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REVIEW

CATECHOLAMINES AND THEIR METABOLITES

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LIST OF ABBREVIATIONS

CI	Chemical ionization
CZE	Capillary zone electrophoresis
DMnPSI	Dimethyl-*n*-propylsilylimidazole
ECD	Electron-capture detection
ED	Electrochemical detection
EDA	Ethylene diamine
EI	Electron impact
FID	Flame ionization detection
GC	Gas chromatography
HE	Hydroxyestrogens
HPLC	High-performance liquid chromatography
MHE	Methoxylated hydroxyestrogens
MS	Mass spectrometry
MSAL	Methoxysalsolinol
NI	Negative ionization
NICI	Negative-ion chemical ionization
PFB	Perfluorobenzylimine
PFP	Pentafluoropropionyl
PICI	Positive-ion chemical ionization
SAM	S-Adenosyl-L-methionine
SIM	Selected ion monitoring
tBDMS	*tert.*-Butyldimethylsilyl
TFA	Trifluoroacetyl
TFAAn	Trifluoroacetic anhydride
THI	1-Methyl-3,5,6-trihydroxyindole
TLC	Thin-layer chromatography
TMS	Trimethylsilyl

Abbreviations of separate compounds and relevant enzymes are given in Table 1.

1. INTRODUCTION

Clinical interest in the levels of catechols in body fluids derives from fundamental issues about the physiology of adaptive responses to stress: assessments of disease severity and prognosis, the diagnosis of altered function of catecholamine-synthesizing tissues and pharmacotherapy.

All living things preserve their internal environment in the face of vicissitudinous experience. One way in which mammals maintain homeostasis is by anticipatory and compensatory recruitment of the sympathoadrenomedullary system. The extent of this recruitment can reflect the magnitude of the external threat as perceived or the internal threat as sensed. An important application of measurements of catecholamines and their metabolites is to provide biochemical indices of these secondary alterations in sympathoadrenomedullary function.

In addition, primary derangements of catecholaminergic function can cause or contribute to disease. This etiological role is most clear in pheochromocytoma, a tumour which secretes catecholamines directly into the bloodstream. The clinical manifestations and laboratory evaluation of pheochromocytoma depend on the rates of release of catecholamines into the bloodstream.

In contrast to pheochromocytoma, where the pathophysiological role of altered catecholaminergic function is straightforward but the clinical disorder is rare, pathophysiological hypotheses based on catecholaminergic systems have been offered to explain a large number of common disorders in cardiology (e.g., essential hypertension, mitral prolapse syndrome, hypertrophic cardiomyopathy and type A behaviour pattern), psychiatry (e.g., depression and schizophrenia) and neurology (e.g., dysautonomias, Parkinsonism, post-endarterectomy hypertension and epilepsy), and these hypotheses about the pathophysiological role of altered catecholaminergic function have been more problematic and have led to controversy and intensive research.

Continuing interest in assessing sympathoadrenomedullary function in health and disease has led to the development of clinical techniques based on measurements of catecholamines and their metabolites. These techniques have concentrated on the sympathetic neurotransmitter norepinephrine (NE) (noradrenaline), the adrenomedullary hormone epinephrine (E) (adrenaline), the central nerve transmitter dopamine (DA), the catecholamine precursor dihydroxyphenylalanine (DOPA) and various metabolites of these compounds in body fluids such as plasma, urine and cerebrospinal fluid.

This paper describes procedures applicable to these body fluids. Other procedures have been used successfully in measuring the levels of these substances in brain tissue and incubation media, but typically high concentrations in the former situation and much cleaner samples compared with biological fluids in the latter can render these other procedures irrelevant in a clinical laboratory. We also do not consider the use of simultaneous assays of enzymatic precursors and products to examine the activity of catecholamine synthetic enzymes.

2. CHEMISTRY AND BIOSYNTHESIS

2.1. Review of compounds of interest

Catecholamines are 1-R-3,4-dihydroxybenzenes, where R contains an amino group. The three catecholamines DA, E and NE are all derivatives of DOPA, which is a catecholic amino acid. Because of its acidic character, DOPA is not always considered along with the other three catecholamines. The latter com-

pounds are well known neurotransmitters and have a large number of metabolites which will be dealt with later. However, because of its importance as a precursor, DOPA should also be considered. Further, a series of DOPA thioethers have become of increasing interest, because they are metabolites from melanocytes and melanoma cells and may be considered as tumour markers. These compounds will be dealt with very briefly. Other catecholamine analogues are of interest as drugs or for use in analytical techniques.

A number of metabolites are of interest, and their abbreviations according to the principles given in earlier reviews [1,2] are given in Table 1.

2.2. *Chemistry and biochemistry, an overview*

The general scheme of the biosynthesis of catecholamines is given in Fig. 1. Tyrosine hydroxylase (TH) first converts the amino acid tyrosine to DOPA. Depending on the organ and cells, subsequent reactions take place to give the different catecholamines and their metabolites. By the action of an aromatic amino acid decarboxylase, DA is formed from DOPA. NE differs from DA in having a β-hydroxy group which is introduced by a dopamine hydroxylase, and finally a methyl group is introduced on to the amino group by the enzyme phenylethanolamine N-methyltransferase (PNMT) to give E. Comparing these four parent compounds on a chemical basis, DOPA is a catecholic amino acid and the other three are catecholamines; DA and NE are primary amines, E is a secondary amine and DOPA and DA lack the hydroxy group on the β-carbon.

A general outline of the metabolism is shown in Fig. 2. By the action of a monoamine oxidase (MAO) and an aldehyde dehydrogenase, acid metabolites are formed, and by the action of catechol O-methyltransferase (COMT) methylation of either the parent compounds or of the metabolites is effected. From a chemical standpoint, the grouping given in Table 2 seems adequate. Such a grouping may help to explain the chromatographic properties of the compounds.

2.2.1. *Dopamine*

The metabolism of DA leads to the production of 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) as well known acid metabolites (Fig. 2). If methoxylation first occurs 3-methoxythyramine (3MT) is formed, which subsequently is transformed to 3-methoxy-4-hydroxyphenylethanol (MHPE). It should be noted that salsolinol (SAL) is also formed as a metabolite from DA, and this compound has α-adrenergic agonist activity. Its formation as a consequence of alcohol consumption has been suggested [3,4].

2.2.2. *Epinephrine and norepinephrine*

The metabolism of E and NE (Fig. 3) is well known. By the action of the two enzymes MAO and COMT their final metabolites are formed. It should be noted that apart from methylation and oxidation the catecholamines can also be conjugated to glucuronic acid and sulphuric acid.

TABLE 1

CATECHOLAMINES AND RELATED COMPOUNDS INCLUDING ENZYMES OF INTEREST

Compound	Abbreviation
Catecholamines	
Dopamine	DA
Epinephrine (adrenaline)	E
Norepinephrine (noradrenaline)	NE
Catecholamine metabolites (and precursors)	
3,4-Dihydroxymandelic acid	DOMA
3,4-Dihydroxymandelic aldehyde	D
3,4-Dihydroxyphenylalanine	DOPA
3,4-Dihydroxyphenylacetic acid	DOPAC
3,4-Dihydroxyphenylethanol	DOPET
3,4-Dihydroxyphenylethylene glycol (DOPEG)	DHPG
Homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid)	HVA
6-Hydroxydopamine	6HDA
Metanephrine (3-methoxyepinephrine)	M
3-Methoxy-4-hydroxymandelic aldehyde	3M4HMAld
3-Methoxy-4-hydroxyphenylethylene glycol (MOPEG)	MHPG
3-Methoxy-4-hydroxyphenylethanol	MHPE
3-Methoxytyramine (3-methoxydopamine)	3MT
3-Methoxytyrosine	3MTyr
1-Methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (salsolinol)	SAL
N-Methylepinephrine	N-ME
N-Methylmetanephrine	N-MM
Normetanephrine (3-methoxynorepinephrine)	NM
Vanilacetic acid	VA
Vanillactic acid	VLA
Vanillylmandelic acid (4-hydroxy-3-methoxymandelic acid)	VMA
Vanilpyruvic acid	VPA
Catecholamine analogues	
3,4-Dihydroxybenzylamine	DHBA
3,4-Dihydroxyphenylpropionic acid	DOPPA
Isoproterenol	IP
α-Methyldopamine	αMDA
α-Methyl-3,4-dihydroxyphenylalanine	αMDOPA
α-Methylnorepinephrine (α-methylnoradrenaline)	αMNE
N-Methyldopamine	N-MDA
Tetrahydropapaveroline	THP
Dopa thioethers (L-forms)	
2-S-Cysteinyl-DOPA	2SCD
5-S-Cysteinyl-DOPA	5SCD
6-S-Cysteinyl-DOPA	6SCD
2,5-S-Dicysteinyl-DOPA	2,5SDCD
5-S-Cysteinylglycine-DOPA	5SCGD
5-S-Glutathione-DOPA	5SGD
Enzymes	
Catechol O-methyltransferase	COMT
Dopamine β-hydroxylase	DBH
Monoamine oxidase	MAO
Phenylethanolamine N-methyltransferase	PNMT
Tyrosine hydroxylase	TH

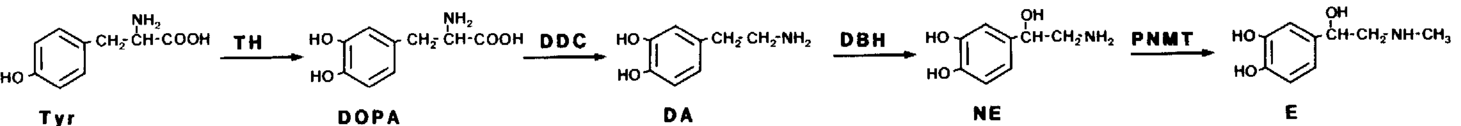

Fig. 1. Synthesis of catecholamines.

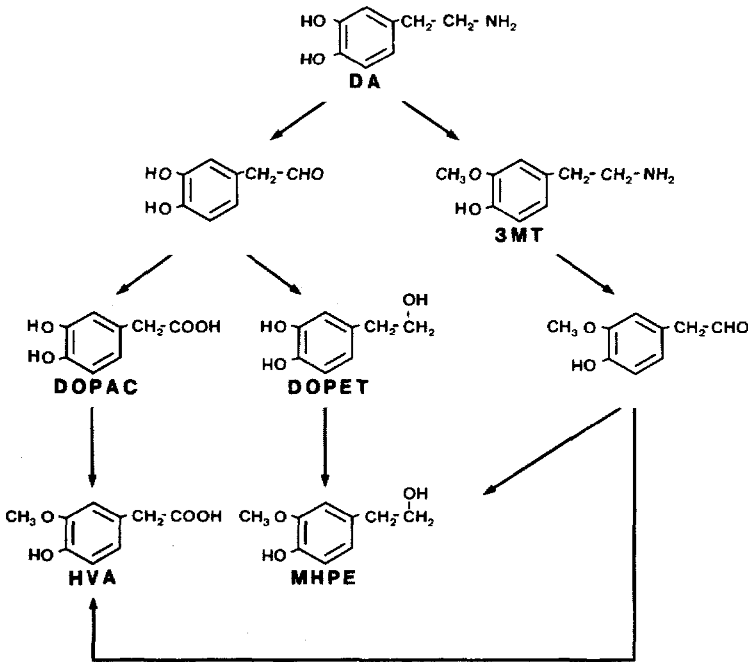

Fig. 2. Dopamine metabolism.

TABLE 2

GROUPING OF COMPOUNDS ACCORDING TO FUNCTIONAL GROUPS IN THE MOLECULES

Type	Compound	Group*					
		1	2	3	4	5	6
Catecholamino acids	DOPA	+	–	+	–	–	+
	DOPA thioethers	+	–	+	–	–	+
	3MTyr		+	+	–	–	+
Basic compounds	Catecholamines:						
	DA	+	–	+	–	–	–
	NE	+	–	+	–	+	–
	E	+	–	$NHCH_3$	–	+	–
	Methoxycatecholamines:						
	3MT	–	+	+	–	–	–
	NM	–	+	+	–	+	–
	M	–	+	$NHCH_3$	–	+	–
Neutral metabolites	Alcohols:						
	DHPG (DOPEG)	+	–	–	OH	+	–
	DOPET	+	–	–	OH	–	
	Aldehydes:						
	D	+	–	–	CHO	+	–
	Alcohols, methoxylated:						
	MHPE	–	+	–	OH	–	
	MHPG (MOPEG)	–	+	–	OH	+	–
	Aldehydes, methoxylated:						
	3M4HMAld	–	+	–	CHO	+	–
Acidic metabolites	DOPAC	+	–	–	COOH	–	–
	DOMA	+	–	–	COOH	+	–
	HVA	–	+	–	COOH	–	–
	VMA (HMM)	–	+	–	COOH	+	–

*1 = Catechol group; 2 = 3-methoxy group; 3 = NH_2 group (α-position); 4 = other group (OH, CHO or COOH) (α-position); 5 = OH group (β-position); 6 = COOH group giving an amino acid compound.

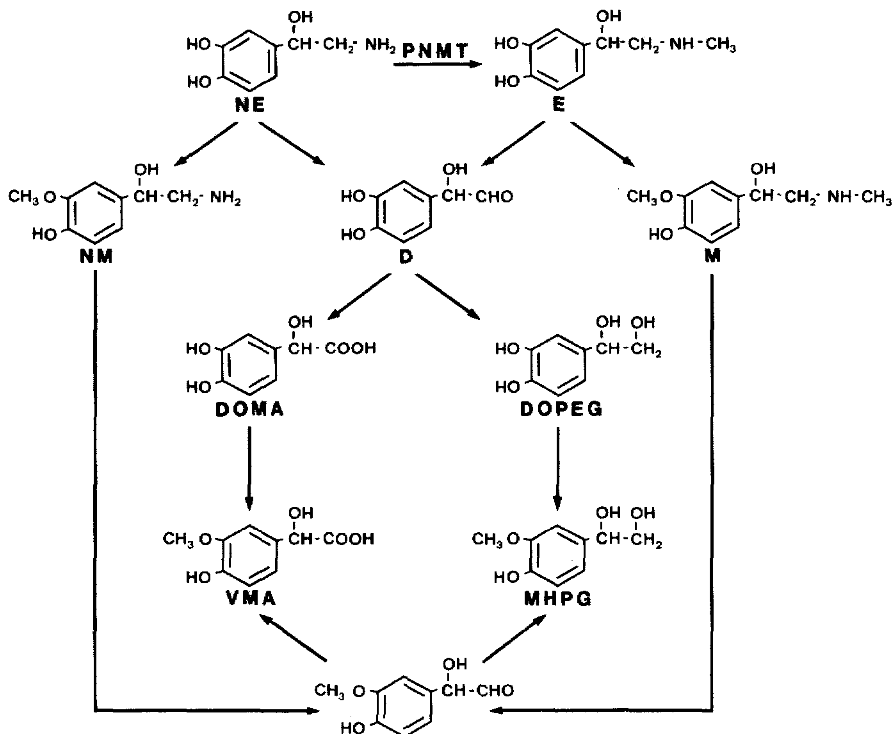

Fig. 3. Metabolism of norepinephrine and epinephrine.

2.2.3. Catecholic drugs

The use of L-DOPA in the treatment of Parkinson's disease has greatly improved our understanding of the function and metabolism of this catechol and its metabolites. A derivative, carbidopa (S,α-hydrazino-3,4-dihydroxy-α-methylbenzenepropanoic acid), is also used in the treatment of this disease and its pharmacokinetics need to be determined [5].

2.2.4. Catecholamine analogues

In the list of abbreviations (Table 2) a few catecholamine analogues are mentioned. Many of these are used as internal standards in analytical procedures.

2.3. Regulation of production

An adequate understanding and interpretation of measurements of catecholamines and their metabolites requires consideration of their regulation, and several regulation mechanisms are depicted in Fig. 4. These mechanisms are summarized below, before considering biomedical applications and technical measurements.

2.3.1. Enzyme regulation

Catecholamine biosynthesis begins with the uptake of the amino acid tyrosine into the sympathetic neuronal cytoplasm and conversion to DOPA by TH. This

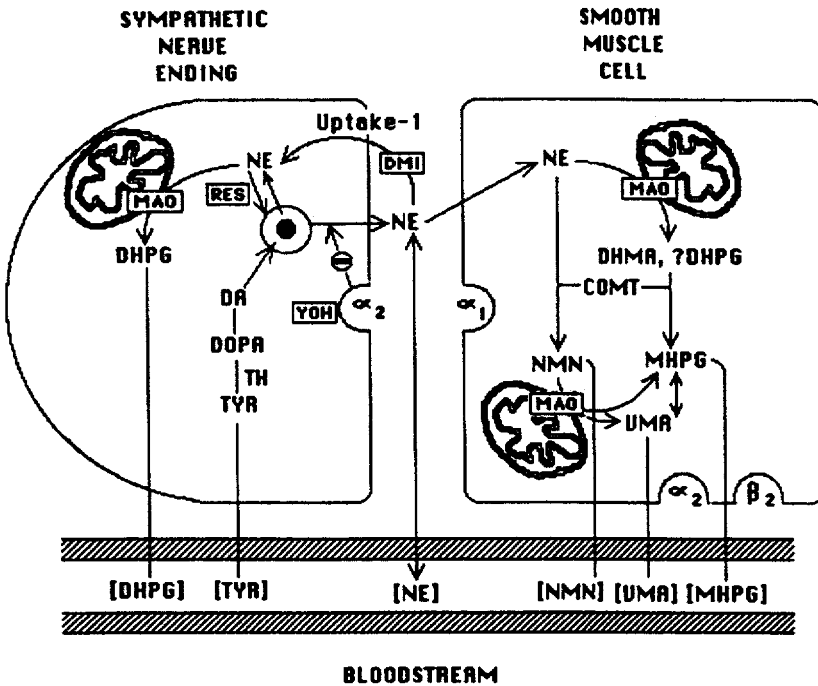

Fig. 4. Mechanisms of norepinephrine synthesis, release, uptake and metabolism. NE = norepinephrine; TYR = tyrosine; TH = tyrosine hydroxylase; DOPA = dihydroxyphenylalanine; DA = dopamine; DHPG = dihydroxyphenylglycol; MHPG = methoxyhydroxyphenylglycol; NMN = normetanephrine; VMA = vanillylmandelic acid; DMI = desipramine; MAO = monoamine oxidase; COMT = catechol-O-methyltransferase; RES = reserpine; YOH = yohimbine.

enzyme is highly localized to catecholamine-synthesizing cells such as in the adrenal medulla, sympathetic nerves and particular nuclei of the brain. TH activity is subject to feedback inhibition by its products, DOPA, NE and DA.

DOPA in the bloodstream can be taken up into neural tissue and into tissues devoid of TH activity, bypassing the rate-limiting enzymatic synthetic step [6]. Uptake of DOPA by the brain is the basis of the beneficial effects of DOPA in the treatment of Parkinson's disease. Uptake of DOPA by proximal tubular cells of the kidney is a substantial source of urinary DA [7,8]. As DA stimulates natriuresis, it is possible that DOPA administration may be beneficial to patients with congestive heart failure [9,10].

DOPA in the axonal cytoplasm is converted to DA by L-amino acid decarboxylase. This enzyme is not specific for DOPA as its substrate, nor is it localized specifically in catecholamine-synthesizing tissues, being especially abundant in parenchymal cells of the liver and kidney. The conversion of DOPA to DA outside the brain can be inhibited by carbidopa, with the result that combined treatment using DOPA and carbidopa delivers more DOPA to the brain than does treatment with DOPA alone.

2.3.2. Vesicular translocation

Dopamine in the neuronal cytoplasm is taken up into vesicles by an energy-requiring process which can be inhibited effectively by reserpine. By what ap-

pears to be the same process, axoplasmic NE can be taken up stereoselectively into the vesicles. The vesicles contain dopamine-β-hydroxylase (DBH), which catalyses the conversion of DA to NE. In the vesicle NE can be stored, can leak back into the cytoplasm or can be released into the synapse. As reserpine inhibits the entry of DA and NE into the vesicles, it also causes a net leakage of DA and NE from the vesicles into the cytoplasm. This eventually depletes vesicular stores of the catecholamines. One of the bases of the catecholamine hypothesis of depression is that reserpine, which is well known to cause depression as a side-effect, depletes neuronal stores of catecholamines. By increasing the cytoplasmic DA level, reserpine increases the plasma levels of the deaminated metabolite DOPAC. By preventing translocation of NE into the vesicles, reserpine acutely increases plasma levels of 3,4-dihydroxyphenylethylene glycol (DHPG or DOPEG), the deaminated metabolite of NE, and chronically decreases the plasma DHPG level as vesicular NE stores become depleted (unpublished observations).

2.4. Release

2.4.1. Exocytosis

According to generally accepted concepts of noradrenergic neurotransmission, sympathetic stimulation causes exocytotic release of the soluble contents of the vesicle, including DBH, NE, adenosine triphosphate and peptides.

The released NE can bind to post- or extra-synaptic receptors to elicit physiological responses; it can bind to pre-synaptic α-2 adrenoceptors to diminish NE release for a given amount of sympathetic nerve traffic; it can be taken up into extaneuronal cells such as smooth muscle or endothelial cells; it can diffuse into the bloodstream; it can be bound non-specifically; it can be sulphate- or glucuronide-conjugated (in humans the sulphate-conjugated form predominates); and it can be taken back up into the axonal cytoplasm.

In the central nervous system, noradrenergic pathways have been identified which appear to be especially important in inhibitory modulation; the exact functions of NE as a central neurotransmitter are much less well understood than its actions as the neurotransmitter of the sympathetic nervous system.

2.4.2. Non-exocytotic release

The sympathomimetic amine tyramine displaces NE from storage vesicles into the cytoplasm and increases NE release without the concurrent release of DBH [11]. It therefore appears that NE can also be released non-exocytotically. This non-exocytotic release may be by way of reversal of the usual inwardly directed process of neuronal uptake, uptake-1 [12].

2.5. Uptake

2.5.1. Neuronal uptake (uptake-1)

The predominant means of removal of endogenously released NE from neuroeffector junctions is by uptake-1, an energy-requiring, non-stereoselective pro-

cess which can be blocked effectively by tricyclic antidepressants such as desipramine and by cocaine and can be attenuated by inhibitors of Na–K ATPase such as ouabain [13,14].

2.5.2. Extraneuronal uptake (uptake-2)

NE also can be removed by extraneuronal uptake (uptake-2), an energy-requiring, non-stereoselective process which can be blocked effectively by adrenal corticosteroids such as corticosterone and by O-methylated metabolites of catecholamines [15].

2.6. Metabolism

2.6.1. Extraneuronal norepinephrine metabolism

NE in extraneuronal cells can be metabolized by COMT to form normetanephrine (NM) or, after the formation of deaminated intermediates, to form 3-methyoxy-4-hydroxyphenylethylene glycol (MOPEG or MHPG) or vanillylmandelic acid (VMA) end-products of NE metabolism. MHPG and VMA can be interconverted [16].

2.6.2. Intraneuronal norepinephrine metabolism

NE in the axonal cytoplasm can be taken up into storage vesicles by the process described above, or can be deaminated oxidatively by MAO, an enzyme which is located in the outer mitochondrial membrane in both neuronal and extraneuronal cells. Because of the location of MAO in the cell, NE in the cytoplasm is subject to deamination, whereas NE in the storage vesicles is not.

The immediate product of this deamination is an aldehyde, which is unstable. It can be reduced by aldehyde reductase to form DHPG, or, theoretically, oxidized by aldehyde dehydrogenase to form 3,4-dihydroxymandelic acid (DOMA). The former process appears to be by far the dominant pathway [17].

Unlike NE, DHPG can easily traverse the cell membrane and diffuse readily into the general circulation. Plasma DHPG appears to be derived virtually exclusively from sympathetic nerve endings [18], and so it has been proposed that the regional rate of release of DHPG can indicate the intraneuronal disposition of NE.

After extraneuronal uptake, DHPG can be metabolized by COMT to form MHPG. As uptake-1 blockade decreases but does not abolish the formation of radioactive MHPG from intravenously infused radioactive NE (unpublished observations), MHPG probably has at least two sources: extraneuronal uptake and metabolism of NE by MAO and COMT and extraneuronal removal and O-methylation of DHPG.

2.6.3. Epinephrine metabolism

In humans, E in plasma and urine is derived from adrenomedullary secretion. The concentration of E in the cerebrospinal fluid of humans is infinitesimally low.

The cytoplasm of adrenomedullary cells contains PNMT, which catalyses the

conversion of NE to E. E is an excellent substrate for uptake-1, and oxidative deamination of E would be expected to result in the formation of DHPG, but incubation of vas deferens tissue in radioactive E does not increase radioactive DHPG production (unpublished observations). The intraneuronal disposition of E may therefore differ from that of NE, but how is unknown.

Extraneuronal metabolism of E by COMT leads to formation of metanephrine (M), VMA and MHPG. Hence the measurement of urinary "metanephrines" in the evaluation of pheochromocytoma includes extraneuronal metabolites of NE and E.

2.6.4. Dopamine metabolism

Whereas sympathetic noradrenergic nerves have been clearly described, the existence and function of dopaminergic nerves have been problematic. DA-containing cells have been identified in association with renal glomeruli [19] and in sympathetic ganglia [20].

DA is the predominant catecholamine in human urine. A substantial proportion of urinary DA appears to derive from circulating DOPA, rather than from neuronal DA [7]. Catecholamines are secreted actively into the urine from renal tubular cells [21]. Therefore, the measurement of total urinary catecholamines is considerably affected by factors other than the release of catecholamines into the bloodstream from sympathetically innervated tissues and the adrenal medulla.

Oxidative deamination of DA leads to the formation of DOPAC. Blockade of the vesicular translocation of catecholamines using reserpine increases the plasma DOPAC level about ten-fold (to be published). This is consistent with the view that, as with NE, DA in nerve endings is actively removed from the cytoplasm into storage vesicles. When this removal is blocked, the continuing formation of DA from DOPA in the cytoplasm (possibly combined with net leakage from the vesicles by DA which has escaped conversion to NE) results in increased amounts of substrate available for oxidative deamination. Whereas DHPG is formed by the reduction of the aldehyde intermediate after deamination of NE, DOPAC is formed by oxidation of the aldehyde intermediate after deamination of DA. The two aldehyde intermediates therefore appear to differ in their relative susceptibilities to oxidation and reduction after the deamination step.

DA is an excellent substrate for uptake-1, so studies based on the administration of radioactive DA allow the examination of neuronal uptake, intraneuronal translocation into vesicles and the formation, release and metabolism of newly synthesized NE.

Extraneuronal metabolism of DA by COMT leads to the formation of 3MT. The main end-product of DA metabolism, resulting from combined operation of MAO and COMT, is HVA. As extracerebral inhibition of MAO using debrisoquine decreases the plasma HVA level (unpublished observations), plasma HVA is probably derived from the metabolism of DA both in the central nervous system and in the periphery.

Whereas the possibility that DA is a neurotransmitter outside the central nervous system is a controversial point, there is convincing evidence that DA is a neurotransmitter in the central nervous system. DA formed in the substantia

nigra traverses a path to the *corpus striatum* where its release influences extrapyramidal control of muscle tone and movement. The main pathological feature of Parkinson's disease, loss of nerve cells of the substantia nigra, appears to be the cause of low levels of DA in the basal ganglia and the cause of the extrapyramidal dysfunction that defines the disease. The remarkably beneficial effects of therapy with DOPA in Parkinson's disease support this dopaminergic etiological hypothesis. The neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) [22] depletes DA in the *corpus striatum* and produces a clinical syndrome similar to Parkinson's disease which responds to DOPA treatment.

DA in human plasma is extensively sulphoconjugated. Ingested catecholamines increase the conjugated DA level without affecting the free levels of catecholamines [23].

2.6.5. *Renal and hepatic clearance*

In contrast to mechanisms of release, uptake, and metabolism of catecholamines, renal and hepatic mechanisms of catecholamine removal from the body have received little attention. The liver excretes acetylated and O-methylated metabolites of catecholamines and DOPA into the bile [24].

In human urine, most of the radioactive species excreted after administration of tracer-labelled exogenous catecholamines are metabolites, whether sulphoconjugated or unconjugated. About 99% of DA in human plasma is sulphoconjugated; about 50–70% of circulating NE and E also are sulphoconjugated [25]. Sulphoconjugation appears to occur by way or phenolsulphotransferase in platelets. Because this sulphoconjugation process is slow, the relationship between changes in the levels of conjugated catecholamines and changes in sympathoadrenomedullary function is obscure. Although it was proposed that urinary excretion of unconjugated and sulphate-conjugated MHPG could indicate metabolism of NE in the central nervous system, this view is no longer accepted [26]. As noted above, in humans the main catecholamine excreted in the urine in unconjugated form is DA, derived to an important extent from circulating DOPA.

The main urinary metabolites of NE in humans are VMA, NM, MHPG and DHPG and the main urinary metabolite of DA is HVA.

The kidney excretes catecholamines by active secretion [21]. This secretory process appears to be more active for E and for the synthetic catecholamine isoproterenol than for NE.

The active role played by the kidney in determining catecholamine excretion, the role of the local production of DA from DOPA by non-neuronal means and the long observation periods required for urine collection limit the applicability of urine analysis for assessing acute changes in sympathoadrenomedullary function.

3. BIOMEDICAL APPLICATIONS OF MEASUREMENTS OF CATECHOLAMINES AND THEIR METABOLITES

Measurements of catecholamines and their metabolites are useful for clinical diagnosis, for examining compensatory recruitment of sympathetic activity as an

aid in determining the severity of an illness and the prognosis, for following therapeutic levels in the body fluids of patients undergoing treatment with biogenic amines and for research purposes.

3.1. Clinical diagnosis

In contrast to the wide spectrum of disorders associated with compensatory recruitment of sympathetic activity, few have been shown to involve primary derangements of catecholamine synthesis or release.

3.1.1. Pheochromocytoma

Pheochromocytoma is a rare cause of clinical hypertension. It is a tumour of chromaffin cells and most commonly is found in the adrenal gland of adults and children or along the pathways of embryological development from the neural crest, i.e., along the aorta to the aortic bifurcation at the organ of Zuckerkandl.

Because the tumour is often benign and surgical removal therefore often curative, and because the associated hypertensive paroxysms or catecholamine-induced cardiomyopathy can be severe and even life-threatening, clinicians commonly wish to exclude the diagnosis of pheochromocytoma by various screening tests based on excessive release of catecholamines. Measurements of urinary excretion of "metanephrines" (NM and M) and of VMA have been shown to be sensitive and specific tests for pheochromocytoma. Patients with pheochromocytoma also usually have high circulating levels of NE, E or both – most commonly of NE.

False-positive results are occasionally obtained in hypertensives undergoing these screening tests, at least partly because individual differences in stress responses result in large inter-individual variabilities of plasma and urinary levels of catecholamines and their metabolites. To decrease the likelihood of false-positive results, clonidine suppression testing is performed [27]. In this test, the α-2 adrenoceptor agonist and central sympatholytic clonidine is administered orally, and blood pressure and antecubital venous blood samples are taken at the start and 3 h after drug administration. Clonidine decreases plasme NE by decreasing the sympathetic neural activity and possibly by stimulating inhibitory α-2 adrenoceptors on pre-synaptic sympathetic nerve endings. In patients with pheochromocytoma, clonidine can fail to decrease high plasma levels of NE, as the tumour can continue to secrete catecholamines into the bloodstream. The failure of suppression of plasma NE by clonidine is therefore a positive result. A similar argument has been applied to suppression testing using the ganglion blocker pentolinium [28].

The occurrence of false-negative results in biochemical screening tests for pheochromocytoma has presented a more difficult problem. Several provocative tests have been devised, including administration of calcium with pentagastrin or administration of glucagon, to detect excessive catecholamine or blood pressure responses in patients harbouring pheochromocytomas [29]. The results have been inconsistent. No comparison of these tests has been published.

Consistent with the intraneuronal source of DHPG, plasma NE-to-DHPG ra-

tios in patients with pheochromocytoma are characteristically increased [30,31]. In our experience, however, the use of this ratio for diagnostic purposes has not been an important improvement over measurements of plasma NE alone, as a small but definite frequency of false-negative results was obtained using this ratio and because, in one instance, a markedly abnormally low plasma NE-to-DHPG ratio was obtained.

In patients with pheochromocytoma, malignancy is defined by the occurrence of metastases, not by the pathological appearance of the tumour. Some patients with malignant pheochromocytoma do not have hypertension and they can have tumours in unusual locations. We recently found that about 60% of patients with malignant pheochromocytomas had high plasma levels of the catecholamine precursor DOPA [32]. Patients with benign pheochromocytomas all had plasma levels of DOPA within the normal range. It is possible that malignant pheochromocytomas are less well differentiated than benign pheochromocytomas, so that the uptake of DA into storage vesicles or the intravesicular conversion to NE is less active.

3.1.2. Neuroblastoma

Neuroblastoma is a cancer which, like pheochromocytoma, is derived from cells of neural crest origin. Neuroblastoma differs clinically from pheochromocytoma in several important respects: first, neuroblastomas occur most commonly in children and represent one of the most frequent paediatric cancers; second, neuroblastomas are usually malignant and lethal; third, they are not typically associated with hypertension; and fourth, levels of catecholamines in plasma or urine are often within the normal range.

We recently found that all patients with neuroblastoma who we tested had very high circulating levels of DOPA [32], confirming the findings of others [33]. It appears that the cancer cells are so poorly differentiated that although DOPA can be produced by them, the remainder of the catecholamine-synthesizing and -releasing apparatus is undeveloped. Successfully treated patients with neuroblastoma can have marked decreases in plasma DOPA levels to within the normal range [33]. Hence plasma levels of DOPA may prove useful in the diagnostic evaluation and treatment of patients with neuroblastoma.

3.1.3. Melanoma

Malignant melanoma is another cancer derived from neuroectodermal tissue. In the melanocyte DOPA is produced from tyrosine by the action of tyrosinase [34]. In contrast to other DOPA-containing cells, melanocytes do not contain DOPA decarboxylase and DA and other catecholamines are therefore not produced in the melanocytes. DOPA may accumulate in the melanocytes, and serum DOPA may be increased in some patients with advanced melanoma metastasis [35]. Only in advanced cases of melanoma disease is there an increased excretion into the urine [35–37]. Several studies have shown increased excretion of DA, DOPAC, HVA and also 3-methoxytyrosine (3MTyr), the O-methylated product of DOPA (for a review, see ref. 34). A metabolite of DOPA, vanillactic acid (VLA),

formed by transamination, reduction and O-methylation of DOPA [2], has also been found in melanoma urines [38].

More interesting in melanoma, however, is the further synthesis of dopaquinone [34] from which indolic compounds and the DOPA thioether 5-S-cysteinyl-DOPA (5SCD) are formed. From the former compounds eumelanins are synthesized, and 5SCD is an intermediate compound formed in the synthesis of pheomelanins. Among the cysteinyl-DOPAs, 5SCD is the most important, but 2SCD, 6SCD and 2,5-S-dicysteinyl-DOPA (2,5SDCD) are also formed. The increased urinary excretion of 5SCD found in patients with metastases of melanoma [39] is of great value for diagnostic studies of melanoma metastases.

3.1.4. Autonomic insufficiency

Autonomic insufficiency can come to clinical attention in many ways, including impotence or decreased libido, decreased sweating, orthostatic hypotension, disturbed micturition, diarrhoea, pupillary abnormalities or constant heart rate. Orthostatic hypotension is especially common and can be disabling or even cause syncope.

Autonomic insufficiency is usually secondary to a systemic disorder. Deficient reflexive vasoconstriction during orthostasis can be the result of defective afferent information to the brain from arterial baroreceptors, abnormal information processing, decreased ganglionic neurotransmission or peripheral sympathetic failure or endorgan hyporesponsiveness. Generalized diseases such as diabetes mellitus and infiltrative diseases such as amyloidosis can involve more than one link in this reflexive chain. Intravascular volume depletion can also cause orthostatic hypotension, but in this instance reflexive tachycardic responses provide a clue that the hypotension does not result from autonomic insufficiency.

Idiopathic orthostatic hypotension is associated with peripheral sympathetic failure and low plasma levels of NE [40]. Patients with this disorder also have a decreased neuronal uptake of radiolabelled NE [41], consistent with a decrease in the number of functional sympathetic nerve endings. Attempts at therapy of this disorder have often been disappointing. Main treatments are a high salt diet, mineralocorticoids and support hose. A relatively new approach is to determine the responses of blood pressure and plasma NE to yohimbine and clonidine. In patients with complete denervation, upregulated post-synaptic adrenoceptors could render the individual responsive to the hypertensive effect of α-2 adrenoceptor agonism with clonidine [42]; in patients with incomplete denervation, pre-synaptic α-2 blockade with yohimbine would be expected to enhance the release of endogenous NE.

3.2. Disease severity and prognosis

The vital homeostatic role of the sympathoadrenomedullary system leads to the suggestion that virtually any clinical disorder not associated with autonomic insuffiency can recruit increased activity of the sympathoadrenomedullary system.

In disease states where the organism senses severe, generalized threats to wellbeing or survival, diffuse, metabolic defenses are aroused, subserved by hor-

mones such as cortisol and E. In other conditions, countered more appropriately by localized responses, regional changes would be expected in activities along nerve pathways such as the parasympathetic and sympathoneural systems.

Consistent with this concept, hypoglycaemia, asphyxiation, haemorrhage, trauma, pain, agitation, syncope and shock all produced marked increases in plasma E. In hypoglycaemia, even mild intensities of this prototypically metabolic stressor produce increased adrenomedullary secretion of catecholamines [43], whereas plasma levels of NE can be increaed to a surprisingly small extent.

In contrast, mild or moderate intensity exercise, orthostasis, cold pressor testing, diving, the post-prandial state and hyperthermia represent challenges which usually can be met adequately with localized changes in blood flow and glandular secretion. These situations are associated with larger changes in plasma NE than in plasma E [44–46]. When the organism is exercised to exhaustion, undergoes forced prolonged standing, develops pain from cold immersion or suffers severe hyperthermia, both adrenomedullary and sympathoneural activation occur, as these situations would pose more global, metabolic threats.

In several common disease states, such as congestive heart failure, myocardial infarction and hypoglycaemia, plasma levels of the catecholamines are often directly related to the severity of the condition. For instance, in congestive heart failure, plasma NE has been found to correlate with the New York Heart Association classification of functional status [47]. Further, the prognosis of patients with congestive heart failure has been related to plasma NE [48]. In patients with acute myocardial infarction, the extent of the cardiac necrosis and the prognosis have been correlated with plasma levels of catecholamines [49,50].

Another situation in which plasma catecholamines can reflect the severity of a threat to survival is in foetal distress. Abnormal patterns of foetal heart rate and foetal acidosis as determined by the pH of scalp vein blood are associated with high plasma catecholamines [51].

Compensatory recruitment of sympathoadrenomedullary activity to maintain organ perfusion can be a double-edged sword, in that the high levels of catecholamines can themselves induce organ damage or arrhythmias. For instance, a septic diabetic can have deranged blood glucose control because of the E response to the sepsis; a patient with congestive heart failure can have a decreased threshold for ventricular arrhythmias due to high circulating levels of catecholamines; a patient with cardiac asthma can develop a myocardial infarction on receiving E as treatment for wheezing; an elderly patient with colonic bleeding could have angina pectoris due to both the anaemia and the reflexive cardiac stimulation; and a patient with heart failure who has down-regulated β-adrenoceptors can fail to respond appropriately to infused pressors such as isoproterenol. More questionable is the supposition that, in an otherwise healthy individual, compensatory recruitment of sympathoadrenomedullary activity produces lasting tissue damage.

3.3. *Pharmacotherapy*

Catecholamines and DOPA are commonly used in pharmacotherapy. In Parkinson's disease, treatment with DOPA has been shown to ameliorate the symp-

toms and signs of the condition and to prolong life. In patients treated with DOPA for prolonged periods, an "on–off" phenomenon can develop, in which the patient suffers fluctuations from signs and symptoms of excess DA (e.g., choreoathetotic movements, slurred speech, disordered behaviour) to signs and symptoms of the Parkinsonian condition. The "off" periods have been found to be associated with trough blood levels of DOPA [52].

Patients in clinical shock are usually treated with intravenously administered pressor amines, including isoproterenol (IP), E, NE, DA and dobutamine. Large individual differences characterize clearances of these drugs, and these differences are augmented in patients with hepatorenal failure, which can prolong catecholamine clearances markedly, with the result that infusion rates based on body weight can predict poorly the blood levels achieved in critically ill patients, even if good relationships are obtained between infusion rates and blood levels in healthy individuals (unpublished observations). The rapidity of currently available methods for measuring plasma levels of these substances introduces the possibility that monitoring of therapeutic levels of these amines can aid in the treatment of the patients.

E is commonly included in local anaesthetic solutions to promote haemostasis and, by vasoconstriction, to prolong the duration of anaesthesia. Injection of E with the local anaesthetic can markedly increase plasma levels of the catecholamine and produce circulatory effects related to the stimulation of adrenoceptors [53,54].

3.4. Research

Measurement of levels of catecholamines and their metabolites is a common research strategy used in evaluating etiological hypotheses about psychiatric disorders, studying neurocirculatory regulation, neuroendocrinology, evaluating the participation of the sympathetic nervous system in the pathophysiology of common illnesses, studies of mechanisms and consequences of stress and the central neural mechanisms that determine sympathetic activity.

3.4.1. Stress

The life work of Walter Cannon, who coined the term homeostasis, centred on the emergency function of the sympathoadrenomedullary system as a vital determinant of the organism's efforts to maintain the milieu interieur during stress [55]. Selye [56] popularized the stress concept, with most of his experimental work involving the pituitary–adrenocortical system.

More recently, the integration of activities of these major stress systems of the body has received more attention. In particular, Frankenhaeuser [57] has conducted a large number of studies of stress where urinary excretion of catecholamines and cortisol have been measured simultaneously. On the basis of this experience, she proposed several concepts that have as their basis the view that perceived characteristics of the situation, rather than the physical conditions themselves, determine adrenomedullary responses to stressors. These concepts include the following: (1) emotional excitement, whether pleasant or unpleasant,

is the cause of adrenomedullary responsiveness; (2) E is more responsive to mental stress than is NE, which increases only when the psychological stress is intense, and NE is more responsive to physical stressors such as exercise and the cold pressor test; and (3) distress is associated with pituitary–adrenocortical activation, whereas effort is associated with catecholamine secretion.

Results of a study recently conducted by us [58] lead us to suggest a few modifications of these concepts. The study was based on haemodynamic and plasma catecholamine responses to a non-distressing, mentally challenging situation, viz., playing a video game. Antecubital venous NE and E, arterial NE, E and corticotropin and total body spillover of NE into arterial blood (based on infusion of tracer-labelled NE) were measured simultaneously during the game. The corticotropin levels did not change, consistent with the above-described association between pituitary–adrenocortical activation and experienced distress. Despite the effort and active engagement of the subjects, however, the plasma levels of E increased relatively little. This finding corroborated a similar finding by another group using a similar laboratory challenge [59]. Further, whereas antecubital venous levels of NE did not change and were unrelated to the haemodynamic responses, the responses of total body NE spillover were more apparent than responses of arterial E and were correlated positively with the magnitudes of the haemodynamic responses. The results therefore did not support the predominance of adrenomedullary over sympathoneural responses in this setting. The finding that the responses of corticotropin and arterial E were strongly positively correlated led to the suggestion that non-distressing mental challenge can activate central neural systems determining sympathoneural outflow, with highly variable pituitary–adrenocortical and adrenomedullary responses. Overall, our current conceptualization is that novel, distressing situations stimulate increases in pituitary–adrenocortical and adrenomedullary activity; situations involving non-distressing, active attention and coping stimulate regional changes in sympathoneural activity; and emotional feelings, both negative (e.g., anxiety) and positive (e.g., elation), increase adrenomedullary activity. Patterns of catecholamine release and excretion may therefore differ depending on perceived aspects of the situation.

3.4.2. Neuropharmacology and neuroendocrinology

An adequate understanding of the pharmacological actions of drugs requires consideration of the multiple potential sites of their action. For instance, the antihypertensive agent clonidine stimulates post-synaptic α-2 adrenoceptors and, in the absence of other effects, produces vasoconstriction. This vasoconstrictor action was the original purpose in developing clonidine as a drug, as it had the potential to be used as a nasal decongestant. Stimulation of pre-synaptic α-adrenoceptors, however, augments the inhibitory modulation of NE release from sympathetic nerve endings. As clonidine does not affect the blood pressure of patients with spinal cord transection, the main mechanisms of its hypotensive actions, however, are in the central nervous system [60]. It appears that clonidine can act at α-2 adrenoceptors in the medulla oblongata to decrease the sym-

pathetic outflow; in addition, it is possible that clonidine can exert hypotensive effects by a central neural mechanism independent of α-2 adrenoceptors [61].

The field of neuroendocrinology has reorganized fundamentally the views about the brain and endocrine systems. The functions of the brain were thought to be behavioural interactions between the organism and the external environment, whereas those of the endocrine system were thought to be hormonal regulation of the internal environment. It is now clear that, via a large number of releasing factors in the brain, the brain controls the endocrine system. This control introduces a new emphasis on coordination of behavioural and endocrine activities to serve adaptive functions.

Several of these releasing factors can be influenced by catecholamines in the brain. The origins and purposes of catecholaminergic pathways in neuroendocrinology, and the relationships of these pathways to sympathoadrenomedullary function, are matters of intense current research.

3.4.3. Pathophysiology

Catecholaminergic systems have been thought to participate in the development of the maintenance of several clinical disorders in psychiatry, neurology and cardiology.

In psychiatry, the catecholamine theory of depression is based on a putative deficiency in catecholamine release in the brain. This has helped to explain why tricyclic antidepressants, which inhibit the neuronal uptake of NE in the brain, and MAO inhibitors, which decrease the metabolism of NE in the brain, have often been shown to be effective anti-depressant drugs, and why reserpine, which depletes NE stores, can cause depression as a side-effect. The theory cannot explain well why cocaine, which both releases NE and inhibits neuronal uptake of NE, is not an effective anti-depressant.

An alternative hypothesis is that depression actually is associated with excessive NE turnover in the brain. Tricyclic antidepressants and MAO inhibitors can decrease, by mechanisms that are not clear, NE turnover in the brain, and reserpine stimulates TH activity [62]. Interactions between central neural mechanisms determining pituitary–adrenocortical activity, which is deranged in a substantial proportion of depressed patients [63,64] and central mechanisms determining catecholaminergic activity are being explored actively.

Dopaminergic systems of the brain have provided the basis for another theory in psychiatry: that thought disorders such as schizophrenia are the result of excessive release of DA in brain. This helps to explain the ameliorating effects of DA receptor blockers in psychotic patients.

The strong association between depletion of DA in the basal ganglia and Parkinsonian syndromes has already been alluded to above.

Disorders of sympathoadrenomedullary regulation have been suspected in several circulatory conditions. The evidence is convincing that idiopathic orthostatic hypotension is associated with loss of peripheral sympathetic function. Excessive sympathoadrenomedullary activity may occur early in essential hypertension [65] and in neurogenic hypertension such as that associated with intracranial bleeding

[66], the Guillain–Barre syndrome [67] and interruption of baroreceptor afferents [68].

4. ANALYTICAL METHODS

Owing to the immense importance of the catecholamines for the regulation of physiological processes in the brain and nerve conduction [1], researchers have strengthened their efforts to improve their analytical methods, and history shows the success of these efforts. Many of the old techniques nowadays seem obsolete, however, because new methods have greatly improved sensitivity and accuracy and suitability for measuring catecholamines in biological fluids and tissues. Earlier methods have been reviewed [69–72].

4.1. Early methods

The analysis of biological extracts for catecholamines has been carried out by a variety of chemical techniques. Von Euler and Hamberg [73] introduced a colorimetric procedure which led to much research on the function of the neuron. The adrenochrome identified by Von Euler could also be chemically converted to an adrenolutin (1-methyl-3,5,6-trihydroxyindole; THI) which had characteristic fluorescent properties [74]. This formed the basis for a sensitive method for the detection and quantification of small amounts of catecholamines in tissues. This assay was then widely used for the determination of catecholamines [75]. An excellent review of early THI methods was given by Callingham [76] and more recently by Miura and Adachi [77]. The second group of commonly used fluorescence assays was based on the condensation of oxidized catecholoamines with ethylenediamine (EDA) [78]. The fluorimetric methods that were developed later using EDA condensation have been excellently reviewed [70]. Recently improved high-performance liquid chromatographic (HPLC) methods have been developed with fluorimetric detection with both the THI and EDA procedurs.

4.2. Radiochemical methods

4.2.1. Radiochemical techniques

Three classes of radiochemical methods can be identified: early methods such as double isotope dilution assays or oxidation methods, radioenzymatic methods and radioimmunoassays. Although the early radiochemical methods by which catecholamines were oxidized by, e.g., iodine-131 [79] and chromium-51 dichromate [80], gave detection limits of about 50 pg, these methods have not come into common usage.

The few investigations on radioimmune methods which have been published are of interest because of their potential for rapid serial determinations and in vivo application for cellular localizations and quantitation in tissue. The early literature was reviewed in detail by Holly and Makin [70].

4.2.2. Radioenzyme techniques

With radioenzyme techniques the labelling of the catecholamine is achieved by the use of an enzyme, PNMT or COMT which transfers the radioactive methyl group (usually [^{3}H]methyl or [^{14}C]methyl) from S-adenosyl-L-methionine (SAM) to the catecholamine.

4.2.2.1. PNMT method for NE and NM. In the PNMT method the labelled methyl group is transferred to the primary amine of NE to give E. In the first PNMT method for the determination of brain NE [81] the [^{14}C]E was separated from the methyl donor by paper chromatography before liquid scintillation counting. A subsequent improvement to methods for NE was obtained by the use of tritiated SAM [82] or purified PNMT [83,84]. Improvements were also obtained by better purification of the labelled product [84] and prepurification of plasma [85–87].

Axelrod [88] found that NE, NM and octopamine and also a variety of phenylethanolamine-derived compounds were substrates for PNMT. This was utilized by Vlachakis and De Quattro [89], who adapted the PNMT–[^{3}H]SAM method for the measurement of urinary NM. The [^{3}H]M formed was selectively isolated by thin-layer chromatography (TLC) and counted. The specificity of the method was mainly due to the fact that other N-methylated derivatives have different chromatographic characteristics. Similar methods for plasma NM were described [90–93]. By including acid hydrolysis before the assay, Vlachakis and Niarchos [94] determined the total concentration of NM in plasma.

4.2.2.2. COMT method for catecholamines. The first COMT method was described by Engelman et al. [95], who used a double isotope technique for the measurement of E and NE. [^{14}C]SAM was used as a methyl donor and [^{3}H]NE was added as a tracer to calculate the recovery. They purified the methylated product by cation-exchange chromatography and converted the methoxy products to vanillin before liquid scintillation counting. A large number of modifications of the COMT method with improved sensitivity and specificity have since appeared and a detailed review has been published [70].

Some of these improvements may be mentioned briefly. Engelman and Portnoy [96] replaced their purification procedure for derivatives with a TLC step and improved the sensitivity of the method. Other procedures such as cellulose acetate electrophoresis have been suggested [97], but TLC became the method of choice.

Passon and Peuler [98], after a thorough evaluation of different steps, introduced a number of improvements which increased the sensitivity ten-fold over that of the method of Engelman and Portnoy [96]. Other methods [99,100] scaled down the reaction mixture and used only 50 μl of plasma. The latter method [100] also included benzoxylamine, a DOPA decarboxylase inhibitor, in the reagents to prevent interference from DOPA in the analysis of DA. This method had detection limits of 1 pg for NE and E and 6 pg for DA. This method formed the basis of a kit (Cat-A-Kit; Upjohn, Kalamazoo, MI, U.S.A.) containing all the reagents necessary for the determination of catecholamines in plasma which became very popular and was widely used. By including DOPA decarboxylase in the assay, DOPA was converted to DA and the sum of DOPA and DA was determined. The

TABLE 3

R_F VALUES FOR COMT REACTION PRODUCTS SEPARATED BY TLC

Reaction products were separated on precoated LK5DF silica gel TLC plates with the solvent system chloroform–ethanol–ethylacetate–acetic acid–water (60:25:5:5:5). Abbreviations: MSAL = methoxysalsolinol; HE = hydroxyestrogens (2-hydroxyestrone, 2-hydroxyestradiol, 2-hydroxyestriol); MHE = methoxylated hydroxyestrogens. Adapted from Dean et al. [107].

Substrate	Reaction product	R_F value
DOMA	VMA	0.20
NE	NM	0.22
E	M	0.26
DA	3MT	0.34
SAL	MSAL	0.42
THP		0.47
DOPEG	MHPG	0.70
DOPAC	HVA	0.83
DOPET	MHPE	0.86
HE	MHE	0.93

amount of DOPA was then determined by difference [101]. In a further extension of the method, sulphate conjugates of DOPA, DA, NE and E were determined. This was achieved by addition of a sulphatase to the incubation mixture. The concentrations of catecholamine and DOPA sulphates were then calculated as the difference between the free catecholamines and DOPA and the "total" amount measured in the presence of the sulphatase. For ten healthy subjects the plasma free fractions on average were 45% for DOPA, <1% for DA, 19% for NE and 12% for E.

The COMT enzyme catalyses the transfer of a methyl group from SAM to the 3-hydroxy position of many catecholic compounds. The COMT method is therefore capable of measuring a number of different catecholamines. Depending on different methodological strategies, such as the choice of separation by TLC, other metabolites have also been determined. Usually the analytes were DA, NE and E [101], but other constellations were also reported for plasma, such as the main catecholamines DA, NE and E together with DOPAC, DOMA, DOPEG and 3,4-dihydroxyphenylethanol (DOPET) [102] or together with DOPA, DOPAC, DOMA, DOPEG and DOPET [103]. Others analysed DOPAC together with DA, NE and E in plasma [104,105] and with minor modifications also DOPA, DOPEG and DOMA [105]. Vlachakis et al. [106] measured NE, E, DOMA and DOPEG in plasma, tissue and cerebrospinal fluid. Radioenzymatic methods have also been described for SAL in plasma and tissue together with DA [107] and together with DA, NE and E [108]. Table 3 gives the R_F values obtained for different catecholamines [107]. It is evident that there is a risk of obtaining interferences from adjacent spots, although precautions are taken to avoid this.

For chromatographic separation, HPLC is superior to TLC. Several attempts have therefore been made to improve the separation of different methylated reaction products from each other and from the radioactive methyl donor [109–

113]. Although good separations have been obtained, this application of HPLC has not gained widespread use. When the advantages of radioenzyme methods (sensitivity, application to specific catecholic drugs) are to be utilized, it seems best performed according to classical means by TLC separation. An excellent description of the application of the radioenzymatic method to the determination of DA, NE and E was recently published [114]. It included views on the strategy for selective extraction and TLC separation of methylated reaction products.

4.2.3. Radioimmune techniques

The application of radioimmunoassays and other immunoligand methods for the determination of low-molecular-weight compounds such as thyroid and steroid hormones has been successful in recent years. However, this is not so with catecholamines and their metabolites, mainly because the antibodies obtained cross-react with compounds similar in structure [115], and these are numerous in this field. Nevertheless, a few methods have appeared for 3MT (3-O-methyldopamine) in plasma and urine [116], DOPA, DA and 3MT in urine [117], free and conjugated M in urine [115,118], E in adrenal gland extracts [115], NE and E in tissues and serum [119,120] and MHPG in brain tissue [121]. However, it must be borne in mind that the specificities of these methods depend on the absence of cross-reacting compounds or their successful removal by enzyme conversion or chromatographic means prior to analysis.

Recently, Iinuma et al. [122] reported radioimmune methods for the determination of M and NM in plasma and urine. They used antibodies against M and NM which also showed 3.1 and 3.7% cross-reactivity to synephrine and octopamine, respectively. These compounds are *p*-hydroxyphenylethylamines which, after labelling with ^{125}I in the 3-position, revealed a 70–150 fold greater affinity for the antibodies than the unlabelled synephrine and octopamine. With these compounds as tracers they obtained satisfactory sensitivity and precision with their methods, and the analytical recoveries were quantitative [122]. This method seems very satisfactory, but we believe that the greatest potential of immunochemical methods lies in their ability to localize catecholamines and their metabolites at the cellular and subcellular levels.

4.3. Gas chromatography

4.3.1. Catecholamines

Several studies on the use of gas chromatography (GC) with electron-capture detection (ECD) were performed during the period 1960–1970 [123–132]. For example, Lhuguenot and Maume [132] in 1974 investigated the use of pentafluorobenzyliminetrimethylsilyl derivatives for the quantitative determination of catecholamines in the picomole range with ECD. The highest sensitivity was obtained with a pulsed electron-capture detector which allowed analysis down to 10 pg of six primary amines of biological interest, viz., tyramine, 3MT, octopamine, DA, NM and NE. They applied the method to extracts of the adrenal gland and measured 180 μg of NE in 1 g of fresh tissue. This result was similar to that obtained earlier [129].

4.3.2. Methoxycatecholamines

A GC method for the determination of methoxylated catecholamines (NM, M and 3MT) in urine was published by Nelson et al. [130]. They improved the purification of urinary clean-up by ion-pair extraction of the amines. After derivatization of the N,O-dipentafluoropropionyl β-O-ethyl derivatives of NM and M and the N,O-dipentafluoropropionyl derivative of MT, they obtained a good separation by GC–ECD. The sensitivity of the method was satisfactory for application to urine determinations.

4.3.3. Neutral and acid catecholamine metabolites

In the late 1970s attempts were made by Muskiet et al. [133] to quantify the urinary acidic (VMA, HVA, DOPAC and VLA) and alcoholic (MHPG, DOPEG and vanilethanol) catecholamine metabolites by GC profiling by use of trimethylsilyl (TMS) derivatives. Further studies were performed on HVA, DOPAC, *p*-hydroxyphenylacetic acid and vanillic acid [134]. The TMS derivatives of catecholamine metabolites could not all be measured simultaneously because VMA was incompletely separated from neighbouring peaks and MOPEG was completely overlapped by citric acid [133,134]. By using *tert.*-butyldimethylsilyl (tBDMS) derivatives instead of the more generally applied TMS derivatives of organic acids, they obtained a considerably improved resolution of urinary organic acids [135]. They used capillary GC with flame ionization detection (FID) and achieved a good separation of the urinary metabolites HVA, vanillic acid, MHPG, VMA, DOPAC and VLA.

From the foregoing examples it is evident that although GC techniques have been improved substantially over the years, sufficient separation of different compounds cannot be achieved with standard GC techniques. When capillary GC with FID is used the number of peaks is very large and problems with peak overlapping and peak identification sometimes occur [136]. The development of GC techniques was reviewed by Schmitter [137].

4.4. Gas chromatography with mass spectrometric detection

Early gas chromatographic–mass spectrometric (GC–MS) methods for the determination of catecholamines [138,139] and HVA [140] were published in 1972, and even before then the fragmentation patterns of their derivatives and derivatives of their metabolites were studied. Many of the early studies were directed at measuring either the catecholamines, the methoxylated catecholamines, the neutral metabolites or the acid metabolites from either DA or from the other two catecholamines E and NE. More recent studies have aimed at the combined analysis of a larger number of the metabolites together with the parent catecholamine compounds. An excellent review on the subject has been published [141]. It also described the function of GC–MS, detailed various parts of the techniques and gave updated references to the possibilities and shortcomings of GC–MS. A number of earlier reviews are also available [142–144].

4.4.1. Catecholamines

Using perfluorobenzylimine (PFB) combined with TMS ether as derivatization agents, Lhuguenot and Maume [132] demonstrated the application of GC–MS with mass fragmentographic detection to the quantitation of DA and NE in extracts from the adrenal gland. Later Muskiet et al. [145] used the trimethylsilyl-N-trifluoroacetyl derivatives as obtained by a modification of the Donike [146] method for the assay of DA, NE and E in urine.

4.4.2. Methoxycatecholamines

Änggård and Sedvall [127] systematically studied the fragmentation pattern of perfluoroacetyl derivatives of M and NM. With both trifluoroacetyl (TFA) and pentafluoropropionyl (PFP) derivatives they obtained satisfactory separations of the two methoxylated catecholamines by GC with XE-60 as the stationary phase without interference from derivatized 3MT. They found the TFA derivatives to be unstable whereas the PFP derivatives were stable. Based on these findings, Robertson et al. [147] developed a quantitative assay for urinary M and NM as the PFP derivatives and based on GC–MS with selected ion monitoring (SIM). Similarly, Muskiet et al. [145], using pentafluoropropionic anhydride for derivatization and deuterated 3-O-methoxycatecholamines as internal standards, obtained quantitative urinary data for the methoxylated compounds M, NM and 3MT. Further improvements of the technique were reported by Canfell et al. [148].

4.4.3. Catecholamines together with methoxycatecholamines

The derivatization of DA, NE, E, 6-hydroxydopamine (6HDA) and 3MT was thoroughly studied by Miyazaki et al. [149] using trifluoroacetic anhydride (TFAAn) and dimethyl-*n*-propylsilylimidazole (DMnPSI) as reagents. With DA and NE as examples the acylation was first performed with TFAAn for 30 min at room temperature. Methanolysis for 1 h at room temperature gave the N-trifluoroacetyl derivative of DA and the 2′-O-methyl-N-trifluoroacetyl derivative of NE. Silylation with DMnPSI then gave the N-trifluoroacetyl-O-dimethyl-*n*-propylsilyl ether derivative from DA and the 2′-O-methyl-N-trifluoroacetyl-O-dimethyl-*n*-propylsilyl ether derivative from NE. With such derivatives from the five catecholamines excellent stability was obtained over seven days and excellent separations were obtained using GC and GC–MS with SIM. The advantages of chemical ionization mass spectrometry (CIMS) were preferred over the electron impact (EI) mode because of its higher sensitivity in the formation of adduct ions. A similar detailed study of the derivatization of catecholamines to give mixed derivatives was published by De Jong and Cramers [150] for urinary catecholamine measurements. Martin et al. [151] showed that GC–MS in the negative-ion chemical ionization (NICI) mode of the PFP derivative of NM yields 200- and 350-fold increases in sensitivity compared with those obtained using the positive-ion chemical ionization (PICI) and EI modes, respectively. They therefore investigated the PFP and tBDMS–PFB derivatives with catecholamines and methoxycatecholamines more closely and compared their application in GC–MS in the negative ionization (NI) mode.

4.4.4. Alcoholic catecholamine metabolites

DOPEG and MHPG, being deaminated neutral metabolites from NE, can be easily extracted and determined by GC–MS in different biological samples. Many methods have been published for MHPG, and a simple routine GC–MS method for the determination of plasma DHPG in combination with MHPG was described by Jimerson et al. [152]. Often the alcoholic metabolites are extracted and measured together with acid metabolites (see below).

4.4.5. Acid catecholamine metabolites

Early papers were in general aimed at methods for the determination of one or two acid metabolits, sometimes together with their parent catecholamine (cf., e.g., refs. 153–156). Faul and Barchas [142] summarized several derivatization methods for acidic catecholamine metabolites for GC–MS. Of particular interest is the recent development of highly sensitive GC–MS techniques. Wood [157] investigated a SIM assay for DA and its metabolites using NICI conditions and found a dramatic increase in sensitivity in comparison with EI and PICI. He estimated that the sensitivity of the analysis in the NICI mode was increased 100–150 fold for DOPAC, 500–750 fold for HVA, 10–15 fold for DA and 10–20 fold for 3MT in comparison with EI. Recent developments in this context were summarized by De Jong et al. [158]. They also found the sensitivity of electron-capture (EC) negative-ion MS to exceed that of EI by two or three orders of magnitude. They were therefore able to measure acidic catecholamine metabolites in cerebrospinal fluid and plasma and urine with good precision (coefficient of variation $< 5\%$) at the low nanomoles per litre level. Their method combined acetylation of the phenolic hydroxy groups in buffered aqueous solution with pentafluorobenzyl ester formation and acetylation of aliphatic hydroxy groups under anhydrous conditions. The resulting per-O-acetyl carboxypentafluorobenzyl esters were subjected to GC–EC negative-ion MS. The mass spectra of acetylpentafluorobenzyl derivatives of acidic catecholamine metabolites are shown in Fig. 5 and the SIM tracing in Fig. 6.

Concomitant with the development of sensitive negative-ion MS methods, ordinary GC–MS methods have been developed. For example, improvements have been made by Michotte et al. [136] and Davis et al. [159] using capillary GC–MS with ECD, so that a number of metabolites can be measured with good accuracy in plasma, cerebrospinal fluid and urine.

4.5. High-performance liquid chromatography

With the advent of HPLC, a general procedure with broad applicability was introduced into both research and routine laboratories during the 1970s. A large number of papers have appeared that define the chromatographic conditions for optimal separations of the different catecholamines and their detection. Although a good separation is mandatory for a successful result, the choice of detector is very important for the sensitivity and performance of the analysis. For the analysis of biological samples and extracts thereof, further purification is often required before HPLC analysis, and to improve the sensitivity and speci-

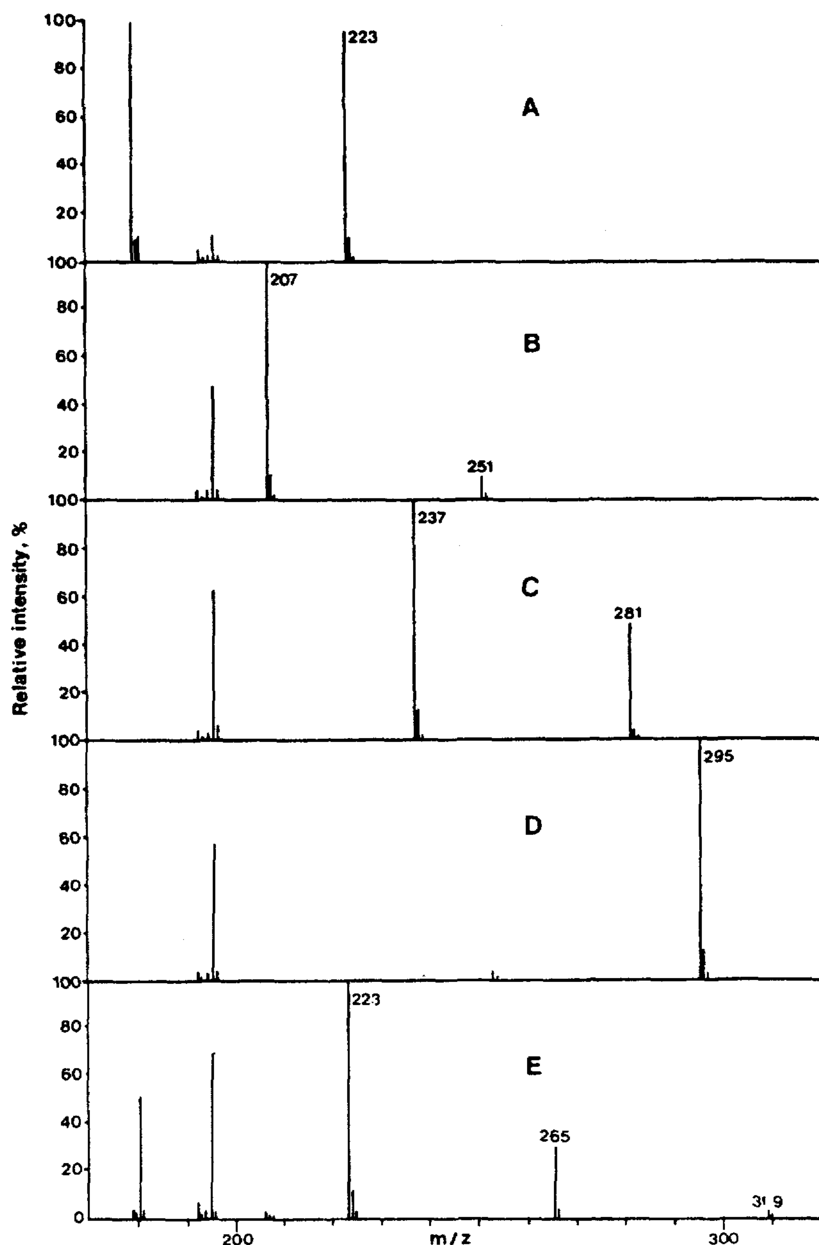

Fig. 5. Electron-capture detection negative-ion mass spectra of acetylpentafluorobenzyl derivatives of (A) HVA, (B) DOPAC, (C) VMA, (D) VLA and (E) DOMA. (From ref. 158.)

ficity of the methods derivatization of the catecholamines has also been used. With improvements in techniques, fully automated procedures have emerged. Although compromises between economy and performance often have to be made, we shall in general neglect the economic aspects in the evaluation of the methods and merely try to evaluate the other different factors and their effects on final

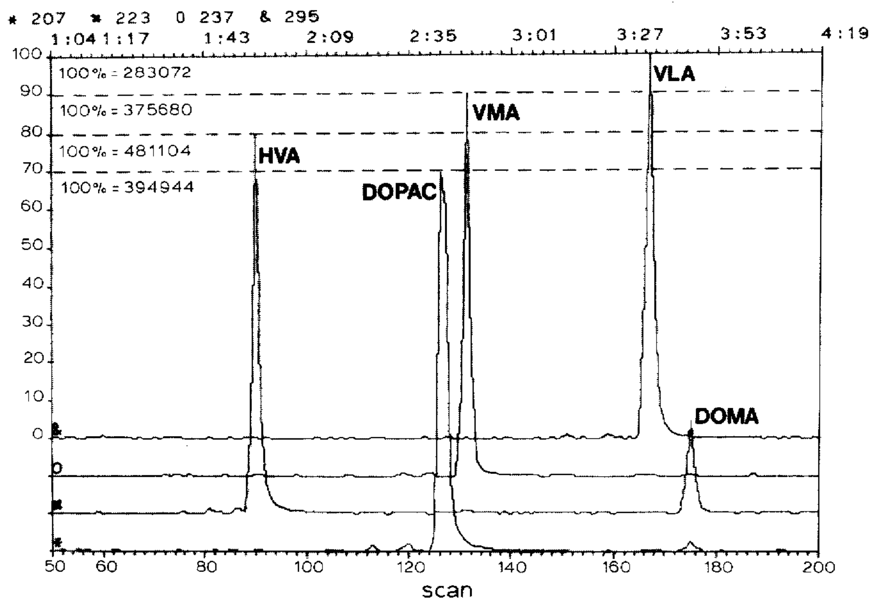

Fig. 6. Selected-ion recordings of standards, HVA, DOPAC, VMA, VLA and DOMA. (From ref. 158.)

analytical outcome and the applicability of the overall procedures to analyses of biological samples.

A number of valuable reviews of HPLC have also appeared [69,71,72,160–166]. The analytical outcome of an HPLC determination depends on factors such as detection, column performance, chromatographic conditions, biological fluid composition, precolumn manipulations for clean-up and/or derivatization, chemical characteristics or properties of the analyte or its derivative and post-column derivatization procedures. These factors have to be considered separately, but can only be evaluated when integrated into an analytical system.

4.5.1. Column

Packing materials for column liquid chromatography have developed dramatically during the last ten years. For both normal- and reversed-phase chromatography improved packing techniques also have given better columns. The size of the silica particles has a great impact on the efficiency of the column. Thus, when high efficiency is needed, 3-μm particles are appropriate. However, one should bear in mind that technical difficulties and problems occur more often with 3-μm than with 5- or 10-μm particle columns. Therefore, if 3-μm particles are not needed, such columns should be avoided. However, as will be discussed later, for the separation of a number of catecholamines and amine metabolites, an improvement in the efficiency of the column may resolve a peak of interest from an impurity peak.

4.5.2. Chromatographic conditions

4.5.2.1. Normal-phase chromatography. Normal-phase chromatography was initially expected to become a standard chromatographic procedure. In this technique the stationary phase is more polar (silica column) than the eluent, which

is an organic phase. However, because of its low reproducibility, the technique has not gained widespread use.

4.5.2.2. Ion-exchange chromatography. Ion-exchange HPLC has been used for the analytical separation and quantitation of the basic (protonated) catecholamines [167–181]. For these compounds cation exchangers are used. This is also the case for the separation of methoxy compounds. Thus Ponzio et al. [182] determined 3MT in brain tissue by cation-exchange HPLC–ED after a simple isolation procedure.

The basis for the chromatographic separation is the retention of the positively charged amino group of the catecholamines. Neutral metabolites are therefore not retained on ion exchangers, and anion exchangers have not been used in the analytical HPLC of acid metabolites other than in the prepurification step [183,184].

Although the current trend is to abandon ion-exchange column liquid chromatography in favour of reversed-phase HPLC, Mefford et al. [185] recently showed the favourable application of a cation-exchange reagent, N-methyl oleyl taurate, to the analytical separation of catecholamines. This reagent semi-irreversibly "loads" the C_{18} reversed-phase material [186], blocking the reversed-phase sites of the column. As a result, only compounds which are separated by ion-exchange mechanisms are retained. The advantage of this method over ion-pair reversed-phase HPLC–ED for the determination of DA, NE and E is inversion of the retention order between E and NE. This gives a higher signal for E, which has a lower concentration than NE in biological fluids. Further, operation of the chromatography at pH 7 increases the electrochemical response, particularly for E.

4.5.2.3. Reversed-phase chromatography. Molnár and Horváth [187] applied reversed-phase chromatography [188] to the separation of catechol compounds. They illustrated very clearly the separation of metabolites in different metabolic pathways of DA and showed the strong influence of pH on retention when biogenic amines and acidic metabolites were chromatographed.

4.5.2.4. Ion-pair reversed-phase chromatography. Ion-pair reversed-phase HPLC has been known for more than fifteen years [189,190] and was applied as early as 1974 [190] for the separation of amines, amino acids, carboxylic acids, biogenic amines and their metabolites. These early studies did not produce great sensitivity. In recent years a number of studies have been conducted [191–195] in which improvements in both the column efficiency and mobile phase condition resulted in very good separations of the compounds.

Several approaches can be used to manipulate chromatographic separations in the reversed-phase ion-pair technique and some of these approaches have been summarized by Kontur et al. [194]. Altering the pH of the mobile phase produces differential changes in retention based on changes in the charges of some of the analytes. Remarkably small changes in pH (between about 3.2 and 3.6) can change the position of the DOPA chromatographic peak with respect to that of NE and the DOPAC chromatographic peak with respect to those of E and DA. Increasing the mobile phase pH decreases the retention of DOPA and DOPAC, because these acids become more extensively ionized.

TABLE 4

REDUCED FACTORIAL DESIGN: CALCULATED MEAN EFFECTS (β VALUES) ON THE RETENTION TIMES OF THE SUBSTANCES WHEN CHANGING THE MOBILE PHASE PARAMETERS FROM + TO –

From ref. 195.

Substance*	Mobile phase variable						
		CH_3CN:	OSA:	pH:	T:	Na_2EDTA:	DEA:
	β_0**	β_1	β_2	β_3	β_4	β_5	β_6
DHPG	0.97	−0.05	−0.01	+0.02	−0.03	0	0
VMA	1.04	−0.05	−0.05	+0.34	+0.03	+0.03	0
L-DOPA	1.43	−0.10	+0.18	−0.52	+0.06	−0.17	−0.04
DOPAC	1.53	−0.13	+0.06	−0.30	−0.08	+0.02	−0.01
NE	1.54	−0.19	+0.37	+0.06	−0.14	+0.6	−0.14
MHPG	1.59	−0.20	−0.05	+0.05	−0.07	+0.02	−0.02
E	2.17	−0.40	+0.63	+0.14	−0.29	+0.13	−0.26
NM	3.10	−0.68	+1.00	+0.21	−0.42	+0.23	−0.41
5-HTP	3.88	−0.51	+0.81	−2.18	+0.07	−0.71	−0.24
DA	3.98	−1.05	+1.43	+0.40	−0.74	+0.36	−0.63
MET	4.93	−1.36	+1.77	+0.47	−0.86	+0.49	−0.85
5-HIAA	4.95	−0.85	−0.18	−0.90	−0.52	+0.17	−0.10
5-HTOL	5.10	−1.06	−0.17	+0.36	−0.68	+0.06	−0.06
HVA	6.38	−1.17	−0.39	−1.87	−0.29	+0.37	−0.24
ISOP	7.80	−2.60	+3.04	+0.99	−1.59	+0.95	−1.47
5-HT	12.6	−4.24	+5.17	+1.80	−3.33	+1.49	−2.59

*Abbreviations: DHPG = 3,4-dihydroxyphenylethylene glycol; VMA = vanillylmandelic acid; DOPA = 3,4-dihydroxyphenylalanine; DOPAC = dihydroxyphenylacetic acid; NE = norepinephrine; MHPG = 3-methoxy-4-hydroxyphenylethylene glycol; E = epinephrine; NM = normetanephrine; 5-HTP = 5-hydroxytryptophan; DA = dopamine; MET = metanephrine; 5-HIAA = 5-hydroxyindole-acetic acid; 5-HTOL = 5-hydroxytryptophol; HVA = homovanillic acid; ISOP = isoproterenol (internal standard); 5-HT = serotonin.
**β_0 is the average retention time.

Wester et al. [195], by factorial design, optimized the mobile phase (Table 4) and described a rapid (<25 min) isocratic system for the complete separation (Fig. 7) of the individual catecholic and methoxycatecholic compounds DOPA, DA, DOPAC, 3MT, HVA, NE, NM, DOPEG, MHPG, E, M and VMA and other biogenic amines, viz., 5-hydroxytryptophan, serotonin, 5-hydroxytryptophol and 5-hydroxyindoleacetic acid, in addition to the internal standard isoproterenol (IP). Their ion-pairing compound was octylsulphate, identified as one of the main variables affecting the retention. Not unexpectedly, an increase in the concentration of the octylsulphate counter ion increased the retention of protonated amines (NE, E, NM, M, DA, IP and serotonin) but had only a small or no effect on neutral substances, whereas acids were influenced slightly in the opposite direction. The retention of the protonated amines decreased on addition of diethylamine, a protonated amine.

Fig. 7. Chromatogram of 15 pmol of standards; 20 μl were injected. The mobile phase consisted of a 0.1 mol/l citrate buffer containing 0.3 mmol/l Na_2EDTA, 0.293 mmol/l octylsulphate and acetonitrile-water (6.3:93.7, v/v) at a pH of 2.35. The guard cell was set at +1.20 V, the first detector cell at +0.20 V and the second detector cell (coupled to the integrator) at +0.80 V. The flow-rate was 1.2 ml/min and the temperature was kept at 25°C. For abbreviations, see Table 4. (From ref. 195.)

4.5.3. Detection

4.5.3.1. UV detection

Biological media contain a large number of UV-absorbing compounds, including catecholamines and their metabolites. Only in urine are the concentrations high enough for UV detection to be applied to the eluate from HPLC. Thus Krstulović et al. [196] obtained the HPLC profile with UV detection (285 nm) of the ethyl acetate extract of urine and found that both VMA and free and total MHPG could be measured adequately in patients with catecholamine-secreting tumours. However, spectrophotometric detection does not provide the sensitivity needed for the analysis of trace amounts of free MHPG [197].

4.5.3.2. Fluorimetric detection

Native fluorescence methods. Catecholamines possess native fluorescence arising from the presence of a catechol group. For measurement of this native fluorescence, an excitation wavelength of 285 nm and an emission wavelength of 325–340 nm are usually employed. This was utilized for the identification of NE and

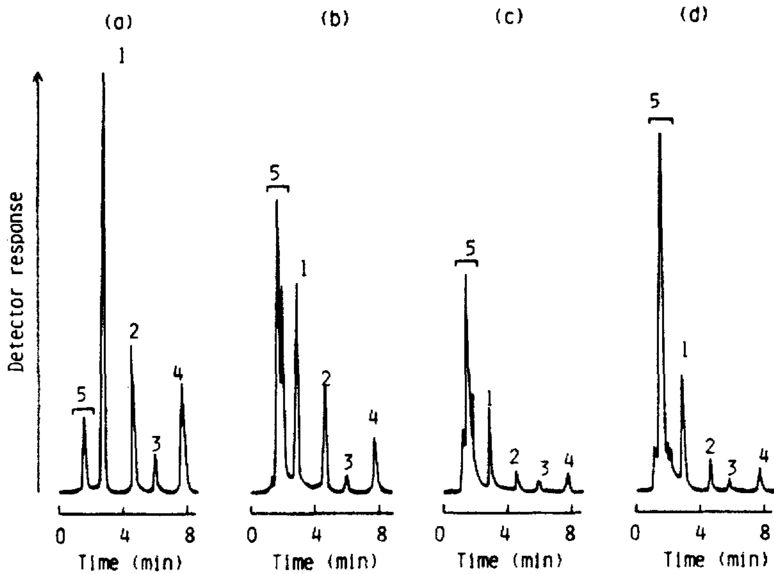

Fig. 8. Chromatograms obtained with a plasma added with IP. Portions (0.5–1.0 ml) of plasma were treated according to the clean-up procedures; (a) chromatography on Toyopak SP, (b) alumina treatment, (c) perchloric acid deproteinization and (d) ultrafiltration through UFO Mini-30 filter, followed by the procedure for the derivatization and HPLC. Peaks (concentrations in pmol/ml in parentheses): 1=NE (1.72); 2=E (0.56); 3=DA (0.21); 4=IP (0.50); 5=unidentified peaks. (From ref. 214.)

DA after HPLC separation from related compounds in brain and heart extracts [198]. Jackman [199] used a one-step cation-exchange purification of urine and detected the native fluorescence of the compounds. The sensitivity of the method was increased when, after ion-exchange purification, the catecholamines were concentrated on alumina [200], and for the determination of methoxycatechols in urine further organic extractions improved the method substantially [201].

Recently, utilizing a column-switching technique with on-line fluorimetric and electrochemical detection, Julien et al. [202] were able to measure simultaneously the parent compounds DOPA, DA, NE and E together with their internal standard IP and the neutral and acidic catecholamine metabolites DOPEG, DOPET, DOPAC and DOMA together with their internal standard 3,4-dihydroxyphenylpropionic acid (DOPPA). Parallel fluorimetric and amperometric detection was utilized to give a measure of peak purity. Although the detection limit of fluorimetry was higher than with ED, except for DOPET the minimum detectable concentrations in urine were far below the concentrations found in human urine.

MHPG and VMA also possess endogenous fluorescence. After clean-up of urine by organic extraction, MHPG is readily determined by HPLC with fluorimetric detection [203]. Utilizing a small reversed-phase column for urine purification followed by ion-pair HPLC, Anderson et al. [204] obtained an excellent separation of VMA from interfering substances in urine. Using both fluorescence

and electrochemical detection, peak purity and possible interferences were evaluated. The authors stated that either fluorimetric or electrochemical detection (or both) may be used, but fewer late-eluting peaks and fewer practical problems were encountered with the fluorimetric method. ED gave a lower detection limit than fluorimetry (50 vs. 400 pg), but the limit with fluorimetry was more than sufficient for the determination of VMA in urine. In other recent studies commercial C_{18} cartridges [205] and ion-exchange resin columns [206] were utilized for sample preparation before HPLC with fluorimetric detection.

Hydroxyindole method. In the hydroxyindole method catecholamines are oxidized and then rearranged to give fluorescent trihydroxyindoles (from E and NE) or a dihydroxyindole (from DA). Usually the oxidation is performed with potassium hexacyanoferrate(III). The application of this procedure to post-column detection after HPLC was reviewed by Imai [207], and more recently the application of automated HPLC procedures was reviewed by Mori [166]. The latter paper contains detailed descriptions of equipment and performance. Applications are surveyed for human plasma, urine and brain extracts with good separation and quantification. The hydroxyindole method seems more selective than ED for NE and E.

Fluorescamine. Pre-column labelling with fluorescamine followed by HPLC and fluorimetric detection is a sensitive method for the determination of DA and NE [208,209] but not for E because fluorescamine does not react with E. 3MT and NM were also determined.

o-*Phthalaldehyde.* HPLC of catecholamines followed by post-column reaction with *o*-phthalaldehyde has been tried [210]. Although the method was sensitive for the detection of DA and NE, E could not be quantified for the same reason as with fluorescamine.

Ethylenediamine condensation. The condensation reaction between catecholamines and EDA has also been tried as a post-column reaction [211,212]. Catecholamines were separated on a reversed-phase column, reacted with EDA at 70°C and determined fluorimetrically. Separation and detection of DOPA, DA, NE and E were obtained with a detection limit of 20–40 pg.

1,2-Diphenylethylenediamine. Nohta and co-workers [213,214] investigated the use of 1,2-diphenylethylenediamine as a derivatization reagent for catecholamines. Of the water-miscible organic solvents tested for acceleration of the derivatization reaction, acetonitrile gave the best results. The maximum peak height was obtained with about 50% acetonitrile in the reaction mixture [213], and the reaction products with DA, NE, E, IP and N-methyldopamine (N-MDA) were stable after reaction with 1,2-diphenylethylenediamine for 30 min. The catecholamine derivatives were separated within 8 min on a reversed-phase column, and their detection limit was ca. 2 fmol in a 100-μl injection volume [214]. The authors investigated different clean-up procedures for plasma – cation-exchange chromatography (Toyapak SP), alumina treatment, deproteinization with perchloric acid and ultrafiltration – and found the cation-exchange procedure most satisfactory (Fig. 8). The method was suitable for the determination of DA, NE and E in plasma [214] and urine [215] and was also adapted for determi-

nation of the free and total catecholamines in human erythrocytes, platelets and plasma [216] and for DOPA in plasma and urine [217].

Glycylglycine post-column reaction. The catecholamines DA, NE and E were separated on a cross-linked vinyl alcohol copolymer with carboxymethyl groups (Asahipak ES-502C) [181]. By post-column reaction at 90°C with a reagent stream containing glycylglycine, boric acid, zinc sulphate, tartaric acid and hexacyanoferrate(III), fluorescent derivatives were obtained from the catecholamines. The precision of the method as applied to urine was satisfactory, and the analytical recovery was quantitative.

Periodate post-column reaction. Flood and co-workers [218,219] reported the specific determination of metanephrines and VMA by coupling HPLC with Pisano's method, using periodate to convert the analytes to vanillin. This reaction for oxidative cleavage of β-glycols was recently applied to the post-column detection of MHPG after HPLC of human urine [220]. The detection limit was 0.08 mg/l for urine, the precision of the method was 2–9% (within-assay coefficient of variation) and the analytical recovery was 72%. The authors claimed that the method was superior to electrochemical and fluorescence detection methods.

4.5.3.3. Electrochemical detection

Liquid chromatography with ED, as pioneered by Adams [221] and Kissinger et al. [222], has become popular in the last decade because it is straightforward and offers good selectivity and sensitivity [162–164,223].

Two types of electrodes have been used. The first to be introduced, and the most popular, is the thin-layer flow cell. The column effluent passes in a thin layer over the surface of a carbon paste of glassy carbon electrode and a small proportion of the oxidizable species is oxidized at the electrode surface. With time the electrode surface becomes coated, and the packing must be replaced or the electrode surface polished or cleaned [224]. An advantage of this arrangement is that it is inexpensive.

The second type of electrode is a flow-through cell where the column effluent passes through a glassy carbon matrix. Because of the large surface area of the cell, all species oxidizable at the set potential are oxidized quantitatively. Therefore, these detectors are often named coulometric. The sensitivity is excellent, but the signal-to-noise ratio may not be improved over the thin-layer arrangement. These electrodes are more expensive and cannot be opened or polished. They may be regenerated by flushing with 6 *M* nitric acid.

An advantage of coulometric cells is that they can be placed in series, so that electrodes early in the series can act on substances eluting from the column and derivatize the substances for detection at another electrode later in the series. For instance, in the measurement of catechols, one can exploit the fact that these biochemicals are reversibly oxidizable, whereas other constituents contributing to the baseline signal may be irreversibly oxidizable. By using the first cell in the series to oxidize and the last cell in the series to reduce and by recording from the last cell, the chromatographic results are improved with respect to both the signal-to-noise ratio and the width of the solvent front.

The series flow-through electrode configuration also allows attenuation of signals from easily oxidized substances which contribute to the background noise,

by including a low oxidation potential at early electodes and a higher oxidation potential at the recording electrode. This can be particularly helpful for assays of metabolites that require high oxidation potentials owing to the lack of adjacent hydroxy groups on the benzene ring. The utility of a dual electrode was illustrated by Minegishi and Ishizaki [225], who determined DOPAC, HVA and 5-hydroxyindoleacetic acid in serum. HVA needed to be detected at an electrode potential of 0.75 V. However, at this potential tryptophan was also oxidized and interfered with the DOPAC peak. By use of a dual electrode, DOPAC (and 5-hydroxyindoleacetic acid) could be determined at 0.6 V while HVA was determined at 0.75 V in the same chromatographic run.

Another example is the determination of MHPG in plasma and cerebrospinal fluid [226]. The potential of the conditioning cell was set at +0.40 V to oxidize most of the MHPG present in the sample. By setting the potential of the first detector of the analytical cell at −0.05 V, the response of the second detector was decreased by only 10%, but this eliminated many interfering substances. The potential of the second detector was set at −0.45 V to obtain the highest signal from the MHPG reduction. This resulted in good baseline stability. HPLC with coulometric detection can be used for all types of biological samples containing different amounts of catecholamines, ranging from a few picograms to tens of nanograms, and the selectivity and resolution are high [227].

4.5.4. Extraction and clean-up procedures

For the determination of catecholamines in tissue and biological fluids a vast number of procedures have been suggested and tried. For the best results one has to consider the chemical nature of the analyte to be determined, the biological matrix and its content of possibly interfering compounds. Further, the selectivities of the chromatography and the detection system should be considered. Therefore, the different references quoted below should be considered as examples of clean-up procedures. Single-step procedures are often used, but it should be borne in mind that often two-step or multi-step clean-up has to be used.

4.5.4.1. Organic solvents and protein precipitation. Although carefully collected cerebrospinal fluid can be injected directly on to a liquid chromatographic column, direct injections of samples can produce poor chromatographic results and decrease the column and detector life. For plasma or urine, sample clean-up procedures are essential. Extraction of catecholamines and their metabolites with organic solvents has been widely used, particularly for plasma and urine [228–232]. Most commonly used is diethyl ether or ethyl acetate extraction with back-extraction into the aqueous phase using perchloric or hydrochloric acid. These procedures eliminate proteins and make the sample ready for further analytical steps. If only protein precipitation is required the sample is usually mixed with perchloric acid or trichloroacetic acid [233,234]. Such procedures do not bring about any selective clean-up or enrichment of the compounds of interest as in other procedures [235].

4.5.4.2. Alumina. The extraction of catecholic compounds with activated alumina was developed by Anton and Sayre [75], and has subsequently been used in a number of studies. The procedure gives clean extracts for DA, NE and E from

serum and tissue homogenates [236–238]. Samples for the assay of catechols such as DOPA, DOPEG and DOPAC in addition to the catecholamines can also be purified by adsorption on alumina [239].

The alumina extraction can be by column or batch. The latter procedure has consistently proved successful [239], but others advocate the column procedure [240].

The sample preparation scheme includes increasing the pH of the alumina to more than 8.0 and vigorous shaking of the sample with the alumina, resulting in adsorption of the catechols by attraction of the hydroxy groups of the catechol nucleus. The alumina can then be washed with water and the catechols are eluted with acid. Gentle rocker-type mixing is inadequate. Slow, completely circular rotation of centripetally oriented sample tubes may be acceptable, but one must take care to ensure adequate tumbling of the alumina and the absence of large air bubbles preventing mixing when the tube is inverted. As catechols are unstable at high pH, the sample should not stay in the Tris–EDTA buffer for long periods.

4.5.4.3. Cation exchanger. A number of clean-up procedures utilizing a cation exchanger have been described [241–243]. With Bio-Rex 70, one of the most commonly used cation-exchange resins in this context, Odink et al. [243] recently showed that all amines tested [NM, M, 3MT, NE, E, α-methylnorepinephrine (αMNE), 3,4-dihydroxybenzylamine (DHBA), DA, N-MDA, SAL, α-methyldopamine (αMDA), IP and tetrahydropapaveroline (THP)] were retained by this resin at pH 6.5. Except for NM, M and 3MT, which do not contain a vicinal hydroxy group, all of them were then readily eluted with boric acid. Alcoholic (DOPEG, MHPG) and acidic metabolites [VLA, vanilpyruvic acid (VPA), HVA, VMA, DOPAC and DOMA] were not retained on the column. This was also the case with the amino acids [3MTyr, DOPA and α-methyl-3,4-dihydroxyphenylalanine (αMDOPA)], presumably because of their amphoteric properties. The properties of the ion exchanger and the specific elution with boric acid of compounds that contain a vicinal hydroxy group makes this clean-up procedure very suitable for complex biological samples such as urine.

For the clean-up of catecholic amino acids from serum and urine, strong ion exchangers have to be used [217,244]. One obvious drawback of this technique is that all other catecholic compounds with weaker affinity are co-eluted. For the catecholic diamino acid 5SCD this method therefore can only be used in a combined clean-up procedure [244].

4.5.4.4. Boric acid affinity chromatography. More than ten years ago an affinity gel was developed where boric acid was covalently bound to a resin matrix [245]. Such gels have become popular and have been used in a number of studies [244,246–250]. A few kinds of different affinity gels have become commercially available (Affigel 601 = immobilized boric acid; Amicon 30 = immobilized phenylboric acid) and they differ in the spacing of the boric acid from the matrix. These gels retain catecholic compounds presumably by forming a boric acid complex with the vicinal hydroxy groups at increased pH [243]. Elution can then be performed by decreasing the pH. In fact, differential elution can be performed by a pH step gradient, as the affinity of different catecholic compounds depends on pH [250]. A number of successful clean-up procedures with immobilized boric

TABLE 5

RETENTION VOLUMES OF THE CATECHOLAMINES AND THE THREE CLASSES OF THEIR DERIVATIVES ON ALUMINA AND DIHYDROXYBORYLSILICA AS A FUNCTION OF pH

Column dimensions: 30 mm × 2.1 mm I.D. Mobile phase: 0.005 *M* phosphate with various pH values. Flow-rate: 1.3 ml/min. Injection of 1-2 μl of a solution of the compounds (50-5000 μg/ml) in 0.05 *M* phosphate buffer (pH 2.5). Detection: fluorimetric, SFM 23 LC. From ref. 255.

Compound	Retention volume (ml)											
	Alumina					Dihydroxyborylsilica						
	pH 2.0	5.0	6.0	7.0	8.0	pH 2.0	3.5	5.0	6.0	7.0	7.5	8.0
A	0.22	0.33	2.2	32	430	0.16	0.20	0.46	1.2	7.6	25	34
NA	0.20	0.35	2.6	40	700	0.16	0.20	0.39	1.0	5.2	16	24
DA	0.20	0.31	1.8	20	330	0.16	0.20	0.40	1.1	6.7	21	33
α-Me-DA	0.20	0.29	1.5	20	350	0.18	0.20	0.46	1.2	7.7	21	33
EPI	0.20	0.29	1.6	14	270	0.18	0.22	0.52	1.7	12	40	52
DHBA	0.20	0.31	2.9	45	520	0.16	0.20	0.44	1.3	9.8	32	43
NMN	0.20	0.25	0.43	0.64	0.79	0.18	0.17	0.23	0.54	0.76	0.82	0.92
MN	0.20	0.21	0.43	0.51	0.61	0.16	0.20	0.25	0.67	0.96	1.3	1.6
3-MT	0.20	0.25	0.38	0.55	0.69	0.18	0.20	0.27	0.71	1.1	1.4	1.7
DOPA	0.20	0.21	0.22	0.73	12	0.18	0.20	0.23	0.43	0.53	1.1	1.5
α-Me-DOPA	0.20	0.23	0.27	0.51	10	0.18	0.20	0.23	0.40	0.51	0.89	1.3
DOPAC	0.20	0.21	0.20	0.20	0.26	0.30	0.46	0.79	1.7	–	–	2.3
DOPEG	0.20	0.21	0.25	0.35	3.0	0.22	0.22	0.29	0.55	0.75	3.1	3.5
DOPET	0.20	0.21	0.22	0.40	2.5	0.26	0.26	0.41	0.70	0.73	5.1	4.9
HVA	0.20	0.21	0.18	0.18	0.18	0.30	0.39	0.37	0.30	0.25	–	0.24
MOPEG	0.20	0.21	0.20	0.20	0.18	0.22	0.20	0.21	0.26	0.26	0.24	0.29

acid have been described for catecholamines in brain tissue, serum and urine. Most of these procedures were combined techniques. One draw-back of using immobilized boric acid seems to be the variation of the properties of different batches of the gels [250].

4.5.5. *Automated methods*

With some catecholic compounds the boronic affinity principle has been applied to the use of a boronic affinity HPLC column [251]. The biological sample is injected into the system without prior purification and the compound of interest is adsorbed on top of the column. By column switching the compound is eluted from the first column and chromatographed. Such methods have been published for the determination of, e.g., DA, NE and E [252,253] and DA, NE, E and DOPAC [254]. An excellent elaboration of the prerequisites for on-line sample pretreatment on small alumina or dihydroxyborylsilica columns (Table 5) was carried out by De Jong et al. [255], which resulted in an automated method for the determination of E, NE and DA in urine. Fig. 9A shows an example of the

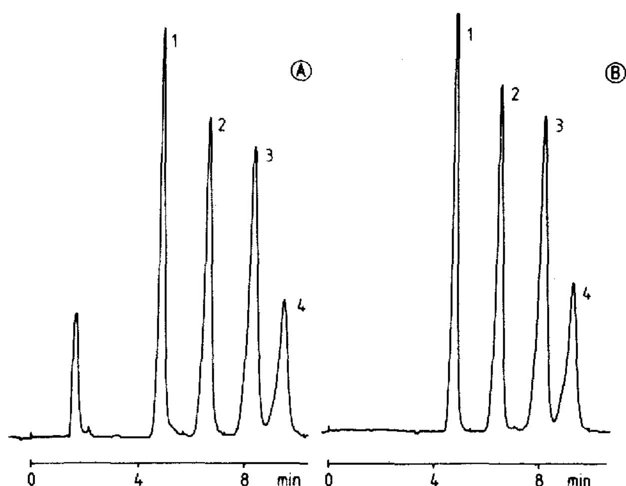

Fig. 9. Comparison between separation of the catecholamines after on-line pre-concentration on dihydroxyborylsilica (A) and direct separation of the catecholamines (B). Separation: system I. Detection: fluorimetric, LS-4. (A) Injection via the pre-column (30 mm×2.1 mm I.D.) of 1 ml of a mixture (5 ng/ml per component) of (1) NE, (2) DHBA, (3) E and (4) DA in mobile phase 1 [0.01 *M* phosphate, 0.01% (w/v) EDTA, pH 8.1]; $w_1 = 1$ ml/min. Mobile phase 2: system I. $w_2 = 1.3$ ml/min. $t_I = 2$ min (indicated in the figure as $t = 0$ min); $t_{II} = 5$ min; elution from the pre-column by backflushing. (B) Direct injection on the analytical column of 50 μl of a mixture containing 100 ng/ml of each component. Mobile phase: system I. Flow-rate 1.3 ml/min. (From ref. 255.)

separation of the catecholamines by means of ion-exchange chromatography after on-line pre-concentration of the column. Compared with direct injection of the same amount (50 ng) of each compound in a small volume (Fig. 9B), there was no significant extra band broadening and peak-height decrease by about 10%.

By similar techniques the acid metabolites VMA and HVA are isolated by anion exchange on a first column and chromatographed on a second or third column [256,257]. The cascade principle was used by Honegger et al. [258] with an analysis time of 25 min. Benedict [259] used C_{18} and C_8 microbore reversed-phase columns in series for the determination of DOPA, DA, NE, E and DOPAC in plasma and urine after clean-up.

4.6. Capillary zone electrophoresis

Capillary zone electrophoresis (CZE) was introduced by Mikkers et al. [260] and Jorgenson and Lukacs [261–263] as a highly efficient separation technique. With proteins, up to 10^6 theoretical plates have been obtained. Although small molecules with higher diffusion coefficients give a smaller number of theoretical plates and a number of obstacles have to be overcome, the technique is of great interest also for the separation of small molecules. Recently Wallingford and Ewing [264] reported the construction of an electrochemical detector to be used with CZE. They obtained a separation efficiency of the order of 180 000 theoretical plates for the separation of 1,2-benzenediol (catechol) from catecholamines, and in an extended study with a 26 μm I.D. column they obtained separation efficien-

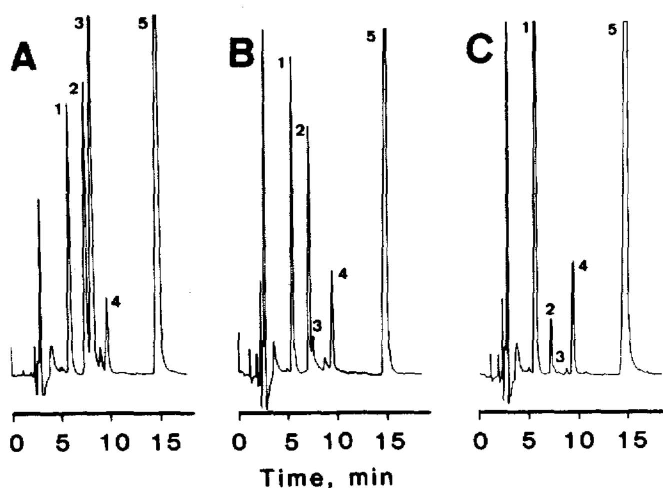

Fig. 10. Chromatograms of extracts from urine samples obtained from a patient with an epinephrine-secreting pheochromocytoma. Samples were collected before tumour resection (A), during the day of the operation (B, collection over 19 h), and one day after surgery (C). Catecholamine concentrations in A, B and C were, respectively: for norepinephrine (peak 1), 0.53, 0.41 and 1.15 μM; for epinephrine (peak 2), 0.69, 0.43 and 0.09 μM; for N-methylepinephrine (peak 3), 1.20, 0.04 and 0.005 μM; for dopamine (peak 5), 2.44, 1.14 and 2.61 μM. Peak 4 is the internal standard dihydroxybenzylamine (DHBA). (From ref. 267.)

cies of more than 300 000 plates for DA, NE, E and catechol [265]. The detection limits for the mentioned catechols were 0.2–0.4 fmol. Although problems with band broadening and other technical difficulties remain to be solved, the technique opens up the possibility of performing ultra-trace sample analyses, especially with samples taken from biological microenvironments, e.g., single cells.

4.7. Analysis of selected catecholic compounds and metabolites

The catecholamine metabolites discussed hitherto are well known and their analysis is of broad interest for those involved in catecholamine research. Other metabolites are discussed more seldom, such as N-methylated metabolites, or are of more selected interest, such as SAL. DOPA and DOPA thioethers are catecholic amino acids, and therefore their chemical and chromatographic properties differ from those of the true catecholamines. With the therapeutic implications in Parkinson's disease, the interest in L-DOPA also differs from that in the catecholamines, and with the DOPA thioethers the field of interest is pigment biochemistry, 5SCD serving as a marker for metastatic melanoma.

4.7.1. N-Methylepinephrine and N-methylmetanephrine

Utilizing HPLC–ED [266] Gerlo et al. [267] detected and quantified N-methylepinephrine (N-ME) in the urine of a patient with E-secreting pheochromocytoma (Fig. 10). The compound was identified by GC–MS. In reversed-phase HPLC N-ME eluted after NE and E but before DHBA and DA. The order of elution was not changed when different mobile phase concentrations of octylsul-

phate were used. The level of the compound in plasma and urine of the patient was increased and similarly the authors found increased urinary excretion in three out of four other pheochromocytoma patients. The urinary excretion was detectable in healthy subjects, and in hypertensive patients the excretion was found to be <70 nmol per 24 h.

When it was shown earlier that administration of N-ME to rats increased the excretion of N-methylmetanephrine (N-MM), the metabolic pathway E→N-ME→N-MM was suggested [268]. However, the intermediate metabolite N-ME was not identified until recently, possibly because in trihydroxyindole methods N-ME would not be oxidized as the reaction requires a primary or secondary function. Also in the radioenzymatic (COMT) method for catecholamines the compound may be undetected or add to the observed concentration of one of the catecholamines. In metanephrine assays based on periodate oxidation to vanillin [269], N-MM would be oxidized and contribute to the total metanephrine results. These possibilities illustrate the drawbacks of the mentioned methods, and the identification by HPLC illustrates their potential. Likewise, the possibility of unidentified peaks in normal and pathological urine samples is a potential pitfall in HPLC procedures.

4.7.2. Salsolinol

DA and acetaldehyde are known to undergo a direct condensation to form 1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline (SAL). This compound has been measured by radioenzymatic methods [106,107], GC [270–272], GC–MS [273,274] and HPLC–ED [243,275]. The compound has been shown to occur after ethanol intake, and it has been questioned whether the compound can be formed in vivo in the absence of drug or alcohol intake. Its presence in different kinds of foods and beverages is well established [274] and the compound is found in the brain [273] and urine [243]. From a methodological standpoint it is of interest that when low SAL concentrations have been found in alcohol-naive animals, a lack of assay specificity and possible artifactual formation have been suggested to be responsible [274]. This question was solved using GC–MS [274] by addition of $[^2H_4]$DA to the sample. Under the conditions adopted, no detectable $[^2H_4]$SAL could be found in the samples.

4.7.3. Dihydroxyphenylalanine

The methods available for the determination of DOPA in plasma and urine were recently briefly summarized by Lee et al. [217]. When L-DOPA was introduced for the treatment of Parkinson's disease a number of methods for its determination in biological material were developed. Many of these methods utilized similar analytical principles as for catecholamines. Thus colorimetric [275], fluorimetric [276–278], gas chromatographic [279,280], radioenzymatic [281–284] and liquid chromatographic [285,286] methods were developed. With HPLC both fluorescence and electrochemical detection methods were used. These methods permit the monitoring of therapeutic L-DOPA, but endogenous L-DOPA in plasma can be determined only by radioenzymatic and HPLC methods with electrochemical or fluorimetric detection [217]. Depending on the clean-up proce-

dure, L-DOPA is often washed away during clean-up of the biological sample. However, when extracts from brain [255] are injected directly on to the HPLC column, L-DOPA is measured quantitatively, and also in methods with clean-up procedures aiming at preserving the compound in the final extract.

4.7.4. Thioethers with DOPA

Early methods for the determination of catechol thioethers were based on oxidation of the compounds to yield a fluorophore that can be quantified [287]. In further studies MS methods were used to identify the different compounds (2SCD, 5SCD, 6SCD and 2,5SDCD) obtained by synthesis in vitro [288], and four different O-methylated cysteinyldopa compounds were found in the urine of a melanoma patient by GC–MS [289,290].

As 5SCD is a major intermediate pheomelanin metabolite suggested as a melanoma marker [34], a number of HPLC methods have been developed [236,237,244,291–294]. Synthetic 5-S-D-cysteinyl-L-DOPA, a diastereomer of 5-S-L-cysteinyl-L-DOPA [295], proved to be a practical internal standard when clean-up of urine [291] and serum [293] was performed with a phenylboronate affinity gel. Using the column-switching technique, 5SCD can be determined in urine by direct injection [294]. 5-S-Glutathione-L-DOPA and 5-S-L-cysteinylglycine-L-DOPA can also be analysed by HPLC [296].

4.8. Comparison of different analytical techniques

4.8.1. General aspects

Prior to the introduction of HPLC–ED the most widely used techniques for measuring catechols were fluorimetry, radioenzymatic assays and GC. As is evident from the large number of papers that have been published, HPLC–ED has now become the method of choice when catecholamines and their metabolites are to be determined. Nevertheless, it is of great value to make clear the advantages and disadvantages of different methods. Some of these aspects are briefly summarized below.

Radioenzymatic procedures, such as those of Henry et al. [84] and Peuler and Johnson [100], were the first to be sensitive enough to measure in vivo levels of catecholamines in plasma samples from laboratory animals and were also widely used for human plasma analyses. These methods are expensive, time-consuming and involve radionuclides. Further, several catecholamine metabolites cannot be measured by these procedures. However, the methods have in many instances served as comparison methods in the validation of other technqiues [180], and their use has increased our knowledge of the physiology and metabolism of catecholamines. The methods have been used, but not extensively, for the diagnosis and follow-up of tumours of neural origin.

The development of radioimmune methods has proved to be difficult, but promising papers have appeared. The potential of radioimmunoassay is for screening and, with other immunometric methods, for tissue localization of catecholaminergic structures and receptors.

GC–MS procedures are time-consuming and cumbersome and require expen-

sive equipment. The procedures have been improved greatly in recent years, and the sensitivity of detection nowadays is high. An outstanding quality of GC–MS is the ability to identify unknown compounds, and with the use of deuterium-labelled internal standards the accuracy of measured levels is high.

HPLC offers the advantages of reasonable simplicity, a high degree of versatility and adequate sensitivity and specificity. After the initial investment, the costs are modest. All the catecholamines and their metabolites can potentially be measured. Recent improvements in sensitivity have allowed measurements of catechols in small plasma volumes, as would be required for the study of laboratory animals. Nowadays the chromatograms usually show distinct narrow peaks and good separations from interfering compounds. HPLC, in particular in the reversed-phase mode with ED, is very versatile in the sense that the separation can be influenced in many ways, such as by varying the pH of the mobile phase or by adding ion-pairing reagents and organic modifiers to the mobile phase. With different detectors some degree of selectivity can also be obtained. These advantages have led to the increasing use of HPLC techniques. However, it should be borne in mind that the versatility in the choice of different modes of operation can also lead to unwanted effects. Thus, manipulation of the retentions of the compounds of interest can also change the retentions of interfering compounds. Care should be taken regarding the specificity of published methods when these are applied in different clinical situations and research projects. Some examples are given below.

4.8.2. Some examples

A plethora of different HPLC–ED methods have been described. This may indicate that this technique is a most promising procedure, but also that inherent methodological difficulties prevail. Recently Seegal et al. [235] described the application of HPLC to extracts from brain, cerebrospinal fluid, urine and plasma. Factors considered in order to verify the identity of the chromatographic peaks were the retention time, change in retention time with alteration of mobile phase pH, ion-pairing reagent and methanol concentrations, voltammetry and the in vivo use of pharmacological agents that modify endogenous metabolite concentrations. All these procedures are indirect, and do not prove the identity of a compound, but could be applied to investigate a possible interfering compound in the chromatogram. The authors used their described principles to identify interferences with the HVA peak and then modified the pH of the mobile phase to obtain a good resolution of the HVA peak even when the properties of the column changed with further use.

In general there is agreement between results obtained by different analytical techniques, but systematic differences have been found between radioenzymatic and HPLC methods, the former method giving higher results for NE [297,298]. It was concluded [298] that the difference depends on the source of the plasma, and that it is not due to differences in recovery, loss of linearity or contaminants, but might be due to a variable level of plasma constituents recognized as NE in the radioenzymatic assay, but not in the HPLC method. To consider an example: N-ME which occurs in urine at about the same concentration as E [267] could

in the radioenzymatic method be converted by the catechol-O-methyltransferase to N-MM, the 3-O-methylated catabolite of N-ME. If this radioactive compound then migrates in TLC to the same position as NM it would give rise to a falsely elevated value for NE.

With plasma analysis by HPLC–ED occasional unknown peaks are found to co-chromatograph with the DA peak. This phenomenon has been described by many workers [298], and is a serious drawback. However, in comparison with the radioenzymatic method, there seem to be only very small differences in the results [298].

The measurement of E by both radioenzymatic methods and HPLC–ED is subject to greater analytical variability than that of E, which makes systematic differences more difficult to detect. However, recent studies did not show any great differences in the results for E, although there could be a risk that interfering peaks from, e.g., dihydrocaffeic acid [300], could give an increased value when HPLC–ED is used.

Contaminating peaks are especially common in plasma from subjects who have not fasted but instead have ingested caffeinated of even decaffeinated coffee. The reason is that caffeic acid, a catechol, exists at a high concentration in coffee beans and other plant matter. Bacteria in the gut convert this to dihydrocaffeic acid, which can co-chromatograph with E, DOPAC or DA [300].

Another example is the analysis of MHPG in plasma [301], where an unidentified peak eluted close to the MHPG peak. The chromatography was run at ambient temperature, and the unidentified peak was more sensitive to changes in temperature than MHPG. When the ambient temperature was above 24–25 °C the unidentified peak eluted rapidly enough to interfere with the MHPG peak.

4.9. Practical aspects of catecholamine analysis

4.9.1. Sampling conditions

Catecholamine measurements, especially in plasma, are subject to extensive inter-individual variability. Some of these include body position, time of day, medication, disease state and mental status. The importance of sampling site is best illustrated by the widespread use of the antecubital venous level of NE to indicate the overall sympathetic tone. This sampling site is by far the most convenient for clinical testing. Several factors must be considered before drawing inferences about sympathetic nerve activity based on antecubital venous NE. First, the sympathetic innervation of the arm contributes substantially to NE in the venous drainage [302]. As a result, in situations where sympathetic neural responses are heterogeneous among the several innervated beds, as may occur in defence reactions [303], changes in antecubital venous NE may not indicate changes in sympathetic activity elsewhere. Second, the state of local haemodynamics determines the proportionate regional removal of NE. In the arm, the percent removal of NE varies inversely with forearm blood flow over a range from 10% to about 90% [58,302].

Even if arterial rather than regional venous blood were sampled, however, changes in sympathetically mediated NE release would be underestimated in sit-

uations where the cardiac output was altered. In the setting of a high cardiac output, the enhanced delivery of catecholamines to excretion sites means that in order to maintain a given arterial level of NE, the rate of spillover of NE into the bloodstream must be increased. During mental challenge, haemodynamic responses have been closely linked to changes in total body spillover of NE, weakly linked to changes in arterial NE, and completely unrelated to changes in antecubital venous NE [58].

The sampling site influences markedly the range of expected concentrations of catecholamines. Levels of DA in human urine average about ten times that of NE, whereas levels of unconjugated DA in human plasma average about one tenth of that of NE and often are below the detection limit. In human cerebrospinal fluid, levels of E are rarely above the detection limit.

The stability of the samples obtained also depends on the sampling site. Aqueous solutions of catecholamines at physiological pH are not stable, oxidizing to a brownish colour. In contrast, catecholamines in plasma can be stable for several hours at room temepratures [178,304]. Catecholamines in cerebrospinal fluid disappear rapidly and so, in order to obtain valid results, cerebrospinal fluid should be collected directly into or else transferred rapidly into sample tubes in dry-ice.

4.9.2. Additives, degradation and storage

As catecholamines are liable to oxidize, antioxidants such as sodium metabisulphite or EDTA are commonly added to the samples before assay. We have been unimpressed by the value of these additives. Our blood samples are routinely collected into heparin or EDTA, the plasma is separated by refrigerated centrifugation, and the samples are stored at $-70\,^{\circ}C$ until assayed. Concentrations of catechols in aliquots of standard plasma appear to be stable for at least several weeks under these conditions. We have not observed differences in catechol concentrations among heparinized plasma, EDTA-treated plasma or serum. Anticoagulation using acid–citrate–dextrose, in contrast, has resulted in poor chromatographic recordings. Haemolysis does not affect HPLC–ED measurements of levels of NE or E [305]. We have assayed urine samples collected without additives or collected into 6 *M* hydrochloric acid and the results seem most consistent in acidified urine. Samples of cerebrospinal fluid require rapid freezing soon after collection. The catechol content remains stable for a few weeks when stored at $-70\,^{\circ}C$ until assayed.

5. REFERENCE VALUES FOR LEVELS OF CATECHOLAMINES AND THEIR METABOLITES

Tables 6–8 summarize reported levels obtained using HPLC–ED for unconjugated, non-protein-bound catechols and catecholamine metabolites in human plasma, cerebrospinal fluid and urine. The columns of mean values represent the averages of the reported normal values in the studies cited, and the columns of ranges show the ranges of mean values among the studies cited.

Results obtained using other assay methods, such as radioenzymatic or GC–MS procedures, have generally agreed with those obtained using HPLC [1], but

TABLE 6

LEVELS OF DOPA, CATECHOLAMINES AND CATECHOLAMINE METABOLITES IN HUMAN PLASMA

Biochemical	Mean	Range	Ref.
Catecholamine precursor			
DOPA	2540 pg/ml	2080–3386 pg/ml	6, 239, 259, 306
Catecholamines			
Norepinephrine	257 pg/ml	187–372 pg/ml	18, 168, 239, 259, 306–309
Epinephrine	35 pg/ml	28–42 pg/ml	58, 168, 306, 307, 309
Dopamine	35 pg/ml	0–66 pg/ml	168, 259, 306
MAO metabolites			
DHPG	990 pg/ml	789–1360 pg/ml	18, 239, 310
DOPAC	1.4 ng/ml	0.7–2.0 ng/ml	225, 239, 259, 306
DOMA	180 pg/ml	0–360 pg/ml	17, 310
COMT metabolites			
NM	458 pg/ml	–	311
M	433 pg/ml	–	311
O-Methyl-DA	413 pg/ml	–	311
O-Methyl-DOPA	17.8 ng/ml	–	311
MAO+COMT metabolites			
MHPG	5.2 ng/ml	3.2–14.5 ng/ml	312–318
HVA	16.8 ng/ml	9.8–31.8 ng/ml	225, 312, 319–321
VMA	10.0 ng/ml	9.0–11.8 ng/ml	312, 322, 323

a few exceptions should be noted. Values for plasma DOPAC using HPLC can be underestimated, because the recovery of DOPAC from alumina can be less than that of the catecholamines commonly used as internal standards, and corrections for the recoveries of external standards may be necessary. Reported values for plasma DOMA using HPLC have been variable, at least partly because the chromatographic separation of DOMA from the solvent front can be difficult. Values obtained using radioenzymatic procedures can be overestimated if care is not taken to exclude co-chromatography with other substances during the thin-layer separation of O-methylated metabolites.

6. CONCLUSION

The great number of analytical methods developed during the years for determination of catecholamines and their metabolites include a number of chromatographic procedures. Particularly GC–MS and HPLC methods have contributed greatly during recent years to give accurate and precise determination of these compounds in biological samples.

TABLE 7

LEVELS OF DOPA, CATECHOLAMINES AND CATECHOLAMINE METABOLITES IN HUMAN CEREBROSPINAL FLUID

Biochemical	Mean	Range	Ref.
Catecholamine precursor			
DOPA			
Catecholamines			
Norepinephrine	100 pg/ml	100 pg/ml	324 (schizophrenics)
Epinephrine			
Dopamine			
MAO metabolites			
DHPG			
DOPAC	4 ng/ml	0.4–11 ng/ml	324–326
COMT metabolites			
NM	164 pg/ml	–	327
M			
O-Methyl-DA			
O-Methyl-DOPA	Not detected		326
MAO + COMT metabolites			
MHPG	13.7 ng/ml	0.9–36 ng/ml	324–326, 328, 329
HVA	34 ng/ml	10–47 ng/ml	324–326, 328, 329
VMA		0.3–0.7 ng/ml	325

TABLE 8

LEVELS OF DOPA, CATECHOLAMINES AND CATECHOLAMINE METABOLITES IN HUMAN URINE

Biochemical	Mean	Range	Ref.
Catecholamine precursor			
DOPA	30 μg	–	202
Catecholamines			
Norepinephrine	31 μg	25–37 μg	202, 330–332
Epinephrine	8 μg	6–10 μg	202, 330–332
Dopamine	201 μg	177–298 μg	202, 330–332
MAO metabolites			
DHPG	60 μg	–	202
DOPAC	1004 μg	–	202
COMT metabolites			
NM	184 μg	30–284 μg	332–335
M	93 μg	34–174 μg	332–335
O-Methyl-DA	87 μg	–	333, 334
MAO + COMT metabolites			
MHPG	1.3 mg	–	315 (total)
HVA	4.7 mg	3.2–6.2 mg	319, 336
VMA	3.7 mg	2.3–4.9 mg	1, 204, 319, 336, 337

7. SUMMARY

The research on biosynthesis, physiology, pharmacology, regulation and degradation of catecholamines has continously increased for more than 50 years. This is not unexpected because of the fact that catecholamines are involved in so many life processes such as nerve conduction, blood circulation and hormone regulations in health and disease.

This demands that methods for their determination should be improved, and in fact during the years a number of analytical methods have been published.

About 20 years ago radioenzyme techniques with thin-layer chromatographic (TLC) separation of radiolabelled catecholamine derivatives were developed which greatly contributed to our knowledge of physiological concentrations of catecholamines in biological media, particularly in plasma and brain.

Radioimmune methods were successful for analysis of a number of analytes, but for catecholamines radioimmunoassays developed slowly. We believe that the greatest potential for radioimmunochemical methods lies in their ability to localize catecholamines and metabolites at the cellular and subcellular levels.

With the advent of gas chromatographic–mass spectrometric (GC–MS) and high-performance liquid chromatographic (HPLC) procedures analysis of catecholamines improved greatly., The equipment for GC–MS is expensive and requires technical skilfulness, but in experienced hands a lot of new biological data have emerged. An outstanding quality with GC–MS is that the method offers the ability to identify unknown compounds and is relatively free from interferences from extraneous compounds.

In comparison with GC–MS, HPLC is versatile and has gained a widespread use. Applications for research in the catecholamine field are numerous. In general, the sensitivity and specificity are satisfactory with HPLC, but it should be borne in mind that a number of pitfalls can obscure the results. This involves both sample handling, clean-up and chromatographic procedures. At present, HPLC is the most expanding field in chromatographic determination of catecholamines and their metabolites. This is particularly the case for HPLC with electrochemical detection which has revolutionized our analytical potential in this field.

These chromatographic procedures continue to develop. The prerequisites for further improved methods such as capillary zone electrophoresis and combined HPLC–MS are at hand and hopefully will soon come into more general use for analysis of catecholamines in biological samples.

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