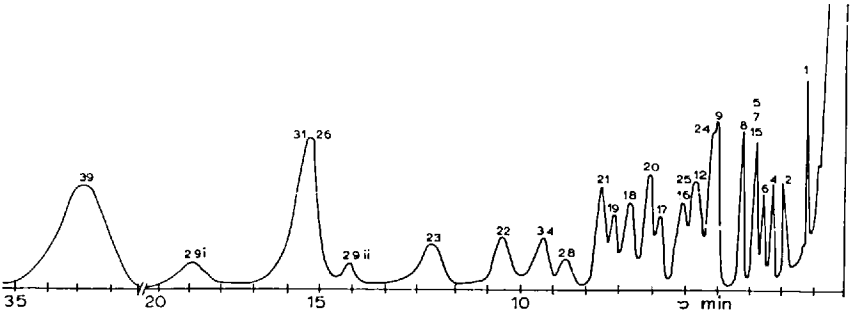


Fig. 3. Chromatogram of methyl ester/TMS ether derivatives of a mixture of phenolic acid reference compounds. About 0.01–0.02 μg of each, prepared as stated in the text, was run on a 7 ft. 10% SE52 column. The peak numbers correspond with those used in Table I.

The detector response produced by the methyl ester/TMS ether derivative of an acid was about ten times that of its corresponding TMS ether/ester, a difference likely to be related to the greater ionisation potential of the former. Detector sensitivity is also related to MU value. Using an amplification setting of 10^{-8} A to give a full scale deflection at an applied voltage of 2250 V, the limits of sensitivity for methyl ester/TMS ether derivatives with MU values between 16–18, 18–20 and 20–22 were 0.5, 1.0 and 2.0 nanograms of parent compound respectively, corresponding to 5, 10

and 20 μg in the aliquot of urine usually used. The sensitivity may be further increased by a factor of 3 to 10 times if amplification settings of 3×10^{-9} A to 10^{-9} A are used. Under these circumstances however, some preliminary purification of the specimen is required, as the high base-line and also the frequent presence of large amounts of hippuric acid derivative tend to make quantitation inaccurate.

TMS ether/ester derivatives of all compounds investigated ran as singlesymmetrical peaks with the exception of the keto acids, which gave rise to two or more peaks; the major had an MU value corresponding with that predicted for the acid itself from considerations of polarity and molecular weight, whilst the minor registered a value consistent with the loss of one or more C atoms from the aliphatic chain.

Dihydroxyphenylpyruvic acid formed secondary derivatives more readily than phenylpyruvic acid, whilst *p*-hydroxyphenylpyruvic acid was of intermediate stability. With three exceptions, VPA which gave two large and multiple small peaks, dihydroxycinnamic acid which gave two peaks, and indolylacetaldehyde which gave a secondary peak corresponding to tryptophol, the methyl ester/TMS ether derivatives of these keto acids were stable and gave single symmetrical peaks. Whether the subsidiary VPA peaks were impurities or decomposition products is unknown. The problem is unlikely to lead to diagnostic difficulty however, as raised keto acid levels are usually accompanied by an increase in the reduced, lactic acid form, which may be readily identified. This association may be seen in Fig. 4 (b), where elevated levels of both *p*-hydroxyphenylpyruvic and *p*-hydroxyphenyllactic acids are present in the urine of a premature infant. These compounds are found in lower concentration in the urine of full term infants (Fig. 4a).

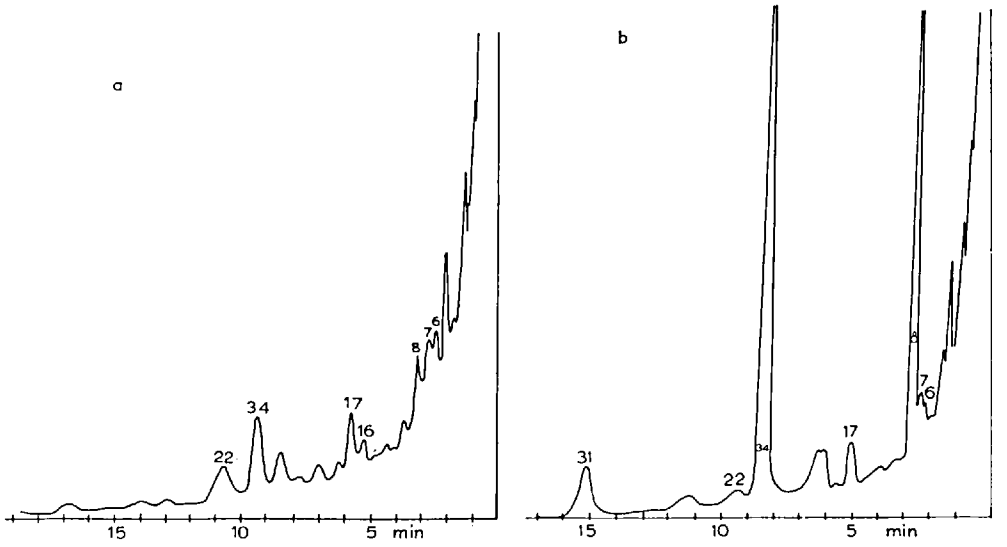


Fig. 4. (a) Chromatogram of methyl ester/TMS ether derivatives of phenolic acids prepared from an ethyl acetate extract of 10 ml urine from a full term newborn infant and run on a 7 ft. 10% SE52 column. The peak numbers correspond with those used in Table I.
(b) Chromatogram of methyl ester/TMS ether derivatives of phenolic acids prepared from an ethyl acetate extract of 10 ml urine from a premature newborn infant, and run on a 7 ft. 10% SE52 column. Attention is drawn to prominent peaks corresponding with *p*-hydroxyphenylacetic, *p*-hydroxyphenyllactic, and *p*-hydroxyphenylpyruvic acids. The peak numbers correspond with those used in Table I.

Some salient features of the application of GLC to the identification and measurement of certain related groups of phenolic compounds, typifying four important pathological conditions, are summarised in Table II.

TABLE II

GLC BEHAVIOUR ON STATIONARY PHASES F-60 AND SE52 OF DERIVATIVES OF SOME PHENOLIC ACIDS EXCRETED IN EXCESS IN CERTAIN DISORDERS

Syndrome	*Acid*	*Comment*
Phenylketonuria (23)	Phenylpyruvic acid	Detected easily when present. The TMS ether/ester derivative is unstable; the methyl ester/TMS ether however is stable and can therefore be measured quantitatively although it has the same MU value as *p*-hydroxymandelic acid (which provides minimal interference in practice as it is poorly extracted into ethyl acetate[9]).
	Phenyllactic acid	Not detected in normal urine. Poorly extracted into ethyl acetate[9] so that ether extraction is necessary. The methyl ester/TMS ether derivative runs together with peaks deriving from *p*-hydroxybenzoic and *m*-hydroxyphenylacetic acids; but a significant increase in size of this mixed peak in association with an increased *o*-hydroxyphenylacetic acid peak, is diagnostic.
	Phenylacetic acid	Very short retention time. Not accurately measurable by this procedure.
	o-Hydroxyphenylacetic acid	Present in small amount in most normal urine samples—an excess is diagnostic. The TMS ether/ester derivative runs together with that of the dietary *m*-hydroxybenzoic acid on SE52 but can be separated from it on F-60 columns. These two acids are well separated as their methyl ester/TMS ether derivative on either column.
Alkaptonuria (24)	Homogentisic acid	Not detected in normal urine. The MU value of its TMS ether/ester derivative coincides with that of DOPAC on both F-60 and SE52 (but DOPAC output is unlikely to be increased in the absence of an HVA rise). The methyl ester/TMS ether derivative is separated from that of DOPAC on both columns, although more satisfactorily on SE52.
Tyrosyluria of prematurity (25)	*p*-Hydroxyphenylpyruvic acid	Decomposes readily giving *p*-hydroxyphenylacetic acid when TMS ether/ester derivative is prepared. The methyl ester/TMS ether derivative is more stable and suitable for quantitative assessment.
	p-Hydroxyphenyllactic acid	The TMS ether/ester derivative emerges with VMA on F-60, but separately on SE52. As its excretion is minimal in the adult, F-60 may be used for VMA estimations in adult urine. Alternatively the methyl ester/TMS ether derivative can be chromatographed on either column.
Catecholamine-secreting tumours (26)	Homovanillic acid (HVA)	The TMS ether/ester derivative is well separated on F-60 but is mixed with vanillic acid on SE52. HVA and vanillic acids are separated on both columns as their methyl ester/TMS ether derivatives.
	3,4-Dihydroxyphenylacetic acid (DOPAC)	The TMS ether/ester derivative can be separated on F-60 but care must be taken not to confuse it with a neighbouring unknown peak (MU value 18.82). The methyl ester/TMS ether may also be separated on SE52 provided DOPAC is first isolated by adsorption on alumina before making the derivative.

4-Hydroxy-3-methoxymandelic acid (VMA)	The TMS ether/ester derivative cannot be estimated quantitatively (see text). The methyl ester/TMS ether derivative is recommended on SE52.
Vanillactic acid (VLA)	Easily identified and measured as either derivative on either column.
Vanilpyruvic acid (VPA)	Neither derivative is stable but the methyl ester/TMS ether derivatives give constant easily detected peaks.
Dihydroxyphenylpyruvic acid	Easily detected as the methyl ester/TMS ether derivative.
4-Hydroxy-3-methoxyphenylglycol (HMPG)	The "methyl ester/TMS ether" derivative (see text) is recommended on SE52.

Reference numbers in parentheses.

REFERENCES

1 M. D. ARMSTRONG, K. N. F. SHAW AND P. E. WALL, *J. Biol. Chem.*, 218 (1956) 293.
2 C. C. SWEELEY AND C. M. WILLIAMS, *Anal. Biochem.*, 2 (1961) 83.
3 C. M. WILLIAMS AND C. C. SWEELEY, *J. Clin. Endocrinol.*, 21 (1961) 1500.
4 C. M. WILLIAMS AND C. C. SWEELEY, *Clin. Chim. Acta*, 7 (1962) 880.
5 C. M. WILLIAMS, *Anal. Biochem.*, 4 (1962) 423.
6 C. M. WILLIAMS AND R. H. LEONARD, *Anal. Biochem.*, 5 (1963) 362.
7 C. M. WILLIAMS, G. E. DORIN AND M. GREER, *Anal. Biochem.*, 6 (1963) 468.
8 C. M. WILLIAMS AND M. GREER, *J. Am. Med. Assoc.*, 183 (1963) 836.
9 C. M. WILLIAMS AND C. C. SWEELEY, in H. SZYMANSKI (Ed.), *Biomedical Applications of Gas Chromatography*, Plenum Press, New York, 1964, p. 225.
10 C. M. WILLIAMS AND M. GREER, *Clin. Chim. Acta*, 11 (1965) 495.
11 R. E. BONDURANT, M. GREER AND C. M. WILLIAMS, *Anal. Biochem.*, 15 (1966) 364.
12 S. WILK, S. E. GITLOW, M. MENDLOWITZ, M. J. FRANKLIN, H. E. CARR AND D. D. CLARKE, *Anal. Biochem.*, 13 (1965) 544.
13 Z. HORII, M. MAKITA, I. TAKEDA, Y. TAMURA AND Y. OHNISHI, *Chem. Pharm. Bull.*, 13 (1965) 636.
14 G. CATROUX AND H. BLACHÈRE, *Compt. Rend., Série D*, 262 (1966) 1345.
15 E. R. BLAKLEY, *Anal. Biochem.*, 15 (1966) 350.
16 J. MENDEZ AND F. J. STEVENSON, *J. Gas Chromatog.*, 4 (1966) 483.
17 C. E. DALGLIESH, E. C. HORNING, M. G. HORNING, K. L. KNOX AND K. YARGER, *Biochem. J.*, 101 (1966) 792.
18 M. G. HORNING, K. L. KNOX, C. E. DALGLIESH AND E. C. HORNING, *Anal. Biochem.*, 17 (1966) 244.
19 M. G. HORNING, E. A. BOUCHER AND A. M. MOSS, *J. Gas Chromatog.*, 5 (1967) 297.
20 S. COOPER, C. M. NIELSON, S. K. SVIHOVEC AND N. WEINER, *Pharmacologist*, 9 (1967) 211.
21 A. I. VOGEL, *A Textbook of Practical Organic Chemistry including Qualitative Organic Analysis*, 3rd ed., Longmans Green, London, 1961, p. 971.
22 C. R. J. RUTHVEN AND M. SANDLER, *Clin. Chim. Acta*, 12 (1965) 318.
23 W. E. KNOX, in J. B. STANBURY, J. S. WYNGAARDEN AND D. S. FREDERICKSON (Eds.), *The Metabolic Basis of Inherited Disease*, 2nd ed., McGraw-Hill, New York, 1966, p. 258.
24 B. N. LA DU, in J. B. STANBURY, J. S. WYNGAARDEN AND D. S. FREDERICKSON (Eds.), *The Metabolic Basis of Inherited Disease*, 2nd ed., McGraw-Hill, New York, 1966, p. 303.
25 H. R. BLOXAM, M. G. DAY, N. K. GIBBS AND L. I. WOOLF, *Biochem. J.*, 77 (1960) 320.
26 M. SANDLER, *Proc. Roy. Soc. Med.*, 60 (1967) 795.

Proc. Assoc. clin. Biochem. 5 (1968) 123

Gas Chromatographic Measurement of Phenolic and Indolic Acids and Alcohols in Human Urine

F. KAROUM AND M. SANDLER

Bernhard Baron Memorial Research Laboratories and Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London, W.6

Gas chromatographic analysis, with its qualities of speed, sensitivity, high resolution and quantitative interpretation, is gradually taking its place in clinical biochemistry as an important analytical tool. So far it is well established in the fields of steroid and lipid assay; it undoubtedly has an important future rôle in the diagnosis and therapeutic monitoring of diseases associated with an abnormal excretion of phenolic and indolic acids and alcohols (Karoum *et al.*, 1968). Some of the conditions which may be profitably studied by this technique, together with characteristic urinary metabolites are listed in Table I.

TABLE I

Disease	Metabolites excreted in excess
Phenylketonuria (Knox, 1966)	phenylpyruvic acid, phenylacetic acid, *o*-hydroxyphenylacetic acid
Alkaptonuria (La Du, 1966)	homogentisic acid
Tyrosyluria of prematurity (Bloxam *et al.*, 1960)	*p*-hydroxyphenylpyruvic acid, *p*-hydroxyphenyllactic acid
Catecholamine secreting tumours (Sandler, 1967)	homovanillic acid (HVA), 4-hydroxy-3-methoxymandelic acid (VMA), 4-hydroxy-3-methoxyphenylglycol (HMPG)
Carcinoid tumours (Sandler, 1968)	5-hydroxyindoleacetic acid (5HIAA)

Trimethylsilyl ether/ester (TMS E/E) and methyl ester/trimethylsilyl ether (ME/TMS E) compounds are the volatile derivatives most suitable for quantitative assay of phenolic acids and alcohols. For indolic acids and alcohols, the ME/TMS E derivative is preferable.

TMS E/E derivatives may be chromatographed on either a 7 feet 10% silicone oil (F-60) or a 10% methyl phenyl silicone gum (SE 52) column. All the phenolic compounds shown in Table I are easily measured on the F-60 column with the important exception of VMA (Fig. 1). The ME/TMS E of VMA however, run on a 7 feet 10% SE 52 column, gives a discrete tracing which is very suitable for quantitation (Fig. 2). In general, the ME/TMS E derivatives of phenolic acids, run under isothermal conditions, tend to give better separations on a 7 feet 10% SE 52 column than on F-60. ME/TMS E derivatives of indolic acids and alcohols are best chromatographed on a 5 feet 6% SE 52 column.

Before chromatography, an aliquot of urine is extracted at pH 1, either with ethyl acetate (for phenolic compounds) or with ether (for phenolic and indolic compounds). After evaporation, an appropriate derivative is prepared. The amount injected into the column varies between 0.5 and 5 μg. depending on the type of detector used. When analysis of phenolic and indolic alcohols is required, the urine has first to be hydrolysed (Ruthven and

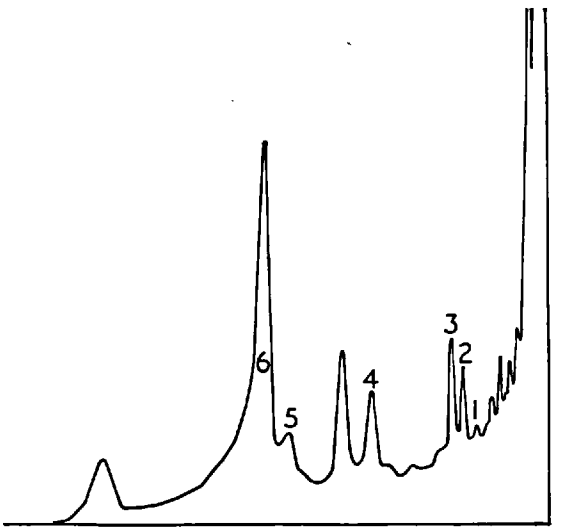


Fig. 1.—Chromatogram of trimethyl silyl ether/ester derivatives of phenolic acids prepared from an ethyl acetate extract of 10 ml. urine from a normal subject and run on a 7 feet 10% F-60 column. (1. *o*-hydroxyphenylacetic acid; 2. *m*-hydroxyphenylacetic acid; 3. *p*-hydroxyphenylacetic acid; 4. homovanillic acid (HVA); 5. 4-hydroxy-3-methoxymandelic acid (VMA); 6. hippuric acid.)

Paper read at the Southern England and South Wales Region Meeting, Halton, May, 1968.

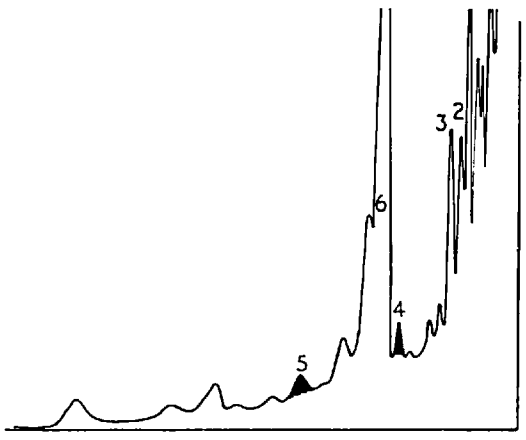


Fig. 2.—Chromatogram of methyl ester/trimethyl silyl ether derivatives of phenolic acids prepared from an ether extract of 10 ml. urine from a normal subject, and run on a 7 feet 10% SE 52 column. (2. *m*-hydroxyphenylacetic acid; 3. *p*-hydroxyphenylacetic acid; 4. homovanillic acid (HVA); 5. 4-hydroxy-3-methoxymandelic acid (VMA); 6. hippuric acid.)

Sandler, 1965) and then extracted with the appropriate solvent at pH 7.5.

Quantitative analysis is carried out by adding internal standards to one of a duplicate of one of the urine specimens in a batch. Measurement of peak height has been found to give consistent results which are comparable with those obtained using calculations involving peak area or the product of peak height and half its width. Most of the technical procedures referred to here are discussed in greater detail by Karoum *et al.* (1968).

Recently, it has become obvious that the scope of this type of gas chromatographic analysis can be greatly enlarged if the instrument is coupled to a mass spectrometer (Dalgliesh *et al.*, 1966). Emerging peaks can be scanned for purity and the identity of unknown compounds can be obtained by this technique.

References

Bloxam, H. R., Day, M. G., Gibbs, N. K. and Woolf, L. I. (1960), *Biochem. J.*, **77**, 320.

Dalgliesh, C. E., Horning, E. C., Horning, M. G., Knox, K. L. and Yarger, K. (1966), *Biochem. J.*, **101**, 792.

La Du, B. N. (1966), in *The Metabolic Basis of Inherited Disease*, ed. J. B. Stanbury, J. S. Wyngaarden and D. S. Frederickson, 2nd Edn., McGraw-Hill, New York, p. 303.

Karoum, F., Ruthven, C. R. J. and Sandler, M. (1968), *Clin. Chim. Acta*, **20**, 427.

Knox, W. E. (1966), in *The Metabolic Basis of Inherited Disease*, ed. J. B. Stanbury, J. S. Wyngaarden and D. S. Frederickson, 2nd Edn., McGraw-Hill, New York, p. 258.

Ruthven, C. R. J. and Sandler, M. (1965), *Clin. Chim. Acta*, **12**, 318.

Sandler, M. (1967), *Proc. R. Soc. Med.*, **60**, 795.

Sandler, M. (1968), in *Advances in Pharmacology*, ed. E. Costa and M. Sandler, Vol. 6B, Academic Press, New York, p. 127.

Reprinted from
Clinica Chimica Acta
Elsevier Publishing Company,
Amsterdam
Printed in The Netherlands

FURTHER OBSERVATIONS ON THE GAS-CHROMATOGRAPHIC MEASUREMENT OF URINARY PHENOLIC AND INDOLIC METABOLITES

F. KAROUM, C. O. ANAH, C. R. J. RUTHVEN AND M. SANDLER
Bernhard Baron Memorial Research Laboratories and Institute of Obstetrics & Gynaecology, Queen Charlotte's Maternity Hospital, London, W.6. (U.K.)

(Received January 2, 1969)

SUMMARY

Previous gas–liquid chromatographic (GLC) studies on urinary phenolic and indolic metabolites and accompanying extraction and preparative procedures have been extended. Whilst ethyl acetate and ether were both suitable for the extraction of phenolic compounds from urine, with a few exceptions, only the latter is recommended for indolic metabolites. Metabolites were selectively extracted into organic solvent by suitably adjusting the pH of the aqueous solution. Thus all acids were extracted at pH 2; between pH 3 and 5 only weaker acids were readily removed; at pH 8 neutral compounds were still extracted but acids now remained in the aqueous phase. Conditions have been devised to minimise *O*-methylation during the preparation of methyl ester derivatives. Quantitation has been improved; indolic compounds at concentrations present in normal urine may now be measured. Using the refined techniques for their estimation, the normal output (20 subjects) of the catecholamine metabolites, homovanillic acid, 4-hydroxy-3-methoxymandelic acid and 4-hydroxy-3-methoxyphenylglycol were (mean ± S.D.) 4.2 ± 1.5 mg/24 h, 5.7 ± 1.3 mg/24 h and 2.4 ± 0.8 mg/24 h respectively.

INTRODUCTION

The application of gas-liquid chromatography (GLC) to the analysis of urinary phenolic and indolic metabolites[1–5] has yielded valuable information about their excretion in normal and pathological conditions. We have previously shown[1,2] that quantitative data can be obtained not only for such compounds as homovanillic acid (HVA), 4-hydroxy-3-methoxymandelic acid (VMA) and 4-hydroxy-3-methoxyphenylglycol (HMPG) for which alternative assay methods are available, but also for others whose relatively unhelpful chemical structure renders specific quantitative estimation difficult, *e.g.* *p*-hydroxyphenyllactic and *p*-hydroxyphenylacetic acids. Certain additional observations are now presented concerning overall recovery, purity and resolution of chromatographic fractions, selection of solvent, choice of pH for

preliminary extraction of metabolites from urine, control of conditions for the formation of derivatives and optimum concentration of stationary phase.

EXPERIMENTAL

Instrumentation (Pye Panchromatograph fitted with an argon ionisation detector), materials and reagents, column preparation and characterisation of peaks were as described previously[1]. The preparation of trimethylsilyl ether/ester (TE/E) and methyl ester/trimethylsilyl ether (ME/TE) derivatives was slightly modified from that used previously by the use of 0.3 ml of a 1:2 mixture of hexamethyldisiloxane and dioxane (instead of 0.2 ml of each) followed by 0.05 ml of trimethylchlorosilane (instead of 0.1 ml). Derivatives of phenolic compounds were separated isothermally as before[1] on a 7-ft. 10% SE52 column at 190°. A more satisfactory separation of ME/TE derivatives of phenolic metabolites with methylene unit values over 20, and of indoles, was obtained on a 5-ft. 5% SE52 column run at 190°.

The effect of solvent and pH on extraction of metabolites was investigated in the following way: authentic compounds (approx. 0.2 mg in 0.5 ml methanol) were added to water (10 ml), and after saturating with NaCl, the pH of successive replicates was adjusted to a series of values (Table II) between 1 and 10, using a glass electrode. Extraction was carried out by vortex mixing ("Whirlimixer") for 1 min with 30 ml solvent (ether or ethyl acetate). Aliquots (25 ml) of the organic phase, separated by centrifugation, were evaporated to dryness at about 40° *in vacuo* and the dry residue from each was retained for the preparation of a ME/TE derivative, or, in the case of phenylpyruvic acid and the neutral compounds, a TE/E derivative. TE/E derivatives were prepared by mixing the dry residue with about 0.5 ml methanol and drying under nitrogen before silylation. Overall recovery was gauged by comparison with data obtained by GLC analysis (assuming 100% recovery of derivatives) of the residue obtained when standard solutions of authentic compounds, equal in amount to those added prior to extraction, were taken to dryness directly.

RESULTS AND DISCUSSION

Conditions suitable for the GLC separation of TE/E and ME/TE derivatives of phenolic acids and alcohols[1] were unsatisfactory for the analysis of indolic compounds at concentrations present in normal urine. This deficiency has been filled by the introduction of a 5-ft. 5% SE52 column operating at 190°, which is suitable for the separation of both mono- and dihydroxyindoles (Fig. 1). Methylene unit values of some indolic and phenolic compounds which can be readily measured in this way are given in Table I.

Experimental conditions employed for the preparation of methyl esters of phenolic acids must be carefully controlled. All monohydroxy aromatic acids and alcohols studied were completely esterified during our standard one-minute exposure to ethereal diazomethane, but dihydroxyphenolic acids underwent minor degrees of *O*-methylation as well. By reducing esterification time to 30 sec, adventitious *O*-methylation was eliminated without serious reduction in yield of methyl esters of monohydroxy compounds. In contrast, mono- and dihydroxyindoles did not form measurable quantities of *O*-methylated derivatives even after 15 min in a solution of

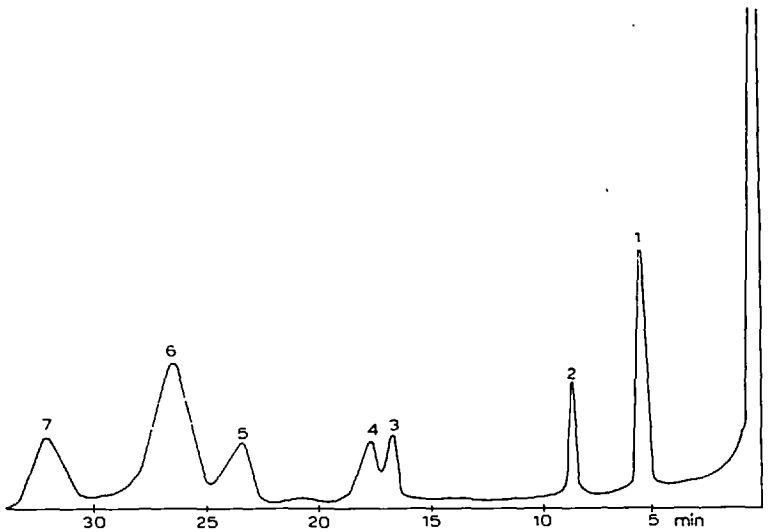


Fig. 1. Chromatogram of derivatives of authentic compounds of biological importance run as a mixture on a 5-ft. 5% SE52 column. Peak numbers correspond to the trimethylsilyl ether/esters (TE/E) of (1) tryptophol, (2) indolylacetic acid, (3) 5-hydroxytryptophol, (5) 5HIAA and the methyl ester/trimethylsilyl ethers (ME/TE) of (4) 5HIAA, (6) 5,6-dihydroxyindolylacetic acid, (7) indolylpyruvic acid.

TABLE I

METHYLENE UNIT VALUES OF ACIDS AND ALCOHOLS WITH RELATIVELY HIGH RETENTION TIME

5-ft. 5% SE52 column maintained at 190°, with inlet pressure of 1500 mm/Hg.

	Methylene unit values of derivatives	
	Methyl ester/TMS ether	*TMS ether/ester*
Indolylacetic acid	18.50	—
Tryptophol	18.34	18.34
5-Hydroxytryptophol	21.25	21.25
5-Hydroxyindolylacetic acid (5HIAA)	21.45	22.16
5-Hydroxy-6-methoxyindolyl-2-carboxylic acid	22.16	—
5,6-Dihydroxyindolyl-2-carboxylic acid	22.55	—
Indolylpyruvic acid	22.95	—
5,6-Dihydroxyindolylacetic acid	24.14	—
Vanilpyruvic acid	21.15	—
Vanillactic acid	19.40	—

diazomethane. Exposure of keto acids (*e.g. p*-hydroxyphenylpyruvic and 4-hydroxy-3-methoxyphenylpyruvic acids) to diazomethane for 2 min rather than 1 min considerably reduced the tendency to form subsidiary peaks. Phenylpyruvic acid was an exception, readily decomposing to give multiple peaks. On the other hand TE/E formation on an SE52 column gave only one derivative and is therefore the procedure of choice (Fig. 2). This recommendation, which contradicts our previous suggestion that the ME/TE derivative is preferable[1], is based on recent detailed investigations using GLC as a screening procedure for phenylketonuria and tyrosyluria.

A comparison of the efficacy of ethyl acetate and ether for extractions at different pH values is summarised in Table II. Whilst in general both were suitable for phenolic compounds, indolic metabolites were much more readily extracted with ether,

which was about twenty times more efficient than ethyl acetate for 5-hydroxyindolylacetic acid (5HIAA). Ether was also more suitable for certain phenolic acids—*p*-hydroxymandelic, phenyllactic, dihydroxyphenylacetic and homogentisic.

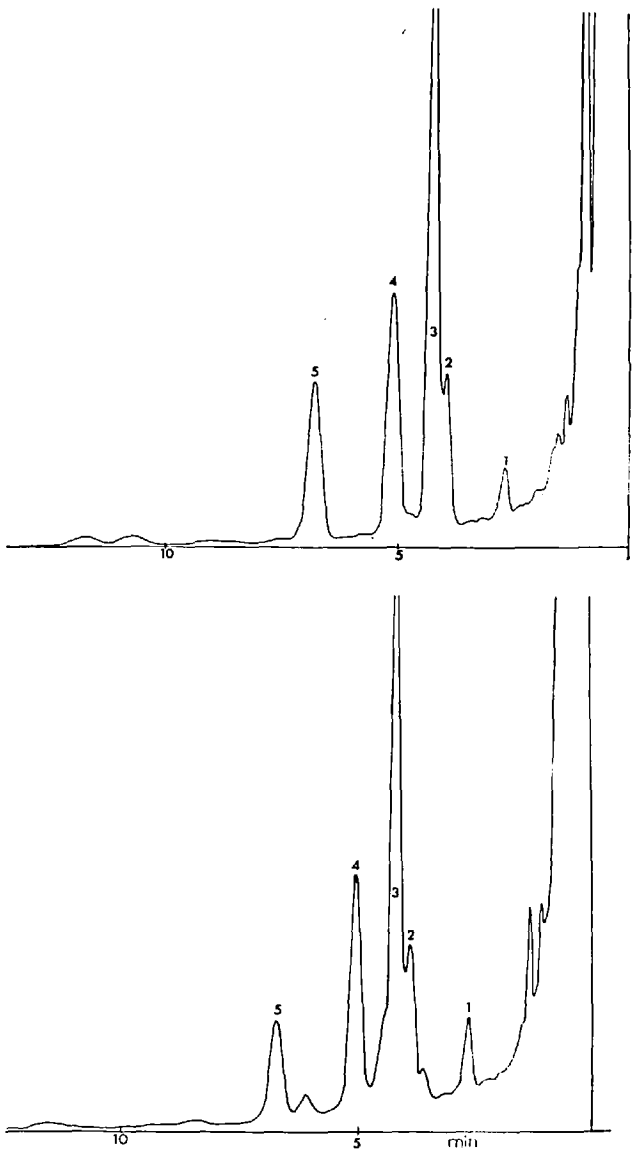


Fig. 2. (a) Chromatogram of trimethylsilyl ether/ester (TE/E) derivatives prepared from an ether extract of 0.2 ml phenylketonuric urine, and run on a 7-ft. 10% SE52 column. (b) Chromatogram prepared and run as in Fig. 2 (a) on an aqueous extract (10 ml) from a square foot of paper-tissue (napkin) previously soaked in a dilution of 0.2 ml of the phenylketonuric urine (Fig. 2 (a)) in water (5 ml) and then dried for 2 days.

The peak numbers (Figs. 2 (a & b)) correspond to the TE/E derivative of (1) phenylacetic acid, (2) *o*-hydroxyphenylacetic acid, (3) phenyllactic acid, (4) *p*-hydroxyphenylacetic acid, (5) phenylpyruvic acid.

TABLE II

RECOVERIES OF PHENOLIC AND INDOLIC COMPOUNDS EXTRACTED WITH ETHYL ACETATE OR ETHER FROM AQUEOUS SOLUTION AT pH VALUES BETWEEN 1 AND 10

Except where stated, ME/TE derivatives were prepared and chromatographed on a 7-ft. 10% SE52 column, apart from 5HIAA and 5-hydroxytryptophol where a 5-ft. 5% SE52 column was used.

	Percentage recovery									
	Ethyl acetate extract					*Ether extract*				
pH value of aqueous phase	1	4	6	8	10	1	4	6	8	10
Phenyllactic acid	30	30	20	N.D*	N.D	75	12	N.D	N.D	N.D
p-Hydroxyphenyllactic acid	73	31	24	13	N.D	84	25	N.D	N.D	N.D
p-Hydroxyphenylacetic acid	90	70	58	N.D	N.D	100	81	12	N.D	N.D
Phenylpyruvic acid+	50	N.D	N.D	N.D	N.D	50	5	N.D	N.D	N.D
p-Hydroxyphenylpyruvic acid	100	5	N.D	N.D	N.D	35	N.D	N.D	N.D	N.D
4-Hydroxy-3-methoxymandelic acid (VMA)	85	10	N.D	N.D	N.D	45	N.D	N.D	N.D	N.D
Homovanillic acid (HVA)	100	100	23	23	—	79	23	10	N.D	N.D
3,4-Dihydroxyphenylacetic acid	10	5	3	N.D	N.D	75	40	3	N.D	N.D
-Hydroxymandelic acid	23	8	1	N.D	N.D	68	19	N.D	N.D	N.D
,4-Dihydroxymandelic acid	63	N.D	N.D	N.D	N.D	13	N.D	N.D	N.D	N.D
omogentisic acid	N.D	N.D	N.D	N.D	N.D	71	N.D	N.D	N.D	N.D
entisic acid	97	28	34	8	8	97	49	13	15	N.D
ippuric acid	64	N.D	N.D	N.D	N.D	47	N.D	N.D	N.D	N.D
-Hydroxy-3-methoxyphenylglycol (HMPG)+	99	72	68	58	51	7	29	47	50	29
-Hydroxy-3-methoxyphenylethanol+	—	—	79	70	—	—	—	—	—	—
-Hydroxyphenylethanol+	—	—	80	80	—	—	—	—	—	—
ndolylacetic acid	53	53	26	N.D	N.D	80	53	26	N.D	N.D
ryptophol+	N.D	28	34	48	44	97	97	97	100	100
-Hydroxyindolylacetic acid (5HIAA)	5	N.D	N.D	N.D	N.D	76	94	9	N.D	N.D
-Hydroxytryptophol+	—	—	—	—	—	94	109	103	106	110

N.D = not detected.
The TE/E derivative was prepared.

At pH values of less than 3, both acidic and neutral compounds were extracted, but with increasing pH, recoveries of stronger followed by those of weaker organic acids fell sharply; recoveries of neutral compounds were not markedly affected. These properties have recently been discussed by Sapira[6]. Gitlow *et al.*[7] took advantage of the relatively strong acidity of VMA to eliminate weaker phenolic acids which interfered during its colorimetric estimation. Dihydroxymandelic and *p*-hydroxymandelic VMA, were effectively extracted by ethyl acetate only when the pH of the aqueous phase was less than 3. Although these investigations (Table II) were carried out on pure solutions, findings were similar when extraction was made from urine, with a tendency for recoveries to be slightly better than from water alone.

Two other examples should be mentioned of how choice of a suitable pH for extraction may help to eliminate unwanted compounds, prior to GLC separation; although pH values of either 1 or 2 were satisfactory for the extraction of most urinary phenolic acids into ethyl acetate, pH 2 must be recommended, as hippuric acid, which sometimes causes interference, was less readily extracted at this pH. Indolic acids however, particularly 5HIAA, were efficiently taken up into ether at pH 3.5, prior to their separation on a 5-ft. 5% SE52 column. Most phenolic acids remained in the aqueous phase at this pH.

Phenolic and indolic alcohols and other neutral compounds were selectively extracted with ethyl acetate or ether at pH 8, leaving acidic compounds in the aqueous phase. The alcohols are excreted into the urine largely as highly polar conjugates which had first to be hydrolysed before extraction. Hydrolysis was conveniently per-

formed by incubating urine (10 ml, pH 6.2) with a sulphatase–β-glucuronidase preparation (suc d'*Helix pomatia*, Industrie Biologique Française, 35 à 49, Quai du Moulin de Cage, 92 Gennevilliers, France) (0.2 ml). Traces of ethyl acetate were found partially to inhibit these enzymes; its use for initial extraction of free aromatic acids

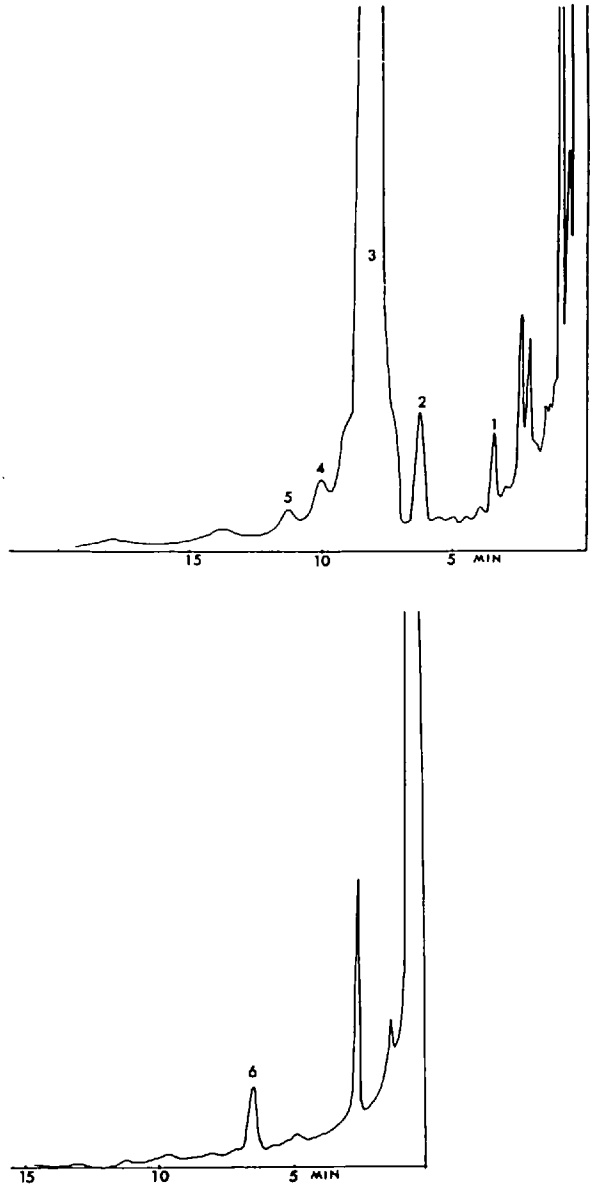


Fig. 3. Chromatograms of methyl ester/trimethylsilyl ether (ME/TE) derivatives prepared from (a) an ethyl acetate and (b) dichloromethane extract of acidified (pH 1) alkaptonuric urine (1 ml), and run on a 7-ft. 10% SE52 column. The peak numbers (Figs. 3 (a & b)) correspond to the ME/TE derivatives of (1) *p*-hydroxyphenylacetic acid, (2) *ortho*-methylated homogentisic acid masking HVA, (3) homogentisic acid, (4) *p*-hydroxyphenyllactic acid, (5) VMA and (6) HVA.

prior to enzymatic hydrolysis of conjugates in the aqueous phase is therefore inadvisable.

Using the extraction procedures outlined above, followed by GLC analysis and quantitation as previously described[1], the urinary excretion of three important catecholamine metabolites, HVA, VMA and HMPG, were determined in 20 normal adult subjects. The results obtained (mean ± S.D.) were 4.2 ± 1.5 mg HVA /24 h,

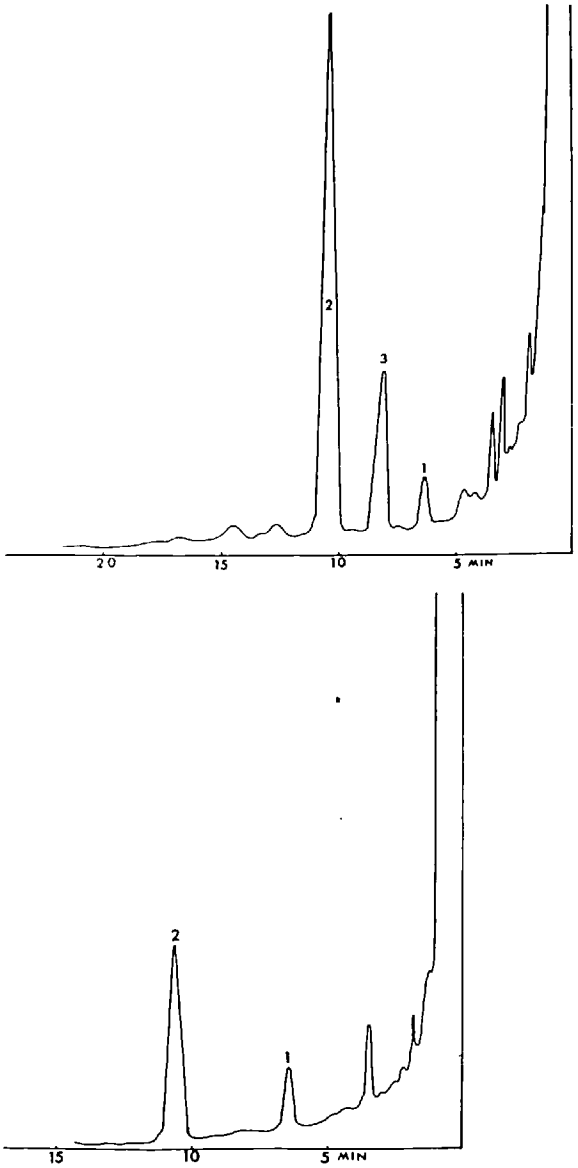


Fig. 4. Chromatogram of trimethylsilyl ether/ester (TE/E) derivatives prepared from (a) ethyl acetate and (b) dichloromethane extract of hydrolysed urine (5 ml) at pH 8 from a patient with neuroblastoma, and run on a 7-ft. 10% SE52 column. The peak numbers (Figs. 4 (a & b) correspond to the TE/E derivatives of (1) 4-hydroxy-3-methoxyphenylethanol, (2) HMPG, (3) HVA.

5.7 ± 1.3 mg VMA/24 h, and 2.4 ± 0.8 mg total HMPG/24 h, values not too dissimilar from those obtained by other techniques[8-11]. Mass spectroscopy confirmed the identity and purity of the GLC fraction associated with each of these metabolites.

The interpretation of chromatograms from the complex mixture of aromatic compounds in urine should always be made with caution, particularly when excretion is grossly abnormal. When ME/TE derivatives of urine extracts from patients with phenylketonuria were prepared, decomposition products of the large amounts of phenylpyruvic acid present gave rise to peaks which masked and might have been mistaken for HVA. This difficulty did not arise when a TE/E derivative was prepared (Fig. 2). In alkaptonuria (Fig. 3), the relatively enormous concentrations of urinary homogentisic acid were responsible for the formation of a small amount of an *O*-methylated analogue during preparation of a ME/TE derivative which at first led to the erroneous conclusion that the artifact was an endogenous metabolite, characteristic of the disease. As in phenylketonuria, the preparation of a TE/E derivative cleared up this confusion. An alternative approach used to obtain an uncontaminated ME/TE derivative of HVA in alkaptonuric urines was to treat the sample with dichloromethane[8] extracting true HVA and leaving the spurious in solution. Dichloromethane was also found to be a useful solvent for 4-hydroxy-3-methoxyphenylethanol (Fig. 4).

ACKNOWLEDGEMENTS

We are grateful to the Smith, Kline & French Foundation for a generous grant for gas chromatography equipment.

REFERENCES

1 F. KAROUM, C. R. J. RUTHVEN AND M. SANDLER, *Clin. Chim. Acta*, 20 (1968) 427.
2 F. KAROUM AND M. SANDLER, *Proc. Assoc. Clin. Biochem.*, 5 (1968) 123.
3 C. M. WILLIAMS AND M. GREER, *Clin. Chim. Acta*, 7 (1962) 880.
4 C. M. WILLIAMS AND M. GREER, *J. Am. Med. Assoc.*, 183 (1963) 836.
5 C. M. WILLIAMS AND M. GREER, *Clin. Chim. Acta*, 11 (1965) 495.
6 J. D. SAPIRA, *Clin. Chim. Acta*, 20 (1968) 138.
7 S. E. GITLOW, M. MENDLOWITZ, E. KRUK AND S. KHASSIS, *Circulation Res.*, 9 (1961) 746.
8 C. R. J. RUTHVEN AND M. SANDLER, *Clin. Chim. Acta*, 14 (1966) 511.
9 J. J. PISANO, J. R. CROUT AND D. ABRAHAM, *Clin. Chim. Acta*, 7 (1962) 285.
10 C. R. J. RUTHVEN AND M. SANDLER, *Clin. Chim. Acta*, 12 (1965) 318.
11 S. WILK, S. E. GITLOW, D. D. CLARKE AND D. H. PALEY, *Clin. Chim. Acta*, 16 (1967) 403.

Fourth International Congress on Pharmacology

July 14 to 18, 1969 in Basel, Switzerland

Abstracts

depressed by PEA (and MAOI), its precursor phenylalanine (and MAOI), its derivative phenylacetaldehyde and its α-methylated homolog amphetamine. In MAOI treated rabbits, dopa and phenylalanine were mutually antagonistic. 5HTP (i.v.) and 5HT (intraventricular) first enhanced and later depressed the slow wave; their effects were partially blocked by MAOI's. Indoleacetaldehydes also affect visual evoked response (Sabelli et al.: Pharmacologist 1968). The locomotor activity of mice was depressed by dopa, epinephrine, isoproteronol, 5HT, T and large doses of PEA. MAOI's did not affect CA or 5HT depression, potentiated T depression and changed the effect of PEA into an amphetamine-like stimulation. Methamphetamine and phenylethylamine antagonized CA, 5HT and T depression; methysergide and LSD antagonized 5HT and T. In newly hatched chicks (lacking blood-brain barrier) not only CA, 5HT and T but also the aldehyde derivatives of 5HT and T produced depression and drowsiness. MAOI partially conteract 5HTP and 5HT sleep (Giardina et al.: Fed. Proc. 1969).

These and other results suggest that the ergotropic-adrenergic vs. trophotropic-serotonergic concepts of Hess and Brodie should be modified as follows: a) catecholamines are ergotropic modulators at least for sensory functions, but PEA may be the modulator of ergotropic hyperactivity; b) sleep mechanisms may involve not only 5HT (Jouvet, 1967) and possibly T, but also indoleacetaldehydes which may be the deaminated metabolites of neuroamines postulated by Jouvet to be responsible for paradoxical sleep.

Supported by Grant MH-14110-01.

Sandler M. Karoum F. Ruthven C.R.J. Southgate J. Calne D.B. (London):
Metabolism of L-DOPA in Parkinsonism

During recent trials of L-DOPA in postencephalitic and idiopathic Parkinsonism (Calne et al., 1969), urine samples were collected before, during and after treatment. They have now been quantitatively assayed isothermally for phenolic acidic and alcoholic metabolites by gas-liquid chromatography.

Comparatively vast amounts of homovanillic acid and dihydroxyphenylacetic acid were excreted as major metabolites but relatively smaller quantities than might have been predicted of 4-hydroxy-3-methoxyphenylethanol. A small increase only in VMA output, together with a marginal decrease in 4-hydroxy-3-methoxyphenylglycol (HMPG) were present, resulting in an increased VMA/HMPG ratio. Increased amounts of *m*-hydroxyphenylacetic acid were observed, presumably deriving from *p*-dehydroxylation of DOPA (DeEds et al., 1957) by gut flora. Appreciable concentrations of 4-hydroxy-3-methoxyphenyllactic acid (VLA) were in evidence, thus establishing transamination as a minor pathway of L-DOPA metabolism. It is possible that the ketoacid precursor of VLA competes for *p*-hydroxyphenylpyruvate oxidase, as increased amounts of *p*-hydroxyphenyllactic acid, the levels of which tended to correlate with VLA output, were also excreted. Despite a putative increase in *p*-tyramine output in Parkinsonism (Boulton et al., 1967), increased levels of its oxidatively deaminated metabolite, *p*-hydroxyphenylacetic acid could not be detected at any stage in the investigation. This paradoxical finding may perhaps be explained by decreased *in vivo* monoamine oxidase (MAO) activity; platelet MAO levels were found to be significantly lower in patients with Parkinsonism than in controls.

References

Boulton A.A., Pollitt R.J., and Majer J.R.: Nature (Lond.) *215*, 132 (1967).
Calne D.B., Stern G.M. and Laurence D.R.: Abstract submitted for this meeting (1969).
DeEds F., Booth A.N. and Jones F.T.: J. biol. Chem. *225*, 615 (1957).

Sedvall G. Nybäck H. (Stockholm):
Effect of LSD on the Disappearance from Brain of Serotonin Formed in vivo from Labelled Tryptophane

H^3-l-tryptophane was administered to conscious mice by constant rate i.v. infusion or injection. Labelled serotonin and tryptophane in brain was isolated by methods involving ionexchange chromatography and organic solvent extraction. Labelled serotonin formed was identified by paper chromatography. During infusion of H^3-tryptophane labelled

THE METABOLISM OF ORALLY ADMINISTERED L-DOPA IN PARKINSONISM

BY

D. B. CALNE, F. KAROUM, C. R. J. RUTHVEN and M. SANDLER

Reprinted from BRITISH JOURNAL OF PHARMACOLOGY, *September*, 1969, *vol.* 37, *No.* 1, *p.* 57.

LONDON
MACMILLAN (Journals) LIMITED
4 LITTLE ESSEX STREET, W.C.2

Br. J. Pharmac. (1969), **37**, 57–68.

The metabolism of orally administered L-DOPA in Parkinsonism

D. B. CALNE, F. KAROUM, C. R. J. RUTHVEN AND M. SANDLER

Medical and Neurological Units, University College Hospital, London W.C.1, and Bernhard Baron Memorial Research Laboratories and Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London W.6

1. Gas-liquid chromatographic methods were used to measure urinary acidic and alcoholic metabolites of L-DOPA, which had been administered in high oral dosage to patients with postencephalitic and idiopathic Parkinsonism.

2. The output of these compounds was normal before treatment. During drug therapy, large quantities of the dopamine metabolites, homovanillic acid and dihydroxyphenylacetic acid, were excreted but traces only of 4-hydroxy-3-methoxyphenylethanol. Noradrenaline metabolites showed little change in output other than a small increase in 4-hydroxy-3-methoxymandelic acid.

3. Information was obtained about a number of minor routes of degradation which might be implicated in the therapeutic action of L-DOPA. A raised output of *m*-hydroxyphenylacetic acid pointed to *p*-dehydroxylation of dihydroxyphenylacetic acid by gut flora. Evidence of transamination as a minor metabolic pathway was obtained by finding appreciable urinary levels of 4-hydroxy-3-methoxyphenyllactic acid. A keto-acid precursor of this compound may act as competitive inhibitor of an enzyme active in the normal degradation route of tyrosine, *p*-hydroxyphenylpyruvic acid oxidase, for increased amounts of *p*-hydroxyphenyllactic acid, the major metabolic derivative of *p*-hydroxyphenylpyruvic acid, accumulated in the urine during DOPA treatment.

The amino-acid L-dihydroxyphenylalanine (L-DOPA) is now firmly established as precursor of the catecholamines in a wide variety of animal species (for review, see Sandler & Ruthven, 1969). A deficiency of its immediate decarboxylation product, dopamine, in the basal ganglia of the brain has been amply demonstrated in Parkinsonism and may be a contributory factor in its pathogenesis (see Hornykiewicz, 1966). Attempts to redress this deficiency thus appear to be a rational approach to treatment of the disease. Dopamine itself does not traverse the blood-brain barrier in appreciable quantity; however, its precursor, L-DOPA, passes across to be decarboxylated within the brain (Gey & Pletscher, 1964).

The clinical material on which the present investigation is based, was obtained during a "between patient", "double-blind" therapeutic trial of oral L-DOPA in Parkinsonism (Calne, Stern, Laurence, Sharkey & Armitage, 1969). Although the clinical effect of the drug has now been studied in a number of trials (Calne *et al.*,

1969) the opportunity thus provided to obtain information on its further metabolism has been largely neglected, apart from some limited studies (McGeer, Boulding, Gibson & Foulkes, 1961 ; Sourkes, Pivnicki, Brown, Wiseman-Distler, Murphy, Sankoff & Saint Cyr, 1965 ; Cotzias, Papavasiliou & Gellene, 1969). Rather more is known about the metabolism of L-DOPA in the normal human subject (Guggenheim, 1913 ; Shaw, McMillan & Armstrong, 1957 ; Sourkes *et al.*, 1965 ; Pletscher, Bartholini & Tissot, 1967 ; Smith, 1967).

Hitherto, assay procedures for many of the urinary metabolites of this amino-acid have been difficult and time-consuming. Gas chromatographic methods for urinary phenolic acids and alcohols have recently become available, however (Karoum, Ruthven & Sandler, 1968 ; Karoum & Sandler, 1968 ; Karoum, Anah, Ruthven & Sandler, 1969) ; they are fast and accurate, and with their help it is possible to measure a number of different compounds on the same chromatogram. We have used these techniques to monitor the urinary excretion of some of these compounds in patients taking part in the therapeutic study described by Calne *et al.* (1969) and have obtained biochemical information which had not previously been available.

Methods

Urine specimens (24 hr), with 25 ml. of 6 N HCl as a preservative, were obtained from forty long-term patients (eighteen men and twenty-two women) with post-encephalitic Parkinsonism who were undergoing a " double-blind " therapeutic trial of oral L-DOPA (dose range 0·5–2·5 g/day in divided doses ; mean 1·3 g, for 47 days). Full details of this trial are provided elsewhere (Calne *et al.*, 1969).

Urine collections were made at four stages in the trial: (1) either before commencing placebo or L-DOPA regimes or while control subjects were on placebo (" pre-treatment ") ; (2) some days after starting oral L-DOPA (" under treatment ") ; (3) within 24–48 hr of stopping the L-DOPA regime (" end of treatment ") ; and (4) 2 weeks later (" follow-up ").

Circumstances prevented the systematic analysis of every 24 hr urine collection ; instead analyses were carried out in two series. In the " homogeneous " series, six female subjects were followed through the four stages enumerated above. In the heterogeneous series, the " pre-treatment " group consisted of three males and eleven females ; seven males and five females were " under treatment ", seven females constituted the " end of treatment " and ten females the " follow-up " groups. Some subjects were common to all groups. The full range of metabolites was not measured in every subject in the heterogeneous series ; the number of subjects from whom the mean excretion rate of each metabolite was calculated is shown in parentheses in Table 1.

Urine samples (24 hr) were also collected for analysis from four patients with idiopathic Parkinsonism who were being given 4·25 to 4·75 g L-DOPA per day, orally in divided doses, almost double the maximum dose given to any of the post-encephalitic patients.

Assay procedure

Urinary phenolic acids and alcohols were measured by isothermal gas liquid chromatography (GLC), essentially as described previously (Karoum *et al.*, 1968 ;

Karoum *et al.*, 1969), using a Pye Panchromatograph with a 7 foot 10% SE52 column at 190° C. The acids were usually run as methyl ester/trimethyl silyl ether (ME/TE) derivatives and the alcohols as their trimethyl silyl ether/esters (TE/E). Chromatographic peaks were characterized in terms of methylene unit values (Dalgliesh, Horning, Horning, Knox & Yarger, 1966) and quantified by comparing peak heights in analyses of duplicate urine samples, to one of which an internal standard was added at the start of the procedure.

In samples from patients in the " pre-treatment " or " follow-up " groups, phenolic acids were isolated from 10 ml. of salt saturated urine at pH 2·0 by extracting twice with 25 ml. of ethyl acetate. Phenolic alcohols were extracted from duplicate 10 ml. specimens of urine after incubating overnight with 0·2 ml. of a sulphatase-glucuronidase preparation (Suc d'*Helix pomatia*, Industrie Biologique Français, Gennevilliers en Seine, France) at 37° C and pH 6·3 to hydrolyse conjugated phenols. The urine sample was adjusted to pH 8, saturated with NaCl and extracted twice with 25 ml. of ethyl acetate. Twenty and 25 ml. portions of the successive extracts from each analysis were pooled, evaporated to dryness under vacuum at 40°–50° C and derivatives prepared from the dry residue.

Analysis of specimens from patients in the " under treatment " or " end of treatment " groups was modified in order to assure satisfactory quantification of the large amounts of homovanillic acid (HVA) and dihydroxyphenylacetic acid (DOPAC) present and prevent their interference with the estimation of lesser metabolites. HVA and DOPAC were assayed following two successive extractions of 0·5 or 1 ml. urine with 25 ml. of diethyl ether AR after diluting with 10 ml. 0·01 N HCl and saturating with NaCl. Portions of the ether extracts, 20 and 25 ml. respectively, were combined and evaporated and, in this case, trimethyl silyl ether/ester derivatives were prepared. To eliminate DOPAC, which interferes with measurement of 4-hydroxy-3-methoxymandelic acid (VMA) and other phenolic acids at concentrations present, 5 ml. of urine was adjusted to pH 8·4, and 2·5 g of alumina ('Camag', MFC, Hopkin & Williams Ltd.) was added and vortex mixed ('Whirlimixer') for 1 min. After centrifuging and adjusting the pH of the supernatant to 2·0, phenolic acids were extracted with ethyl acetate, isolated and analysed as before. For the estimation of phenolic alcohols a 10 ml. portion of urine adjusted to pH 1 with 6 N HCl was extracted twice with 25 ml. of diethyl ether in order to remove phenolic acids. The extracted urine was heated at 60° C for a few minutes under vacuum to expel traces of solvent and then incubated (37° C) with the sulphatase-glucuronidase preparation after adjusting the pH to 6·3. Following incubation the pH was raised to 8·4, alumina added, mixed, centrifuged, and the phenolic alcohols isolated from the supernatant and analysed as before. The preliminary extraction of the phenolic acids is also likely to remove free alcohols. The amount of free HMPG lost in this way is probably negligible compared with the output of its conjugate for although the derivatives of free HMPG (methylene unit value 18·37) and VMA (methylene unit value 18·43) run closely to each other during GLC, there was neither an alteration in the retention time of VMA nor the presence of a shoulder on the ascending slope of its peak in any sample.

Statistical tests

Means and standard errors (S.E.) of means were calculated for the 24 hr urinary excretion values of each metabolite. *t* Tests were carried out to ascertain the signi-

ficance of differences between treatment groups as well as their relation to the dose of L-DOPA. In the homogeneous series the paired *t* test was used, the subjects serving as their own controls. Within each group, an examination of the degree of correlation was made for the following variables: selected metabolites, urine volume, dose of L-DOPA in the previous 24 hr and total dose to date of collection of specimen under test.

Each group was regarded as a random sample from its appropriate population and no attempt was made to match patients who appeared in more than one group. It was assumed, but not tested, that these samples were not subject to selection. The results of statistical tests on the small homogeneous series of six patients analysed during all four phases of the investigation support those found in the larger but heterogeneous series.

The " between group " analysis in the heterogeneous series was based on the logarithms of the recorded data as this transformation appeared to create greater similarity of variance within each of the groups. For the analysis of the homogeneous series, the actual readings were used as their range was smaller compared with the unmatched groups.

Differences between means and between correlation coefficients have been accepted as statistically significant if the likelihood of their occurring by chance was equal to or less than 5% ($P \leqslant 0{\cdot}05$).

Results

The results of G.L.C. analysis on the heterogeneous series expressed as mean 24 hr excretion values $\pm$ S.E. of means, together with observed ranges, are given in Table 1 and grouped according to the four collection schedules. Values are given for urinary phenolic acids and 4-hydroxy-3-methoxyphenylglycol (HMPG) and for the ratio of VMA to HMPG. A parallel but extended set of results from the homogeneous series is shown in Table 2.

The urinary excretion of metabolites in postencephalitic Parkinsonian subjects in " pre-treatment " and " follow-up " groups (Tables 1 and 2) did not differ from the normal range (Karoum *et al.*, 1969).

Compared with " pre-treatment " values, a several-hundredfold rise in HVA excretion was observed in both series of analyses while the patients were " under treatment ". It is likely that a small percentage of this high output was represented by the isomer 3-hydroxy-4-methoxyphenylacetic acid the ME/TE derivative of which cannot be separated from that of HVA under the GLC conditions used. However, the 4-hydroxy group of HVA is resistant to methylation during the 1 min exposure to diazomethane used whereas O-methylation tends to occur more readily in the 3-position. The small amount of 3,4-dimethoxyphenylacetic acid which was noted on the gas chromatogram (Fig. 1b) after subjecting the urine extract to the ME/TE procedure but not after the TE/E procedure indicates that the HVA peak was contaminated by a small amount of 3-hydroxy-4-methoxyphenylacetic acid. At the " end of treatment ", the high mean HVA concentrations fell (significantly in the homogeneous series, $P<0{\cdot}025$) to about two-thirds of the " under treatment " mean value, and had returned to normal in all subjects tested by " follow-up ".

TABLE 1. *Excretion of urinary phenolic acids and alcohols in the heterogeneous series*

Metabolite	" Pre-treatment " mg/24 hr		" Under treatment " mg/24 hr		" End of treatment " mg/24 hr		" Follow-up " mg/24 hr	
	Mean ±S.E. of mean	Observed range	Mean ±S.E. of mean	Observed range	Mean ±S.E. of mean	Observed range	Mean ±S.E. of mean	Observed range
HVA	3·1± 0·33 (14)	1·6–6·0	201±38 (12)	51–460	140±66 (7)	12–505	2·4±0·41 (10)	1·0–5·1
Free DOPAC	<2·0	—	195±33 (12)	30–390	124±45 (7)	13–365	—	—
VMA	4·6±0·54 (14)	1·4–9·3	7·3±0·75 (11)	2·7–11·8	2·6±0·83 (6)	0·6–5·1	3·4±0·37 (10)	1·4–5·1
Total HMPG	2·2±0·32 (9)	0·8–3·5	1·5±0·28 (10)	0·2–3·2	1·0±0·16 (7)	0·7–1·4	1·2±0·14 (10)	0·6–2·0
VMA/HMPG ratio	2·5±0·5 (9)	0·4–4·9	6·6±1·20 (10)	2·3–14·0	2·5–0·60 (6)	0·8–3·6	3·2±0·52 (10)	0·9–6·0

Number of patients in parentheses.
HVA, 4-hydroxy-3-methoxyphenylacetic acid, homovanillic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; VMA, 4-hydroxy-3-methoxymandelic acid; HMPG, 4-hydroxy-3-methoxyphenylglycol.

TABLE 2. *Excretion of urinary phenolic acids and alcohols in the homogeneous series*

Metabolite	" Pre-treatment " mg/24 hr		" Under treatment " mg/24 hr		" End of treatment " mg/24 hr		" Follow-up " mg/24 hr	
	Mean ±S.E. of mean	Observed range	Mean ±S.E. of mean	Observed range	Mean ±S.E. of mean	Observed range	Mean ±S.E. of mean	Observed range
HVA	2·7±0·27	1·8–3·8	271±48	135–460	161±73	33–505	2·9±0·53	1·7–5·1
Free DOPAC	<2·0	—	217±50	151–336	142±21	13–365	<2·0	—
VMA	4·6±0·27	3·5–5·3	6·6±0·92	3·0–9·1	2·6±0·83	0·6–5·1	3·9±0·40	2·4–4·9
Total HMPG	1·4±0·16	0·9–1·9	1·1±0·25	0·5–2·2	1·0±0·18	0·4–1·4	1·2±0·20	0·6–1·5
p-Hydroxyphenylacetic acid	7·4±1·52	3·2–13·9	8·8±2·18	2·1–16·7	8·5±1·79	2·1–15·6	7·5±2·30	3·0–18·5
m-Hydroxyphenylacetic acid	1·7–0·51	0·9–4·0	5·1±1·32	2·6–10·4	5·8±1·88	2·1–14·3	2·1±0·24	1·1–2·5
VLA	<0·1	—	27·5±16·3	2–108	12·3±10·6	0·5–66	<0·1	—
VMA/HMPG ratio	3·3±0·33	2·7–4·8	6·3±0·85	4·1–9·0	2·5±0·60	0·8–4·5	3·6±0·78	1·8–6·1

Six patients. HVA, 4-hydroxy-3-methoxyphenylacetic acid, homovanillic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; VMA, 4-hydroxy-3-methoxymandelic acid; HMPG, 4-hydroxy-3-methoxyphenylglycol; VLA, 4-hydroxy-3-methoxyphenyllactic acid.

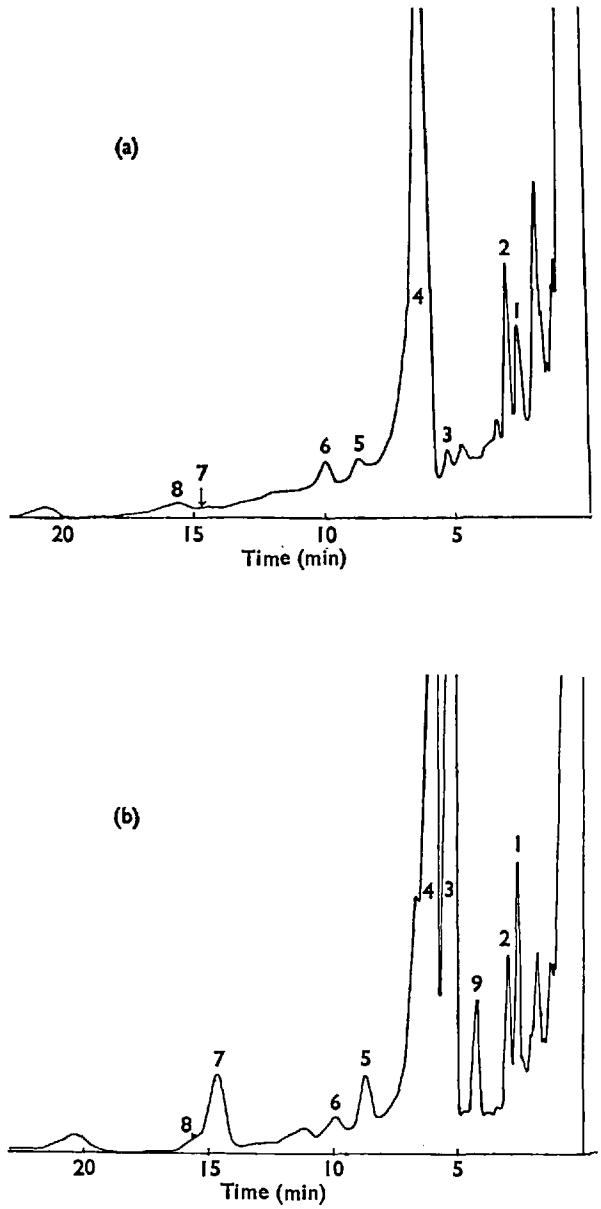


FIG. 1. (a) Gas chromatogram (7 foot, 10% SE 52 column) of methyl ester/trimethyl silyl ether derivatives of phenolic acids prepared from an ethyl acetate extract of urine (10 ml.) from a patient with postencephalitic Parkinsonism before treatment with L-DOPA. Peak identification: 1, *m*-hydroxyphenylacetic acid; 2, *p*-hydroxyphenylacetic acid; 3, homovanillic acid (HVA); 4, hippuric acid; 5, *p*-hydroxyphenyllactic acid; 6, 4-hydroxy-3-methoxymandelic acid (VMA); 7, 4-hydroxy-3-methoxyphenyllactic acid (VLA); 8, *p*-hydroxyphenylpyruvic acid. (b) Gas chromatogram (7 foot, 10% SE 52 column) of methyl ester/trimethyl silyl ether derivatives of urinary phenolic acids from the same patient as in (a) while under treatment with L-DOPA. Urine (10 ml.) at pH 8·4 was shaken with alumina to remove DOPAC before extracting at pH 2 with ethyl acetate. Peak identification: 1–8 as in (a); 9, 3,4-dimethoxyphenylacetic acid (artefact from O-methylation of 3-hydroxy-4-methoxyphenylacetic acid—see text).

There was a very similar high output of free DOPAC " under treatment " which fell considerably at the " end of treatment ", returning to normal in the " follow-up " samples. In both the " under treatment " and " end of treatment " groups, there was a strong positive correlation ($P<0{\cdot}01$) between output of HVA and DOPAC. The total dose of the drug up to the time of urine sampling and the dose in the 24 hr period preceding urine collection were positively correlated with HVA and DOPAC excretion values in both the " under treatment " and " end of treatment " groups. There was also a positive correlation ($P<0{\cdot}02$) between urinary HVA or DOPAC output and urine volume in the " end of treatment " group.

Although 4-hydroxy-3-methoxyphenylethanol (HMPE) excretion in the " under treatment " or " end of treatment " groups was small compared with output of HVA or DOPAC, it represents a substantial increase over the small quantities (less than 100 μg/24 hr) found in normal urine. In four subjects from the heterogeneous series " under treatment ", excretion rates of 0·5, 1, 2 and 2 mg HMPE/24 hr were recorded, whilst three others belonging to the " end of treatment " group in this series had an output of 0·25, 0·5 and 0·5 mg HMPE/24 hr. Althougth the absolute increase in urinary VMA was not as dramatic in patients " under treatment " when compared with that of HVA and DOPAC, it was still significant (heterogeneous series $P<0{\cdot}02$; homogeneous series $P\simeq 0{\cdot}05$).

There was a positive correlation ($P<0{\cdot}02$) between HMPG and VMA output in patients in the heterogeneous series " under treatment ", although a similar relationship was not observed in any of the other groups. The mean ratio of the excretion values of VMA to HMPG in " under treatment " groups in both series was higher than in any of the other groups (Tables 1 and 2). In the homogeneous series, this rise was significant ($P<0{\cdot}02$) for comparisons between " under treatment " and " pre-treatment " or " end of treatment " groups. Inspection of ratios in the heterogeneous series suggested a similar relationship.

4-Hydroxy-3-methoxyphenyllactic acid (VLA) was almost undetectable (less than 100 μg/24 hr) in urine from " pre-treatment " and " follow-up " groups but was excreted in large amount (Table 2) by patients " under treatment " (Fig. 1). 4-Hydroxy-3-methoxyphenylpyruvic acid (VPA) could not be detected in any sample.

Oral L-DOPA did not affect the excretion of *p*-hydroxyphenylacetic acid which remained within the range previously encountered by us (unpublished) in normal

TABLE 3. *Excretion of urinary phenolic acids and alcohols in high dosage* (4·25–4·75 *g* L-*DOPA per day*) *series*

Metabolite	Mean mg/24 hr	Observed range mg/24 hr
HVA	1,707	1,290–2,820
Free DOPAC	2,135	1,110–3,400
VMA	10·0	9·5–10·5
Total HMPG	4·5	2·9–7·1
p-Hydroxyphenylacetic acid	10	5–13
m-Hydroxyphenylacetic acid	14	12–16
VLA	41	32–54
HMPE	1·5	0·4–3·3
VMA/HMPG ratio	2·5	1·4–3·4

Four patients. HVA, 4-hydroxy-3-methoxyphenylacetic acid, homovanillic acid; DOPAC, 3,4-dihydroxyphenylacetic acid; VMA,4-hydroxy-3-methoxymandelic acid; HMPG, 4-hydroxy-3-methoxyphenylglycol; VLA, 4-hydroxy-3-methoxyphenyllactic acid; HMPE, 4-hydroxy-3-methoxyphenylethanol.

individuals throughout the investigation. In contrast there was a significant increase in output of *m*-hydroxyphenylacetic acid (Table 2, Fig. 1) in both "under treatment" and "end of treatment" groups when compared with "pre-treatment" ($P<0.03$) and "follow-up" ($P<0.05$) groups.

The range of metabolites measured in the homogeneous series was also estimated in a small group of patients with idiopathic Parkinsonism on a higher dosage of L-DOPA (Table 3). Apart from *p*-hydroxyphenylacetic acid, the value of each was greater than after treatment with the lower dosage. *m*-Hydroxyphenylacetic acid excretion was so increased as to be greater than that of its *p*-isomer; its output is normally considerably less (Fig. 1).

The excretion of *p*-hydroxyphenyllactic acid (*p*HPLA) was measured in the homogeneous series and in the four patients on higher dosage of L-DOPA. With the exception of one atypical case, patients in the homogeneous series excreted less than 0·5 mg *p*HPLA/24 hr in the "pre-treatment" and "follow-up" periods. Whilst "under treatment", however, four of these patients put out increased amounts of the acid, ranging from 0·9–4·8 mg/24 hr (mean 2·4 mg/24 hr). The patients on higher dosage tended to excrete slightly more, with values ranging from 1·6 to 4·7 mg/24 hr (mean 2·8 mg/24 hr). The atypical patient excreted about 2 mg *p*HPLA daily, even when not on L-DOPA, the output rising as high as 23 mg/24 hr "under treatment" and dropping to 11 mg/24 hr at the "end of treatment". This patient also excreted by far the highest concentration of VLA whilst "under treatment". In general, the excretion of *p*-HPLA tended to rise in parallel with VLA output.

Discussion

Since the first sighting observations on L-dihydroxyphenylalanine (L-DOPA) metabolism in man and rabbit by Guggenheim (1913), its major pathways of degradation have become well established (Sandler & Ruthven, 1969). The greater proportion is decarboxylated to dopamine which is either β-hydroxylated to noradrenaline or, in common with the other catecholamines, inactivated by two alternative routes, involving the primary action of either catechol O-methyltransferase or monoamine oxidase. Homovanillic acid (HVA) which results from the action of both enzymes on dopamine, is quantitatively the most important endogenous DOPA metabolite in normal human urine.

The "pre-treatment" data presented here which do not appear to differ from normal values (Karoum *et al.*, 1969), together with the previous finding of a normal HVA output in this disease group (Greer & Williams, 1963), argue against there being a generalized disturbance of dopamine metabolism in Parkinsonism. The claim of Barbeau, Murphy & Sourkes (1961), pointing to a contrary conclusion, has never been confirmed. Whether certain unidentified chromatographic peaks noted in "pre-treatment" and "follow-up" urine samples but not in normal urine are in any way connected with the pathogenesis of the disease is still under investigation.

Sourkes, Pivnicki, Brown, Wiseman-Distler, Murphy, Sankoff & Saint Cyr (1965) gave L-DOPA to one postencephalitic Parkinsonian subject and noted some delay in the rise in urinary HVA excretion compared with controls; but, in general, our own findings in patients with this disease during treatment are not too dissimilar from those following L-DOPA administration to normal subjects (Shaw, McMillan & Armstrong, 1957; Sourkes *et al.*, 1965). There was a very large increase in urinary excretion of HVA which was equalled (Table 1), and at high dosage even exceeded

(Table 3), by 3,4-dihydroxyphenylacetic acid (DOPAC) output. This increase in relative concentration of DOPAC appears to depend to some extent on route of administration (Sourkes *et al.*, 1965).

It is of interest that children with dopamine-secreting tumours may sometimes put out as much HVA as the patients "under treatment" (Table 1). DOPAC excretion, on the other hand, although well above normal concentrations, forms a proportionately smaller part of the total metabolite output (von Studnitz, 1960; Sourkes, Denton, Murphy, Chavez & Saint Cyr, 1963). This finding implies that the site of metabolic degradation of this endogenously secreted dopamine differs from that generated from exogenously administered DOPA.

There are other points of difference from dopamine-secreting tumours. The intermediate aldehyde produced by oxidative deamination of dopamine is preferentially oxidized further to HVA rather than reduced to 4-hydroxy-3-methoxyphenylethanol (HMPE) (Breese, Chase & Kopin, 1969); during the present investigation, an even smaller proportion of HMPE was found after DOPA treatment than is observed in cases of dopamine-secreting tumour (Karoum, Anah, Ruthven & Sandler, unpublished). von Studnitz (1967) was unable to detect the excretion of the 3-hydroxy-4-methoxy isomer of HVA in patients with such tumours but there was indirect chemical evidence that small amounts were put out in the Parkinsonian subjects "under treatment". This latter finding agrees with a previous claim (Smith, 1967).

A further difference concerns the degree of β-hydroxylation of the side chain. Apart from metabolites derived from the direct metabolism of dopamine, patients with dopamine-secreting tumours almost invariably have a large output of the β-hydroxylated (noradrenaline) series of metabolites with a relatively large contribution from the aldehyde reduction product, 4-hydroxy-3-methoxyphenylglycol (HMPG). Oral DOPA treatment, however, resulted in only a comparatively small increase in 4-hydroxy-3-methoxymandelic acid (VMA) and an increased VMA/HMPG ratio. In the light of these differences, it seems likely that further metabolism of dopamine involving β-hydroxylation takes place largely within the tumour tissue of affected subjects and not at sites remote from it (Sandler & Ruthven, 1966).

The gas chromatographic techniques used have permitted accurate quantitative studies to be performed for the first time on a number of other minor metabolic routes of L-DOPA metabolism in addition to that of HMPE formation. DeEds, Booth & Jones (1957) found that DOPA administration to rabbits gave rise to an increased urinary output of *m*-hydroxyphenylacetic acid, presumably by *p*-dehydroxylation of DOPAC brought about to a large extent by gut flora (Scheline, 1968). Although Shaw *et al.* (1957) were not able to obtain any evidence for the existence of this pathway in man, Booth, Emerson, Jones & DeEds (1957) and Shaw, Gutenstein & Jepson (1961) produced evidence pointing to *p*-dehydroxylation of another catechol acid, caffeic acid, and we have now been able to show that a small but significant proportion of L-DOPA is degraded by this route. There was no evidence of *m*-dehydroxylation, however, as the output of *p*-hydroxyphenylacetic acid was unchanged.

Shaw *et al.* (1957) discussed the possible existence of an alternative pathway of DOPA metabolism via transamination, although they were not able to provide any direct evidence for it. For many years, it has been known that L-DOPA can participate in transamination reactions (Cammarata & Cohen, 1950), and Fonnum, Haavaldsen & Tangen (1964) have characterized several DOPA transaminases in

brain. The evidence now seems to point fairly strongly to an *in vivo* metabolic sequence initiated by DOPA transamination. The immediate product, 3,4-dihydroxyphenylpyruvic acid (DHPPA), is probably O-methylated to 4-hydroxy-3-methoxyphenylpyruvic acid (VPA). An alternate route to VPA would involve the initial O-methylation of DOPA to 3-O-methylDOPA, followed by transamination to VPA. Smith (1967) described an increased urinary excretion of the unstable VPA but had no evidence of its more stable reduction product 4-hydroxy-3-methoxyphenyllactic acid (VLA), after administration of L-DOPA to volunteers. Despite the sensitivity of our methods and the considerably higher dosage range of L-DOPA employed, we were unable to detect VPA in any sample. There was a considerable increase, however, of VLA, its reduction product (Weber & Zannoni, 1966 ; Zannoni & Weber, 1966), confirming earlier observations in patients with DOPA-secreting tumours (Gjessing, 1963 ; Smith, 1965). We would even postulate that its presence is indicative of a sufficient production of DHPPA or VPA to interfere with the normal metabolism of tyrosine. The major metabolite of tyrosine metabolism is *p*-hydroxyphenylpyruvic acid (*p*-HPPA) which is normally further metabolized by *p*-HPPA-oxidase (La Du, 1966), undergoing the recently described "NIH shift" (Guroff, Daly, Jerina, Renson, Witkop & Udenfriend, 1967), to 2,5-dihydroxyphenylacetic acid (homogentisic acid). Our finding of an increased output of the immediate reduction product of *p*-HPPA, *p*-HPLA, which tends to correlate with VLA output, suggests to us that DHPPA or VPA may compete with *p*-HPPA for *p*-HPPA-oxidase. If this were so, DHPPA or VPA might itself be expected to be metabolized to 2,4,5-trihydroxyphenylacetic or 2,5-dihydroxy-4-methoxyphenylacetic acid. We hope to make a careful search for compounds of this type as soon as authentic reference samples become available to us. It has been suggested that compounds with a 2,4,5-substitution pattern which appear to possess high psychotomimetic activity may be implicated in the pathogenesis of schizophrenia (Shulgin, Sargent & Naranjo, 1969).

Whilst it might seem that we have placed disproportionate stress on the existence of minor pathways of L-DOPA metabolism, it must not be forgotten that the time course of the therapeutic response to this drug (Calne *et al.*, 1969 ; Cotzias, Papavasiliou & Gellene, 1969) is slow. As there is indirect evidence to indicate that dopamine generation within the human central nervous system is rapid (Pletscher, Bartholini & Tissot, 1967), we cannot rule out the possibility that the clinical effect derives not from dopamine replacement but from the build-up of some minor metabolite unconnected with the main route of DOPA degradation.

The studies described in this paper were confined to a series of measurements on the acidic and alcoholic end-results of L-DOPA metabolism in the whole organism. We are well aware, however, that any therapeutic effect is likely to derive from metabolic changes localized to a small area of the brain. Pletscher *et al.* (1967) have observed appreciable amounts of labelled HVA in cerebrospinal fluid after ^{14}C-DOPA administration in man and it seems likely that metabolite concentrations in cerebrospinal fluid may mirror the metabolism of DOPA in the brain more faithfully than urinary levels. Parallel studies on amino-acid and amine excretion in these patients are in progress.

We are grateful to the Smith, Kline & French Foundation for a grant for gas chromatography equipment ; to Mr. G. B. Newman for help with the statistical evaluation ; and to the Wellcome Trust, who defrayed the salary of D. B. C.

REFERENCES

BARBEAU, A., MURPHY, C. F. & SOURKES, T. L. (1961). Excretion of dopamine in diseases of basal ganglia. *Science, N.Y.*, **133**, 1706–1707.

BOOTH, A. N., EMERSON, O. H., JONES, F. T. & DEEDS, F. (1957). Urinary metabolites of caffeic and chlorogenic acids. *J. biol. Chem.*, **229**, 51–59.

BREESE, G. R., CHASE, T. N. & KOPIN, I. J. (1969). Metabolism of some phenylethylamines and their β-hydroxylated analogs in brain. *J. Pharmac. exp. Ther.*, **165**, 9–13.

CALNE, D. B., STERN, G. M., LAURENCE, D. R., SHARKEY, J. & ARMITAGE, P. (1969). L-DOPA in postencephalitic Parkinsonism. *Lancet*, **1**, 744–747.

CAMMARATA, P. S. & COHEN, P. P. (1950). Scope of transamination reaction in animal tissues. *J. biol. Chem.*, **187**, 439–452.

COTZIAS, G. C., PAPAVASILIOU, P. S. & GELLENE, R. (1969). Modification of Parkinsonism: chronic treatment with L-DOPA. *New Engl. J. Med.*, **280**, 337–345.

DALGLIESH, C. E., HORNING, E. C., HORNING, M. G., KNOX, K. L. & YARGER, K. (1966). A gas-liquid-chromatographic procedure for separating a wide range of metabolites occurring in urine or tissue extract. *Biochem. J.*, **101**, 792–810.

DEEDS, F., BOOTH, A. N. & JONES, F. T. (1957). Methylation and dehydroxylation of phenolic compounds by rats and rabbits. *J. biol. Chem.*, **225**, 615–621.

FONNUM, F., HAAVALDSEN, R. & TANGEN, O. (1964). Transamination of aromatic amino acids in rat brain. *J. Neurochem.*, **11**, 109–118.

GEY, K. F. & PLETSCHER, A. (1964). Distribution and metabolism of DL-3,4-dihydroxy (2-^{14}C) phenylalanine in rat tissues. *Biochem. J.*, **92**, 300–308.

GJESSING, L. R. (1963). Studies of functional neural tumours V. Urinary excretion of 3-methoxy-4-hydroxy-phenyl-lactic acid. *Scand. J. clin. Lab. Invest.*, **15**, 649–653.

GREER, M. & WILLIAMS, C. M. (1963). Dopamine metabolism in Parkinson's disease. *Neurology, Minneap.*, **13**, 73–78.

GUGGENHEIM, M. (1913). Dioxyphenylalanin, eine neue Aminosäure aus Vicia faba. *Hoppe-Seyler's Z. physiol. Chem.*, **88**, 276–284.

GUROFF, G., DALY, J. W., JERINA, D. M., RENSON, J., WITKOP, B. & UDENFRIEND, S. (1967). Hydroxylation-induced migration: the NIH shift. *Science, N.Y.*, **157**, 1524–1530.

HORNYKIEWICZ, O. (1966). Dopamine (3-hydroxytyramine) and brain function. *Pharmac. Rev.*, **18**, 925–964.

KAROUM, F., ANAH, C. O., RUTHVEN, C. R. J. & SANDLER, M. (1969). Further observations on the gas chromatographic measurement of urinary phenolic and indolic metabolites. *Clinica chim. Acta*, **24**, 341–348.

KAROUM, F., RUTHVEN, C. R. J. & SANDLER, M. (1968). Gas chromatographic measurement of phenolic acids and alcohols in human urine. *Clinica chim. Acta*, **20**, 427–437.

KAROUM, F. & SANDLER, M. (1968). Gas chromatographic measurement of phenolic and indolic acids and alcohols in human urine. *Proc. Ass. clin. Biochemists*, **5**, 123–124.

LA DU, B. N. (1966). Tyrosinosis. In *The Metabolic Basis of Inherited Disease*. Ed. Stanbury, J. B., Wyngaarden, J. B. and Fredrickson, D. S., 2nd ed., pp. 295–302. New York: McGraw-Hill.

MCGEER, P. L., BOULDING, J. E., GIBSON, W. C. & FOULKES, R. G. (1961). Drug-induced extrapyramidal reactions. *J. Am. med. Ass.*, **177**, 665–670.

PLETSCHER, A., BARTHOLINI, G. & TISSOT, R. (1967). Metabolic fate of L-(^{14}C) DOPA in cerebrospinal fluid and blood plasma of humans. *Brain Res.*, 4, 106–109.

SANDLER, M. & RUTHVEN, C. R. J. (1966). 4-Hydroxy-3-methoxyphenylglycol and other compounds in neuroblastoma. In *Recent Results in Cancer Research. II Neuroblastomas. Biochemical Studies*, ed. Bohuon, C., pp. 55–59. Berlin: Springer-Verlag.

SANDLER, M. & RUTHVEN, C. R. J. (1969). The biosynthesis and metabolism of the catecholamines. In *Progress in Medicinal Chemistry*, ed. Ellis, G. P. and West, G. B., vol. 6, pp. 200–265. London: Butterworth.

SCHELINE, R. R. (1968). Drug metabolism by intestinal microorganisms. *J. pharm. Sci.*, **57**, 2021–2037.

SHAW, K. N. F., GUTENSTEIN, M. & JEPSON, J. B. (1961). Intestinal flora and diet in relation to *m*-hydroxyphenyl acids of human urine. *Proc. 5th int. Cong. Biochem., Moscow*, vol. 9, p. 427. Oxford: Pergamon.

SHAW, K. N. F., MCMILLAN, A. & ARMSTRONG, M. D. (1957). The metabolism of 3,4-dihydroxyphenylalanine. *J. biol. Chem.*, 226, 255–266.

SHULGIN, A. T., SARGENT, T. & NARANJO, C. (1969). Structure–activity relationships of one-ring psychotomimetics. *Nature, Lond.*, **221**, 537–541.

SMITH, P. (1965). Pathological excretion of 4-hydroxy-3-methoxyphenyllactic acid. *Nature, Lond.*, **205**, 1236.

SMITH, P. (1967). Metabolism of dihydroxyphenylalanine in human subjects. *Nature, Lond.*, **213**, 802–803.

SOURKES, T. L., DENTON, R. L., MURPHY, G. F., CHAVEZ, B. & SAINT CYR, S. (1963). The excretion of dihydroxyphenylalanine, dopamine and dihydroxyphenylacetic acid in neuroblastoma. *Pediatrics, Springfield*, **31**, 660–668.

SOURKES, T. L., PIVNICKI, D., BROWN, W. T., WISEMAN-DISTLER, M. H., MURPHY, G. F., SANKOFF, I. & SAINT CYR, S. (1965). A clinical and metabolic study of dopa (3,4-dihydroxyphenylalanine) and methyldopa in Huntington's chorea. *Psychiatria Neurol.*, **149**, 7–27.

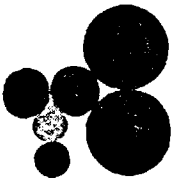
VON STUDNITZ, W. (1960). Methodische und klinische Untersuchungen über die Ausscheidung der 3-Methoxy-4-hydroxymandelsäure im Urin. *Scand. J. clin. Lab. Invest.*, **12**, suppl. 48, 1–73.

VON STUDNITZ, W. (1967). Zur Frage des Vorkommens von 3-Hydroxy-4-methoxy-mandelsäure beim normalen und gesteigerten Katecholaminstoffwechsel. *Klin. Wschr.*, **45**, 307–308.

WEBER, W. W. & ZANNONI, V. G. (1966). Reduction of phenylpyruvic acids to phenyllactic acids in mammalian tissues. *J. biol. Chem.*, **241**, 1345–1349.

ZANNONI, V. G. & WEBER, W. W. (1966). Isolation and properties of aromatic α-keto acid reductase. *J. biol. Chem.*, **241**, 1340–1344.

(*Received April* 14, 1969)



VIIth International Congress of Clinical Chemistry
VII. Internationaler Kongress für klinische Chemie
VIIe Congrès international de chimie clinique

Genève/Evian, September 8–13, 1969

Abstracts

S. Karger Basel (Switzerland) New York 1969

and disc gel electrophoresis. Studies on the nature of the aggregation of PNMT in the particulate fraction are in progress.

2.10.03 Catecholamine and Metabolite Content of Adrenal Medullary Tissue in Hypertension and Other Clinical Conditions

H. Hinterberger, R. H. Steele and R. J. Bartholomew: Division of Clinical Chemistry, Department of Pathology, Prince Henry Hospital, Sydney, Australia

Adrenalin (A), noradrenalin (NA) and 3,4-dihydroxyphenylethylamine (DA) were estimated by fluorimetry, and 4-hydroxy-3-methoxymandelic acid and 4-hydroxy-3-methoxyphenylglycol (HMMA+HMPG) by absorption spectrophotometry in necropsy specimens of human adrenal medullary tissue from more than sixty subjects.

No correlation was found between amine content and time elapsed between death and removal of specimens within 48 h.

Adrenalin content appeared to vary over a wider range than noradrenalin content when different areas of the two glands were sampled separately.

No correlation of amine content with age (15–83 years) or sex of the subject could be established.

Specimens from normotensive subjects and hypertensives treated with reserpine for at least 2 weeks immediately prior to death showed no statistically significant differences of A and NA content, but DA could not be detected in reserpine-treated hypertensives. Hypertensives who had not been treated with reserpine prior to death had DA values comparable to those of normotensives, but their NA and A content was significantly lower ($P < 0.005$ and < 0.0005, respectively).

2.10.04 The Study of Catecholamine Secreting Tumours by Gas-liquid Chromatography

C. R. J. Ruthven, F. Karoum, C. O. Anah, J. Chapman and M. Sandler: Bernhard Baron Memorial Research Laboratories, Queen Charlotte's Maternity Hospital, London,
and Associated Electrical Industries Ltd., Manchester, England

Gas-liquid chromatography (GLC) was found to be a sensitive tool for the rapid appraisal of phenolic acid and alcohol metabolites excreted by patients with catecholamine secreting tumours. Characteristic urinary excretion patterns were obtained by quantitative assay of two major and one minor metabolite of dopamine, 3,4-dihydroxyphenylacetic and homovanillic acids and 4-hydroxy-3-methoxyphenylethanol (HMPE), and two major and two minor metabolites of noradrenaline and adrenaline, 4-hydroxy-3-methoxymandelic acid, 4-hydroxy-3-methoxyphenylglycol, 3,4-dihydroxymandelic acid and 3,4-dihydroxyphenylglycol. Output of p-hydroxyphenylpyruvic and p-hydroxyphenyllactic acids, and vanylpyruvic and vanyllactic acids, metabolite pairs deriving from transamination of tyrosine and DOPA, was also measured. Further identification of some GLC peaks by mass spectrometry (AEI MS 902), showed that a compound excreted in some cases of phaeochromocytoma, with a similar GLC mobility to HMPE, was different from it in nature.

Eng. J. med

ANXIETY PRECIPITATED BY LACTATE

To the Editor: Pitts and McClure (NEJM 277:1329-1336, 967) reported that intravenously administered sodium lactate precipitated a severe anxiety attack in 93 per cent of atients with the diagnosis of anxiety but in only 20 per cent f normal controls. They were also able to minimize the ffects of lactate by intravenous calcium.

We undertook to repeat this study, using their technics nd their lactate solutions. In five patients given lactate, cute anxiety developed between the eighth and twelfth inutes of infusion, peaked at 15 minutes, and remained vere for 15 to 30 minutes. Each patient experienced aftereffects" of irritability, dysphoria, tension, fatigue and eakness for two to seven days.

The response to lactate-calcium infusion was similar to at of lactate alone, but clearly less intense and of shorter uration; there were no "aftereffects." There was no patient esponse to dextrose in saline.

In four control subjects the response to lactate was characerized by tachycardia and restlessness, but without anxiety n three subjects, and with no "aftereffects" in all four. Anxiety was elicited in one control. The controls became drowsy nd bored with lactate and calcium and dextrose in saline.

As observed by Pitts and McClure, a "blind" investigator vas able to name correctly 25 of 27 solutions (92.5 per cent), he patients 13 of 15 solutions (87 per cent), and the controls 6 of 12 solutions (50 per cent).

In concurrent scalp-recorded electroencephalograms (EEG), ve observed changes with lactate in the patients, but not in he controls nor after dextrose in saline and lactate and calium solutions. The EEG exhibited increased beta and decreased alpha abundances and a decreased alpha amplitude. The findings are consistent with the electroencephalographic hanges usually seen in anxiety states.

Hypotheses relating anxiety and lactate are well summarized by Pitts and McClure. We would also add to their ormulations two interesting applications. A lactate tolerance est, we suggest, provides an objective means of identifying a roup of mentally ill with common characteristics of anxiety neurosis, for both therapeutic and prognostic purposes. The intagonism of calcium to lactate precipitated anxiety and its uccessful therapeutic trial (according to a personal communication from Dr. Pitts) suggests that other antianxiety agents may be tested by means of this model.

MAX FINK, M.D.
MICHAEL ALAN TAYLOR, M.D.
JAN VOLAVKA, M.D.
Department of Psychiatry
New York Medical College
Flower and Fifth Avenue Hospitals
New York, N.Y.

METABOLISM OF DOPA IN PARKINSONISM

To the Editor: In a letter to the *Journal* on February 1, 1968, Arras and Bailey[1] reported considerable amounts (2 to 3 gm per 24 hours) of homogentisic acid in a darkly pigmented urine from a patient with Parkinsonism who was receiving 16 gm of dopa per day. Cotzias et al.[2] had previously observed that urine from such patients on dopa therapy turned black on keeping.

Because of the pathologic sequelae of high tissue levels of homogentisic acid in alkaptonuria, it is important to know whether patients on long-term dopa therapy run a similar risk. Our recent gas chromatographic studies on the metabolism of L-dopa in Parkinsonism[4] have provided us with an opportunity to look for homogentisic acid in the urine from four patients with idiopathic Parkinsonism receiving 4.25 to 4.75 gm of L-dopa per day. We were unable, however, to detect the presence of homogentisic acid (less than 0.1 mg per 24 hours). The procedure used, which is described in detail elsewhere,[5,6] was briefly as follows. Salt-saturated urine at pH 2.0 was extracted with ether, the separated solvent evaporated under vacuum, and the phenolic acids in the residue converted to their methyl ester trimethyl silyl ether derivatives. These were separated by isothermal gas chromatography at 190°C with the use of a Pye Panchromatograph with a 7 foot 10 per cent SE 52 column. The position of any peak due to homogentisic acid could have been located and quantified by comparison of peak height with that of internal standards of the authentic acid and with known alkaptonuric urine; methylene unit value provided confirmation of identity. To eliminate any possible masking of homogentisic acid by the large quantities of 3,4-dihydroxyphenylacetic acid (dopac) present in these urines after L-dopa treatment, dopac was removed by pretreatment of urine at pH 8.4 with alumina, after it had first been confirmed that homogentisic acid was not also adsorbed.

The difference between our findings and those of Arras and Bailey[1] does not seem to be explained by the very high drug dosage that they used (16 gm per day), presumably of the D,L-form: apparently, they were still able to detect urinary homogentisic acid at a dose of 10 gm per day — that is, after giving 5 gm of the L-isomer. It is possible that the difference between our two sets of findings stems from some as yet uncharted pathway of D-dopa metabolism, but this is purely conjectural. (It is even conceivable that their patient was also alkaptonuric.) Arras and Bailey suggested that the darkening of the urine that they observed primarily derived from homogentisic acid. The oxidation of dopa itself, of dopamine or of its oxidatively deaminated metabolite, dopac, are likely to have made an even larger contribution. Any oxidation of catechol metabolites would, of course, have been greatly increased if the urine specimens had not been preserved with acid.

M. SANDLER, M.D.
F. KAROUM, B.Sc.
C. R. J. RUTHVEN, PH.D.
Queen Charlotte's Hospital
London, England

1. Arras MJ, Bailey GWH: Metabolism of DOPA in Parkinsonism. New Eng J Med 278:280, 1968
2. Cotzias GC, Van Woert MH, Schiffer LM: Aromatic amino acids and modification of Parkinsonism. New Eng J Med 276:374-379, 1967
3. La Du BN: Alcaptonuria. The Metabolic Basis of Inherited Disease. Second edition. Edited by JB Stanbury, JB Wyngaarden, DS Fredrickson. New York, McGraw-Hill Book Company, 1966, pp 303-323
4. Calne DB, Karoum F, Ruthven CRJ, et al: The metabolism of orally administered L-DOPA in Parkinsonism. Brit J Pharmacol 37:57-68, 1969
5. Karoum F, Ruthven CRJ, Sandler M: Gas chromatographic measurement of phenolic acids and alcohols in human urine. Clin Chim Acta 20:427-437, 1968
6. Karoum F, Anah CO, Ruthven CRJ, et al: Further observations on the gas chromatographic measurement of urinary phenolic and indolic metabolites. Clin Chim Acta 24:341-348, 1969

BCG VACCINE (CONTINUED)

To the Editor: The editorial, "The Paradox of BCG" (NEJM, September 4, 1969), should be applauded. However, there are two replies (NEJM, October 23, 1969) obliquely critical emanating from the United States Public Health Service. BCG vaccination has always been anathema to the Public Health Service. It has depreciated the efficacy of this procedure in the prevention of pulmonary tuberculosis in spite of the overwhelming epidemiologic evidence to the contrary as cited in the editorial.

Tuberculosis is most prevalent among the racial minorities and the poor. This segment of the population is completely dependent on the Public Health Service for treatment and prevention of the disease. As long as the Public Health Service on the federal and state level does not recognize BCG vaccination as an effective tool in the fight against tuberculo-

Reprinted from
12 December 1969, volume 166, pages 1417–1418

SCIENCE

m-Hydroxyphenylacetic Acid Formation from L-Dopa in Man: Suppression by Neomycin

Abstract. *The increased excretion of* m-*hydroxyphenylacetic acid in the urine of patients with parkinsonism being treated with* L-*dopa was reduced by gut sterilization with neomycin. The* p-*dehydroxylation step is thus brought about solely by the action of gut flora; the pathway is unlikely to be involved in the events within the brain leading to the therapeutic benefit effected by* L-*dopa.*

A substantial proportion of patients with parkinsonism obtain more therapeutic benefit from L-dopa (dihydroxyphenylalanine) than from any drug previously available (*1*). While it has been assumed that clinical improvement stems from dopamine generation within the central nervous system, Calne *et al.* (*2*) have pointed out that such a chemical transformation is likely to be rapid (*3*) whereas the time course of the therapeutic response to the drug is slow (*1*). The possibility cannot therefore be ruled out that the clinical effect derives not from dopamine replacement but from the buildup of some minor metabolite unconnected with the main route of dopa degradation. Therefore we charted minor pathways of dopa metabolism revealed by the large doses of drug employed (up to 8 g/day).

The existence of one such pathway, terminating in an increased urinary output of *m*-hydroxyphenylacetic acid (*m*-HPAA), was noted during a trial of dopa in patients with parkinsonism (*2*). DeEds *et al.* (*4*), who made a similar observation after feeding DL-dopa to rabbits, considered that *m*-HPAA might derive from the *p*-dehydroxylation of an intermediate in the reaction sequence, 3,4-dihydroxyphenylacetic acid. However, the possibility that the transformation occurs at some other stage, perhaps by *p*-dehydroxylation of dopa itself, of dopamine, or even of dihydroxyphenylpyruvic acid, with the remaining metabolic steps taking place after absorption of the dehydroxylated product, cannot be ruled out. A human stool suspension can bring about *p*-dehydroxylation in vitro of a variety of phenolic acids (*5*). If *p*-dehydroxylation of the catechol moiety by gut flora (*6*) were an essential step in the production in vivo of *m*-HPAA from L-dopa in man, gut sterilization with neomycin might decrease the urinary output of *m*-HPAA.

Six patients with idiopathic parkinsonism, receiving their maximum tolerated oral dosage of L-dopa (Fig. 1), were given oral doses of neomycin (1 g) daily. L-Dopa metabolism is unlikely to differ in healthy subjects and subjects with parkinsonism (*2*). Urine samples were collected before and during day 3 of neomycin treatment. The *m*-HPAA was isolated from urine saturated with salt (3 ml diluted to 10 ml with 0.01*N* HCl) at *p*H 2.0 by extracting twice (25 ml) with ethyl acetate. Portions (20 and 25 ml, respectively, pooled) of the extracts were evaporated to dryness under vacuum at 40° to 50°C, and the methyl ester–trimethylsilyl ether derivative was prepared. This was separated from other phenolic acid derivatives by isothermal (190°C) gas chromatography (*7*) on a Pye Panchromatograph with a 210-cm 10 per-

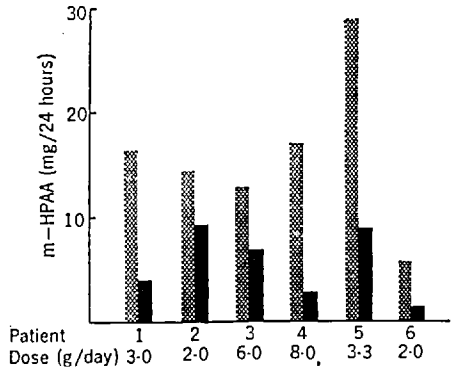


Fig. 1. Excretion of *m*-hydroxyphenylacetic acid (*m*-HPAA) before (hatched columns) and during (solid columns) day 3 of oral administration of neomycin (1 g/day). The subjects were six patients with parkinsonism being treated orally with L-dopa at the dosage shown.

cent SE52 column. The peak corresponding to *m*-HPAA was characterized by its methylene unit value and quantitated by comparing peak heights before and after the addition of the authentic acid as internal standard to duplicate urine samples.

Even on the comparatively low dosage scale of antibiotic employed, a significant ($P < .01$) decrease in *m*-HPAA output was detected (Fig. 1), from a mean ± S.E. of 16.0 ± 3.10 mg per 24 hours before neomycin to one of 5.6 ± 1.35 mg per 24 hours during its administration. Thus, an as yet unidentified intestinal microorganism or group of microorganisms sensitive to neomycin is apparently responsible for the formation of *m*-HPAA from L-dopa. Neomycin suppresses the excretion in human urine of a miscellaneous group of *m*-hydroxylated phenolic acids, presumably derived from dietary catechols (*8*). Similarly the increased excretion of *m*-hydroxylated acids which follows the oral administration of certain catecholic acids both in man (*9*) and the experimental animal (*10*) can be abolished by neomycin.

The *p*-dehydroxylation of L-dopa or one of its catechol derivatives apparently takes place by the action of gut flora within the gastrointestinal tract. The possibility is remote that this pathway is concerned in the train of events culminating in the clinical improvement observed during L-dopa treatment of parkinsonism; the question might be settled however by ascertaining whether a more prolonged trial of neomycin results in clinical deterioration. Such a trial might also indicate whether any of the side effects of L-dopa therapy are eliminated by gut sterilization and thus perhaps stem from the production of *m*-hydroxylated amines by gut flora.

M. SANDLER, F. KAROUM
C. R. J. RUTHVEN
Bernhard Baron Memorial Research Laboratories and *Institute of Obstetrics and Gynaecology, Queen Charlotte's Maternity Hospital, London, England*
D. B. CALNE
Medical and *Neurological Units, University College Hospital, London*

References and Notes

1. D. B. Calne, G. M. Stern, D. R. Laurence, J. Sharkey, P. Armitage, *Lancet* **1969-I**, 744 (1969); G. C. Cotzias, P. S. Papavasiliou, R. Gellene, *N. Engl. J. Med.* **280**, 337 (1969).
2. D. B. Calne, F. Karoum, C. R. J. Ruthven, M. Sandler, *Brit. J. Pharmacol. Chemother.* **37**, 57 (1969).
3. A. Pletscher, G. Bartholini, R. Tissot, *Brain Res.* **4**, 106 (1967).
4. F. DeEds, A. N. Booth, F. T. Jones, *J. Biol. Chem.* **225**, 615 (1957).
5. A. N. Booth and R. T. Williams, *Biochem. J.* **88**, 66P (1963).
6. R. R. Scheline, *Acta Pharmacol. Toxicol.* **26**, 189 (1968).
7. F. Karoum, C. R. J. Ruthven, M. Sandler, *Clin. Chim. Acta* **20**, 427 (1968); F. Karoum, C. O. Anah, C. R. J. Ruthven, M. Sandler, *ibid.* **24**, 341 (1969).
8. A. M. Asatoor, M. J. Chamberlain, B. T. Emmerson, J. R. Johnson, A. J. Levi, M. D. Milne, *Clin. Sci. (London)* **33**, 111 (1967).
9. K. N. F. Shaw, M. Gutenstein, J. B. Jepson, *Proc. Int. Congr. Biochem.* **9**, 427 (1961); J. Dayman and J. B. Jepson, *Biochem. J.* **113**, 11P (1969).
10. J. C. Dacre and R. T. Williams, *J. Pharm. Pharmacol.* **20**, 610 (1968); J. C. Dacre, R. R. Scheline, R. T. Williams, *ibid.*, p. 619.
11. We thank the Smith Kline and French Foundation for a grant for gas chromatography equipment and the Wellcome Trust for support of D.B.C.

7 July 1969; revised 22 September 1969