CATECHOLAMINE AND 5-HYDROXYTRYPTAMINE METABOLISM IN IMMUNOSYMPATHECTOMIZED ANIMALS AND SOME RELATED STUDIES

by

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ABSTRACT

The 24 hour excretion of the principal catecholamine and 5-hydroxyindole metabolites, plus dopamine and 5-hydroxytryptamine, has been estimated in the urine of immunosympathectomized and normal rats. The urinary output of 4-hydroxy-3-methoxyphenylglycol (HMPG) was significantly reduced in immunosympathectomized rats ($P < 0.001$) whereas, expressed in terms of body weight, the excretion of 4-hydroxy-3-methoxymandelic acid (VMA) was increased ($P < 0.05$) and that of 5-hydroxytryptophol tended to decrease ($P = 0.05$). The excretion of other compounds investigated was unchanged. Over 60% of VMA present in normal rat urine is conjugated as a glucuronide, with less than 20% excreted in the free state.

Urinary catecholamine metabolism was also investigated in adrenalectomized rats, in which HMPG was significantly increased ($P < 0.001$) compared with controls. Daily homovanillic acid output was similarly increased ($P < 0.05$), although to a lesser extent. Hydrocortisone treatment abolished these increases. Metadrenaline was detected in 4 out of 6 urines of adrenalectomized rats, providing further evidence for extra-adrenal production of adrenaline.

Subcellular monoamine oxidase (MAO) activity was measured in tissues of immunosympathectomized, reserpinized

and 6-hydroxydopamine-treated mice, and adrenalectomized rats. After immunosympathectomy, mitochondrial, microsomal and supernatant MAO activity in mouse liver was significantly reduced, whilst in the heart, the decrease was restricted to the microsomal fraction alone. 6-Hydroxydopamine treatment also resulted in significant reduction of heart and liver microsomal MAO activity. Changes in subcellular MAO activity of liver, heart and brain after reserpine were complex and time-dependent. The main effect of adrenalectomy was an increase in the mitochondrial MAO activity of rat heart, brain and vas deferens.

On incubation of ^{14}C-noradrenaline or ^{14}C-DOPA with a continuous cell line of neuroblastoma in tissue culture, no convincing evidence was obtained for any further metabolism of these compounds.

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ABBREVIATIONS

Noradrenaline - (-)-1-(3,4-dihydroxyphenyl)-2-aminoethanol

Adrenaline - (-)-1-(3,4-dihydroxyphenyl)-2-methylaminoethanol

Dopamine - β-(3,4-dihydroxyphenyl) ethylamine

DOPA - β-(3,4-dihydroxyphenyl) alanine

NMA - normetadrenaline; 1-(4-hydroxy-3-methoxyphenyl)-2-aminoethanol

MA - metadrenaline; 1-(4-hydroxy-3-methoxyphenyl)-2-methylaminoethanol

VMA - 4-hydroxy-3-methoxymandelic acid

DHMA - 3,4-dihydroxymandelic acid

HMPG - 4-hydroxy-3-methoxyphenylglycol

DHPG - 3,4-dihydroxyphenylglycol

HVA - homovanillic acid; 4-hydroxy-3-methoxyphenylacetic acid

DOPAC - 3,4-dihydroxyphenylacetic acid

HMPE - 4-hydroxy-3-methoxyphenylethanol

DHPE - 3,4-dihydroxyphenylethanol

DHPPA - β-(3,4-dihydroxyphenyl) pyruvic acid

DHPLA - β-(3,4-dihydroxyphenyl) lactic acid

VPA - vanylpyruvic acid; 4-hydroxy-3-methoxyphenylpyruvic acid

VLA - vanyllactic acid; 4-hydroxy-3-methoxyphenyllactic acid

MAO	- monoamine oxidase
COMT	- catechol-O-methyltransferase
PNMT	- phenylethanolamine N-methyltransferase
5-HT	- 5-hydroxytryptamine
5-HTP	- 5-hydroxytryptophan
5-HIAA	- 5-hydroxyindoleacetic acid
5-HIAAl	- 5-hydroxyindoleacetaldehyde
5-HTOH	- 5-hydroxytryptophol
ACTH	- adrenocorticotrophic hormone
ATP	- adenosine 5^{l}-triphosphate
NAD	- nicotinamide - adenine dinucleotide
$NADH_2$	- reduced nicotinamide-adenine dinucleotide
$NADPH_2$	- reduced nicotinamide-adenine dinucleotide phosphate

CHAPTER I

INTRODUCTION

Historical Aspects

Towards the end of the 19th century, Oliver and Schäfer (1895) first demonstrated the profound cardiovascular effects of intravenous adrenal medullary extracts in the dog. Other early landmarks in early catecholamine research were the subsequent chemical identification of adrenaline (Abel and Crawford, 1897; Aldrich, 1901; Takamine, 1901), and the synthesis of both adrenaline and noradrenaline (Stolz, 1904).

The concept of chemical transmission at synapses was put forward by Elliot (1904, 1905), after the similarity between the effects of administered adrenaline and sympathetic stimulation (Lewandowsky, 1899; Langley, 1901) was recognized. For many years there was little progress in this field, although a significant event, viewed in retrospect, was the demonstration by Barger and Dale (1910) that noradrenaline reproduced sympathetic effects more faithfully than adrenaline. In 1921, Loewi and, independently, Cannon and Uridil, both demonstrated the validity of Elliot's concept of neurohumoral transmission. It was generally accepted at this time that the substance 'sympathin' released by sympathetic nerves was identical with adrenaline, an idea which persisted until the 1940's. However, discrepancies obviously existed between the effect of sympathetic nerve stimulation and the

actions of adrenaline. Indeed, it was to explain these differences, that Cannon and Rosenblueth (1933) postulated the formation of two different compounds, 'sympathin E' and 'sympathin I', at various effector organs by the combination of adrenaline with different substances in the cells. Bacq (1934) suggested that noradrenaline might be identical with 'sympathin E'. In 1937, Stehle and Ellsworth had again noted the close similarity between the effects of sympathetic nerve stimulation and the actions of noradrenaline, rather than adrenaline. However, several observations in the 1930's, including the finding that postganglionic stimulation of the nerves of the rabbit ear released what appeared to be adrenaline (Gaddum and Kwiatkowski, 1939) seemed to provide support for the original adrenaline hypothesis.

Noradrenaline was finally shown to be the predominant catecholamine in peripheral tissues and adrenergic nerves by von Euler (1946a,b,c, 1948). Using more refined methods for the purification and biological and chemical analysis of catecholamines, he extracted a substance identical, in every way, with noradrenaline from the hearts of cattle, horses and cats and from bovine splenic nerves. In 1947, Gaddum and Goodwin showed noradrenaline to be the transmitter in hepatic nerves. At about the same time, and independently, noradrenaline was noted to occur in the adrenal medulla (Holtz, Credner and Kroneberg, 1947). The

presence of noradrenaline in nerves and tissues was confirmed by Bacq and Fischer (1947). Direct evidence for the release of noradrenaline from postganglionic sympathetic nerve endings was provided initially by Peart (1949) in cat spleen, and later, by other workers using a variety of tissues (West, 1950; Mann and West, 1950, 1951; Outschoorn and Vogt, 1952).

The autonomic nervous system.

The autonomic nervous system (vegetative, visceral or involuntary nervous system) regulates the activities of the heart, blood vessels, glands, viscera and smooth muscle of the body, (see Burn, 1963). On the basis of anatomical, physiological and pharmacological differences, it can be sub-divided into sympathetic and parasympathetic divisions. The primary function of the autonomic nervous system is to permit the animal to adjust to sudden changes in its environment, thus enabling the body to preserve an internal environment maintained within relatively narrow limits (Cannon, 1930).

Although the autonomic nervous system has been defined in terms of its efferent (motor) pathways (Gaskell, 1916; Langley, 1921), it also includes certain visceral afferents and centres which connect with the efferent pathways (Mitchell, 1953). The central elements of the autonomic nervous system are located in the cerebrum, cerebellum,

brain stem, hypothalamus and spinal cord, interconnected by nerve tracts. The peripheral elements consist of nerves, ganglia and plexuses, providing innervation to the organs. The autonomic afferent fibres from these structures are concerned with the transmission of visceral sensation, with the integration of visceral activities, and with circulatory, respiratory and visceromotor reflexes.

The efferent outflow of the sympathetic nervous system

The cell bodies of the preganglionic sympathetic neurones are located in the lateral horn of the grey matter, primarily in the first thoracic to second lumbar spinal cord segments (human). The preganglionic fibres emerge in the ventral (anterior) root of the segment from which they arise, and enter the mixed spinal nerve, which divides into a small dorsal and a large ventral ramus. The preganglionic fibre continues in the ventral ramus, but soon leaves it as a white ramus communicans, which passes to a sympathetic ganglion.

Three groups of sympathetic ganglia exist; the paravertebral ganglia of the sympathetic chain, the prevertebral ganglia of the abdominal plexuses, and the terminal ganglia which are closely related to the target organ. On entering the sympathetic chain, some preganglionic axons end in the first ganglion they reach: others pass through a paravertebral ganglion and communicate with a more remote prevertebral or terminal ganglion. Some preganglionic fibres

pass cephalad or caudad for varying distances within the sympathetic trunk, before they end in a synapse. Each sympathetic preganglionic fibre forms a synaptic junction with numerous postganglionic neurones. The postganglionic fibres take various routes to the periphery. Preganglionic autonomic fibres are myelinated (medullated), whereas postganglionic fibres are unmyelinated (non-medullated).

The innervation of the urogenital tract

Recent studies have indicated that a special type of short postganglionic sympathetic neurone occurs extensively in the mammalian urogenital tract. (Sjöstrand, 1965; Sjöberg, 1967) These short adrenergic neurones have been shown to differ functionally in some respects from the normal long postganglionic neurones which innervate most peripheral organs. Thus their response to reserpine and nerve growth factor-antiserum and the rate of noradrenaline release from granules isolated from them are all different compared with the response of long adrenergic neurones. These properties are discussed in greater detail in the relevant sections of this thesis.

The organs involved include the internal male genital organs (Falck, Owman and Sjöstrand, 1965; Owman and Sjostrand, 1965; Sjostrand, 1965; Owman and Sjostrand, 1966), the urinary bladder (Hamberger and Norberg, 1965a,b), the urethra (Owman, Owman and Sjöberg, 1967) and the female

reproductive tract (Owman, Rosengren and Sjöberg, 1966; Owman and Sjöberg, 1966; Owman, Rosengren and Sjoberg, 1967; Rosengren and Sjöberg, 1967; Sjöberg, 1967) The female reproductive glands have a rich supply of adrenergic nerves which are related both to the vascular bed and to the smooth muscle coats. The vascular nerves to the genital tract, and also non-vascular nerves to the ovary, arise in the sympathetic chain. The uterus and the vagina are innervated by short adrenergic neurones; the fallopian tube (oviduct) is innervated by both types of neurones.

The sympathetic transmitter

The evidence that noradrenaline is the sympathetic transmitter at postganglionic nerve terminals was summarized by von Euler in 1956. It can be stated briefly under six headings:

(a) Noradrenaline is present in sympathetic nerves Analysis of extracts of adrenergic nerves by reliable assay methods for adrenaline and noradrenaline shows that the active substance is noradrenaline, rather than adrenaline. In a mixed nerve the noradrenaline content depends on the number of adrenergic fibres in the nerve. Thus, the highest value is found in the splenic nerve which has a very rich sympathetic component and, in general, the amount of noradrenaline in a given organ is directly proportional to the density of its sympathetic innervation.

(b) Degeneration of sympathetic nerves is

accompanied by disappearance of noradrenaline from the organ supplied. With regeneration of nerve fibres, tissue noradrenaline content returns to normal.

(c) Noradrenaline is released into the blood stream by stimulation of the sympathetic nerves.

(d) The effects of nerve stimulation are matched by the effects of authentic noradrenaline.

(e) Antagonists of noradrenaline such as dibenamine and tolazoline also antagonize the effects of nerve activity. Since the actions of adrenaline and noradrenaline are similarly antagonized, this type of observation does not differentiate between them.

(f) Noradrenaline in the urine is attributable to the adrenergic nerves and not to the adrenals, as the urinary level is not decreased by adrenalectomy.

Role of the sympathetic nervous system

The sympathetic nervous system is not essential to life. Animals, which have undergone as complete a removal of the sympathetic nervous system as possible appear to live normally if they are kept in a protected environment (Cannon, Newton, Bright, Menkin and Moore, 1929), but do not survive for long if exposed to adverse conditions such as cold or loss of blood.

A detailed account of the physiological functions of the sympathetic nervous system is beyond the scope of

the present thesis (see Keele and Neil, 1965). The physiological and pharmacological properties of noradrenaline and adrenaline have been reviewed by Innes and Nickerson (1965).

Structure of sympathetic innervation apparatus

The main axons in nerve trunks branch into preterminal axons which, in turn, give rise to the adrenergic terminals. The latter, however, do not consist of discrete nerve endings, but have been described in terms of a 'ground plexus' (Hillarp, 1946, 1959). This plexus consists of a network, with meshes of different size, made up of anastomosing strands of Schwann cells. Within the plexus a dense network of very fine fibres, often present in bundles of two to four axons, run together in close contact with the effector cells which they innervate. At intervals, the nerve fibres form a series of varicosities, or beaded enlargements, where the Schwann sheath is incomplete or even absent at the most distal sites (see Grillo, 1966).

Conventional histological evidence has been obtained for the presence of the varicosities (Garven and Gairns, 1952; Hillarp, 1959) and, using the fluorescence histochemical technique (Falck, 1962), noradrenaline has been demonstrated in high concentration in the varicosities (Norberg and Hamberger, 1964; Malmfors, 1965; Falck and Owman, 1966). The noradrenaline content in a single varicosity of a rat has been estimated to be about 5×10^{-9} µg (Dählstrom,

Häggendal and Hökfelt, 1966). Hillarp (1959) advanced the concept that the transmitter substance is released along the length of the terminal fibres so that several effector cells are innervated by each fibre en passage.

The distribution of noradrenaline in the central nervous system

Using biological assay methods, Vogt (1954) reported an irregular distribution of noradrenaline in the brain, with a high concentration in the brain stem, particularly in the hypothalamus. The distribution of the amine in the brain was later confirmed and extended by others (Bertler and Rosengren, 1959a; Laverty and Sharman, 1965a; Glowinski and Iverson, 1966). Noradrenaline may play a role in adrenergic transmission within the central nervous system (see Salmoiraghi, 1966), and histochemical findings have revealed the presence in the brain of fine nerve fibres containing high concentrations of catecholamine with characteristic varicosities (Carlsson, Falck and Hillarp, 1962; Dahlström and Fuxe, 1964; Fuxe, 1965).

The adrenal medulla

The adrenal medulla consists of masses of polyhedral cells separated by large blood sinuses (see Coupland, 1965). The cells contain granules staining blue with ferric chloride or brown with salts of chromic acid (chromaffin tissue). The adrenal medulla is richly supplied by a plexus of sympathetic nerve filaments derived mainly from the greater splanchnic nerve.

The activity of the adrenal medulla may be regarded as intermittent, arising in emergency situations, and therefore limited to periods of struggle or adaptation (Cannon, 1931; Cannon and Rosenblueth, 1933), or as a continuous secretion, with periods of reinforcement in need (Tournade and Chabrol, 1922, 1925). The latter hypothesis, which would involve medullary catecholamines in the maintenance of various homeostatic mechanisms, is controversial and experimental evidence concerning its validity has been reviewed by Malmejac (1964).

On a functional level the adrenal medulla is equivalent to a sympathetic ganglion (Elliot, 1912). Its innervation, like all preganglionic paths, has no relay in its extramedullary pathway (Hermann and Jourdan, 1937). Excitation of the nerves to the adrenal medulla releases acetylcholine (Feldberg and Minz, 1932).

Until 1947, when the presence of noradrenaline was demonstrated (Holtz, Credner and Kroneberg, 1947), adrenaline

was considered to be the only hormone present in the adrenal medulla. The finding of Holtz, Credner and Kroneberg (1947) has been confirmed many times in a variety of species (see West (1955) and Malmejac (1964)), including man (Holtz and Schümann, 1950; Osaki, 1952). There is now both histochemical evidence, employing the light microscope (Hillarp and Hökfelt, 1953; Eränko, 1955), as well as electron microscopic evidence (Tramezzani, Chiocchio and Wasserman, 1964; Coupland and Hopwood, 1966; Wood, 1967) that at least some of the medullary chromaffin cells may primarily store and secrete either adrenaline or noradrenaline. There are distinct species differences in the proportion of adrenaline to noradrenaline present in the gland, or in the efferent adrenal blood (Malmejac, 1964). For example, the proportion of noradrenaline in the efferent adrenal blood or glands of the cat is always much greater than in the dog. The differential release of either adrenaline or noradrenaline can arise as a result of a variety of different stimuli, such as carotid sinus occlusion (Holtz, Engelhardt, Greef and Schümann, 1952), electrical stimulation of either the hypothalamus (Folkow and von Euler, 1954), or the splanchnic nerve (Celander, 1954), and, pharmacologically, in response to a wide variety of drugs (e.g. Duner, 1954; Douglas and Poisner, 1965; Jaanus, Miele and Rubin, 1967; Kayaalp and McIsaac, 1968; Rubin and Miele, 1968). Several studies (Feldberg, Minz and Tsudzimura, 1934; Douglas and Poisner, 1965; Kayaalp and McIsaac, 1968; Rubin and Miele, 1968) have

demonstrated that muscarinic receptors, in addition to nicotinic ones, are present in the adrenal medulla and that both types of cholinergic receptor are involved to a different degree according to the experimental conditions, in catecholamine release by drugs or stimulation of the nerves.

Small concentrations of dopamine are normally present in the adrenal glands of a number of mammalian species (Goodall, 1951; Shepherd and West, 1953; Dengler, 1957; Vandermeulen, Cession-Fossion and Peeters, 1968), including man (Vandermeulen, Cession-Fossion, Lecomte, Orban and Lejeune, 1969). Association of this catecholamine with a granular fraction has been reported (Eade, 1958; Puppi, Benedeczky, Tigyi and Lissák, 1965; Lishajko, 1968). Hempel and Männl (1967) showed that dopamine in the adrenal gland could be secreted into the venous blood under in vivo conditions.

Storage of catecholamines in intracellular particles.

The storage of adrenal medullary catecholamines within specific granules was first demonstrated in 1953 by Blaschko and Welch and by Hillarp, Lagerstedt and Nilson. This finding was followed a few years later by the demonstration of noradrenaline-storage particles in bovine splenic nerves and rat spleen (von Euler and Hillarp, 1956). Other investigations have since revealed the presence of nora-

drenaline-containing particles in peripheral tissues (Potter and Axelrod, 1962; Campos and Shideman, 1962; von Euler and Lishajko, 1965) after centrifugation. Purified preparations of noradrenaline storage particles prepared from homogenates of rat heart (Michaelson, Richardson, Snyder and Titus, 1964; Snyder, Michaelson and Musacchio 1964; Potter, 1966) appeared to differ markedly in sedimentation properties from those isolated from bovine splenic nerve. A recent comparative study (Roth, Stjärne, Bloom and Giarman, 1968) revealed, however, that the rat heart contains a hitherto unrecognized major particulate fraction for noradrenaline storage, which has sedimentation properties similar to the noradrenaline storage particles in bovine splenic nerves. In addition a particle fraction 'peaking' at a lower molarity of sucrose density gradient was present in the rat heart, though not in splenic nerve. The properties of catecholamine storage granules have been extensively investigated since their discovery. A summary of certain pertinent features is given below.

Homogenization experiments appear to indicate that about 75% of total noradrenaline in the suspension of granules is bound in the granules themselves (Campos and Shideman, 1962; Schümann, Schnell, and Philippu, 1964). The <u>in vivo</u> proportion of particulate noradrenaline in bovine splenic nerves may, however, be higher (von Euler, 1968) because of granular breakdown during homogenization.

von Euler and Lishajko (1962) have suggested that the remaining soluble noradrenaline in the cytoplasm may be important as the source of transmitter released by nerve stimulation.

Bovine splenic nerve granules are smaller and more resistant to osmotic changes and to freezing and thawing than the medullary granules (von Euler and Lishajko, 1961a, Stjärne, 1964). Both types of granule, however, have a high ATP content with the same molar ratio of catecholamine to ATP of about 4:1 (Falck, Hillarp and Högberg, 1956; Schümann, 1958a; von Euler, Lishajko and Stjärne, 1963). On incubation in vitro, the bovine splenic nerve granules retain their noradrenaline firmly at low temperatures, but release it rapidly at 37°C, (von Euler and Lishajko, 1963a) whereas adrenal medullary granules release their catecholamines slowly, even at 37°C (Hillarp and Nilson, 1954). Granules from vas deferens and the vesicular gland of the bull release catecholamines at a rate intermediate between that of bovine splenic nerve and medullary particles (von Euler and Lishajko, 1966), a finding which has been related to the special nature of the adrenergic neurones in this area (Owman and Sjöstrand, 1965). The release rate from bovine splenic nerve particles depends on several factors, of which temperature, pH and noradrenaline concentration in the medium appear to be most important (von Euler, 1968). Thus

the rate of release of noradrenaline from bovine splenic nerve granules was lowered when noradrenaline was added to the suspension medium (von Euler and Lishajko, 1963a), and addition of radioactive noradrenaline showed that the release was compensated for by an uptake of exogenous noradrenaline (von Euler, Stjärne and Lishajko, 1963). The ability of isolated storage particles from the adrenal medulla to accumulate small amounts of adrenaline or noradrenaline, when incubated with high concentrations (10-20 µg/ml) of these amines, had previously been demonstrated by Carlsson and Hillarp (1961). This uptake was potentiated by small amounts of ATP and Mg^{++} ions (Kirshner, 1962a, Carlsson, Hillarp and Waldeck, 1963). Similarly the amine uptake mechanism in bovine splenic nerve granules is potentiated by ATP and Mg^{++} (von Euler and Lishajko, 1963b; 1967a; Stjärne, 1964). The catecholamine turnover rate, however, as determined by measurement of the rate of exchange of endogenous for exogenous catecholamine on incubation <u>in vitro</u> in the presence of tritiated noradrenaline, is about 10 times higher in the nerve than in the medullary granules (Stjärne, 1964). In addition, the two types of granule have different reactions to a variety of drugs. For example, phenoxybenzamine and prenylamine in concentrations which retard noradrenaline release from bovine splenic nerve granules increase the release rate in adrenal medullary granules (von Euler, Stjärne and Lishajko, 1964). The

effect of reserpine on isolated granules is discussed later.

The splenic nerve particles obtained from adrenergic fibres may not have precisely the same properties as those found at nerve terminals because of their different location. The properties of rat heart granules (Potter and Axelrod, 1963), for example, are similar to those of bovine splenic nerve granules regarding stability in various media, spontaneous amine release rate and the molar ratio of catecholamine/ATP. On the other hand, spontaneous amine release was not inhibited by reserpine (see page 115) and amine uptake was moderately potentiated by ATP, and inhibited by reserpine only at excessively high concentrations.

Dahlström (1965) has postulated that granular vesicles in peripheral sympathetic nerves are manufactured in nerve cell bodies, and are transported via the axons to the nerve terminals. A good deal of evidence is available to support this hypothesis. Accumulation of noradrenaline above a nerve ligature in various species can be detected by fluorescence microscopy (Dahlström, 1965; Kapeller and Mayor, 1967; Boyle and Gillespie, 1968) and by biochemical extraction (Dahlström and Häggendal, 1967; Geffen and Rush, 1968; Laduron and Belpaire, 1968a). Electron-dense granules and mitochondria also accumulate (Kapeller and Mayor, 1967). In accordance with the demonstration of small quantities of

proteins in the granular vesicles of sympathetic nerve terminals (Austin, Chubb and Livett, 1967), Livett, Geffen and Austin (1968) have demonstrated a rapid proximo-distal movement of noradrenaline and protein along the splenic nerve after injection of labelled noradrenaline and leucine into the coeliac ganglia of cats. The accumulation proximal to ligatures of dopamine-β-hydroxylase, an enzyme which is associated with catecholamine containing particles in various tissues (see page 25), has been demonstrated biochemically in dog splenic nerves (Laduron and Belpaire, 1968a) and by immunofluorescence histology in sheep sympathetic nerves (Livett, Geffen and Rush, 1969).

The concept of transport of adrenergic vesicles within sympathetic nerveshas, however, been challenged by Pellegrino de Iraldi and de Robertis (1968), who have put forward the hypothesis that nerve ligation may cause budding off from enlarged neurotubules to form dense core vesicles, which may not be related to catecholamine containing vesicles. In support of this theory, granulated vesicles are also formed in cholinergic nerves after ligation.

Catecholamine biosynthesis

In 1939 Blaschko proposed a sequence of reactions by which noradrenaline and adrenaline might be formed from tyrosine *in vivo*. Substantial experimental evidence has since accumulated to show it to be the main pathway for catecholamine biosynthesis in mammalian tissues:

L-tyrosine → L-DOPA → dopamine → (-)noradrenaline → (-) - adrenaline.

The pathway is shown in more detail in Fig. 1 with phenylalanine as starting point. Direct evidence for this series of reactions is available from both *in vitro* and *in vivo* studies of catecholamine biosynthesis in the adrenal medulla (Gurin and Delluva, 1947; Demis, Blaschko and Welsh, 1955; Hagen, 1956; Leeper and Udenfriend, 1956; Masuoka, Schott, Akawie and Clark, 1956; Udenfriend and Wyngaarden, 1956; Goodall and Kirshner, 1957; Pellerin and D'Iorio, 1957) in homogenates of sympathetic nerves or ganglia (Goodall and Kirshner, 1958) and, centrally, both in rat and cat brain slices (Masuoka, Clark and Schott, 1961; Masuoka, Schott and Petriello, 1963) and in cat brain in vivo (McGeer, Ling and McGeer, 1963). Catecholamine biosynthesis has also been demonstrated in the hearts of several animal species both *in vivo* (Goldstein, Musacchio and Contrera, 1962) and *in vitro* (Chidsey, Kaiser and Braunwald, 1963; Musacchio and Goldstein, 1963 a,b; Spector, Sjoerdsma, Zaltzman-Nirenberg, Levitt and Udenfriend, 1963).

Enzymes involved in biosynthesis.

The para-hydroxylation of phenylalanine to tyrosine is catalysed by a phenylalanine-4-hydroxylase (EC1.14.3.1) but the reaction is not an essential part of catecholamine biosynthesis if tyrosine is available from dietary sources. Liver phenylalanine hydroxylase (Udenfriend and Cooper, 1952) has been found in many vertebrates, but there are marked species variations in activity (Voss and Waisman, 1966). It consists of two protein fractions (Mitoma, 1956; Kaufman, 1957) and requires a pteridine cofactor, $NADH_2$ and oxygen (Kaufman, 1957, 1958, 1959; Kaufman and Levenberg, 1959). There is evidence to suggest that in adrenal medulla and brain, phenylalanine and tyrosine are hydroxylated by the same enzyme (Ikeda, Levitt and Udenfriend, 1965; Udenfriend, 1966; Ikeda, Levitt and Udenfriend, 1967), which is different from liver phenylalanine hydroxylase (Ikeda, Levitt and Udenfriend, 1965).

Tyrosine hydroxylase (EC 1.14.3.?) catalyses the meta-hydroxylation of L-tyrosine to L-DOPA and is likely to be the rate-limiting enzyme for the formation of catecholamines(Nagatsu, Levitt and Udenfriend, 1964a; Levitt, Spector, Sjoerdsma and Udenfriend, 1965). Bovine adrenal medullary tyrosine hydroxylase requires O_2, Fe^{++} and a tetrahydropteridine for activity (Nagatsu, Levitt and Udenfriend, 1964a). The enzyme occurs in sympathetic nervous tissue, adrenal medulla (Nagatsu, Levitt and Udenfriend 1964a), brain

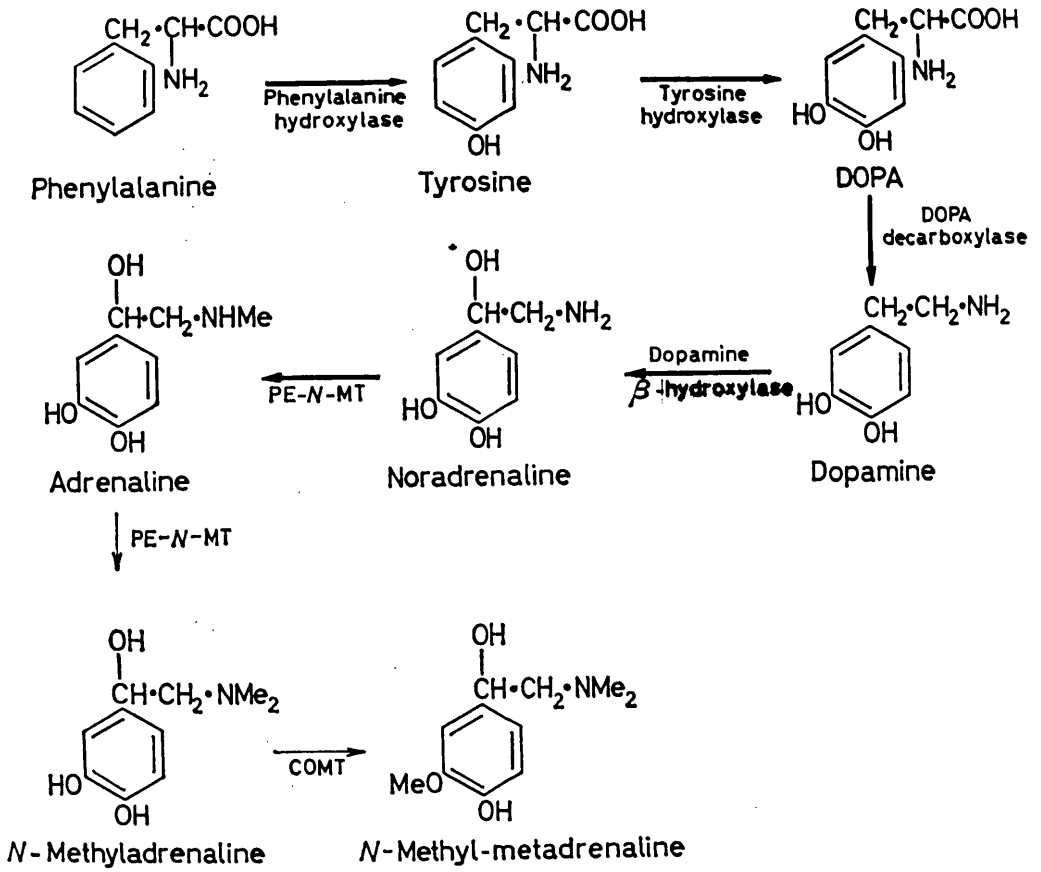


Fig. 1. Main pathway for catecholamine biosynthesis shown in heavy arrows.
N-Methylation of adrenaline and
O-methylation of N-methyladrenaline
lightly arrowed.

(Nagatsu, Levitt and Udenfriend, 1964a, Nagatsu, Levitt and Udenfriend, 1964b ; Bagchi and McGeer, 1964), salivary gland and superior cervical ganglion (Sedvall and Kopin, 1966, 1967a) of a variety of animal species and exhibits a high degree of specifity (Nagatsu, Levitt and Udenfriend, 1964a). D-tyrosin DL-m-tyrosine, tyramine and L-tryptophan are not substrates.

The subcellular localization of tyrosine hydroxylase is controversial since doubt exists as to whether it is a soluble enzyme or is bound to vesicles. Bovine adrenal tyrosine hydroxylase was originally reported to be a particulate enzyme (Nagatsu, Levitt and Udenfriend, 1964a; Petrack, Sheppy and Fetzer, 1968). Other workers have reported it to be present in the adrenal cytoplasm. (Laduron and Belpaire, 1968b; Musacchio, 1968). In bovine splenic nerve homogenate, Stjärne and Lishajko (1967) reported that the enzymatic hydroxylation of tyrosine occurs in the particle-free high speed supernatant. This finding is supported by the observation of Laduron and Belpaire (1968c) that the tyrosine hydroxylase activity of a constricted axon does not increase when synaptic residues accumulate above the constriction. Bagchi and McGeer (1964) found that the enzymic activity in rat and bovine caudate nucleus is associated with a particulate fraction, but no requirement for a tetrahydropteridine was reported. According to Cote and Fahn (1968) however, brain tyrosine hydroxylase requires the presence of a tetrahydropteridine as a co-factor for optimum activity.

In an effort to explain the discrepancy between soluble and particulate tyrosine hydroxylase, Musacchio and Wurzburger (1969) reported that soluble bovine adrenal hydroxylase is unstable and tends to aggregate upon standing or during various preparatory procedures. Whether tyrosine hydroxylase may, in fact, have a bimodal distribution is at present unknown.

Several inhibitors of tyrosine hydroxylase have been described, the most important being α-methyl-p-tyrosine, a competitive inhibitor which reduces tissue levels of catecholamines (Spector, Sjoerdsma and Udenfriend, 1965). Certain iodotyrosines, including 3-iodo-L-tyrosine, also inhibit tyrosine hydroxylase competitively *in vitro* (Goldstein and Weiss, 1965) and *in vivo* (Spector, Ortega-Mata, Sjoerdsma and Udenfriend, 1965) but disappear more rapidly from tissues (Spector, 1966). Catechol compounds including (-)-noradrenaline itself, and 3,4-dihydroxyphenylpropylacetamide in particular, inhibit tyrosine hydroxylation *in vitro* (Udenfriend, Zaltzman-Nirenberg and Nagatsu, 1965) apparently by interaction with the pteridine co-factor.

In 1938, Holtz, Heise and Lüdtke described the *in vitro* decarboxylation of 3,4-dihydroxyphenylalanine (DOPA) to dopamine, catalysed by DOPA decarboxylase (EC.4.1.1.26). The transformation was shown to occur *in vivo* with the detection of dopamine in urine after injection of DOPA (Holtz, Credner and Koepp, 1942). Studies with other sub-

strates and purified enzyme preparations have since revealed that the enzyme acts on many L-isomers; the alternative name aromatic L-amino acid decarboxylase was proposed to cover this relative lack of specifity (Lovenberg, Weissbach and Udenfriend, 1962). Although the enzyme is known to be identical with 5-HTP decarboxylase (Westermann, Balzer and Knell, 1958; Bertler and Rosengren, 1959b; Yuwiler, Geller and Eiduson, 1959) and can readily decarboxylate o- and m- tyrosine and some positional isomers of DOPA (Blaschko, Holton and Sloane-Stanley, 1949; Fellman, 1959; Ferrini and Glässer, 1964) the claim that it has some action on histidine, tryptophan, phenylalanine and p-tyrosine (Lovenberg, Weissbach and Udenfriend, 1962) has been questioned (Awapara, Sandman and Hanly, 1962; Awapara, Perry, Hanly and Peck, 1964).

The decarboxylase is widely distributed in mammalian tissues (Holtz, 1959; Hagen and Cohen, 1966) including those regions of the brain known to contain catecholamines or 5-hydroxytryptamine in high concentration (Holtz and Westermann, 1956). In sympathetic nerves and adrenal medulla, activity is mainly found in the supernatant fraction after centrifugation of tissue homogenates (Blaschko, Hagen and Welch, 1955; Schümann, 1958b; Stjärne and Lishajko, 1967; Laudron and Belpaire, 1968b). It has been reported, however, that some decarboxylase activity is associated with the particulate fraction isolated from bovine splenic nerves

(Stjärne and Lishajko, 1967) although insufficient was present to be quantitatively important for catecholamine biosynthesis. In the brain Rodríguez de Lorez Arnaiz and de Robertis (1964) found decarboxylase activity in the synaptosome fraction.

Pyridoxal phosphate is a co-factor for DOPA decarboxylase (Green, Leloir and Nocito, 1945; Blaschko, Carter, O'Brien and Sloane-Stanley, 1948; Schales and Schales, 1949; Sourkes, Heneage and Trano, 1952; Clark, Pogrund, Akawie, Drell and Hartman, 1955; Buxton and Sinclair, 1956; Buzard and Nytch, 1957; Weissbach, Bogdanski, Redfield and Udenfriend, 1957).

A large number of compounds can inhibit DOPA decarboxylase (Clark, 1959) including competitive inhibitors such as α-methylDOPA (Oates, Gillespie, Udenfriend and Sjoerdsma, 1960), and strong reducing agents such as decaborane (Merritt and Schultz, 1966). The catecholamine-depleting (Hess, Connamacher, Ozaki and Udenfriend, 1961) and hypotensive (Oates, Gillespie, Udenfriend and Sjoerdsma, 1960) actions of α-methylDOPA are not, however, related to decarboxylase inhibition (Hess, Connamacher, Ozaki and Udenfriend, 1961). The compound can itself undergo decarboxylation with the ultimate formation of α-methylnoradrenaline (Carlsson and Lindqvist, 1962; Maître and Staehelin, 1963) which can be released by nerve stimulation (Muscholl and Maitre, 1963) thus acting as a 'false transmitter' (Carlsson and Lindqvist, 1962). The evidence that

α-methylDOPA suppresses adrenergic function in experimental animals is, however, conflicting (see Muscholl, 1966).

The β-hydroxylation of dopamine to noradrenaline is catalysed by the enzyme dopamine-β-hydroxylase (EC.1.14.2.1 This reaction was first demonstrated in vitro by the use of homogenates, aqueous extracts and slices of adrenal gland (Hagen, 1956; Neri, Hayano, Stone, Dorfman and Elmadjian, 1956; Goodall and Kirshner, 1957) and in vivo using labelled precursors (Leeper and Udenfriend, 1956). The enzyme is also present in brain (Udenfriend and Creveling, 1959) and heart (Chidsey, Kaiser and Braunwald, 1963), and is associated with catecholamine-containing granules in all tissues investigated (Kirshner, 1957; Potter and Axelrod, 1963; Musacchio, Kopin and Snyder, 1964; Oka, Kajikawa, Ohuchi, Yoshida and Imaizumi, 1967; Stjärne and Lishajko, 1967; Belpaire and Laduron, 1968). In the adrenal medulla the enzyme has been reported to be surrounded by a semi-permeable membrane (Belpaire and Laduron, 1968). The findings of Stjarne and Lishajko (1967) indicate that dopamine-β-hydroxylase is the only enzyme involved in catecholamine biosynthesis which is localized in bovine splenic nerve vesicles.

Dopamine-β-hydroxylase has been purified and has a molecular weight of about 290,000 (Friedman and Kaufman, 1965; Goldstein, Lauber and McKereghan, 1965). The enzyme requires ascorbic acid as an external electron donor, fumaric acid and O_2 (Levin, Levenberg and Kaufman, 1960)

and contains approximately 2 μmoles of Cu^{++} per μmole of enzyme (Friedman and Kaufman, 1965). The Cu^{++} in the enzyme undergoes cyclic reduction and oxidation during the overall hydroxylation reaction (Friedman and Kaufman, 1965).

Dopamine-β-hydroxylase is not specific for dopamine: it will-β-hydroxylate many phenylethylamines (Goldstein and Contrera, 1962; Creveling, Daly, Witkop and Udenfriend, 1962) including epinine (N-methyldopamine) which is converted to adrenaline (Bridgers and Kaufman, 1962).

Many inhibitors of the enzyme have been described. These include various copper chelating agents (Goldstein, Lauber and McKereghan, 1965), such as disulfiram which has been found to be an effective inhibitor of dopamine-β-hydroxylase *in vivo* (Musacchio, Goldstein, Anagnoste, Poch and Kopin, 1966) as well as *in vitro* (Goldstein, Anagnoste, Lauber and McKeregan, 1964).

The final step in the biosynthesis of adrenaline is the N-methylation of noradrenaline to adrenaline. In 1949, Bülbring first demonstrated that adrenaline is formed from noradrenaline in minced adrenal gland. The enzyme responsible for the conversion is phenylethanolamine-N-methyltransferase (PNMT) (EC.2.1.1.?). Axelrod has partially purified and described the properties of the enzyme (Axelrod, 1962a) which requires S-adenosylmethionine

as methyl donor (Kirshner and Goodall, 1957) and is present in the high-speed supernatant fraction of adrenal medullary homogenates (Kirshner and Goodall, 1957; Axelrod, 1962a; Laduron and Belpaire, 1968b). Recently, however, PNMT activity has been detected in the particulate fraction as well as in the supernatant fraction of bovine adrenal medulla and phaeocromocytoma tumour tissue (Goldstein, Gang and Joh, 1969), suggesting that adrenaline synthesis might also occur within the granule.

The enzyme has been investigated in various mammalian species (Axelrod, 1962a; Fuller and Hunt, 1967a) and was recently purified from human adrenal glands (Kitabchi and Williams, 1969). Although PNMT is primarily located in the adrenal medulla, other tissue sources of the enzyme have been identified. Thus PNMT activity was detected in rabbit heart (Axelrod, 1962a) and de Potter, Bogaert, and Schaepdryver (1966) have shown that labelled noradrenaline can be converted to adrenaline in rat hearts. Other workers have demonstrated that brain tissue can form adrenaline (McGeer and McGeer, 1964; Ciarancllo, Barchas, Byers, Stemmle and Barchas, 1969; Pohorecky, Zigmond, Karten and Wurtman, 1969) and low endogenous concentrations of the catecholamine have been identified in the brain (von Euler, 1946c; Holtz, 1950; Vogt, 1954; Gunne, 1962).

PNMT is specific for phenylethanolamine and its derivatives but phenylethylamines are not N-methylated.

Both dextro- and laevo-compounds are N-methylated but the laevo-isomers have a higher affinity for the enzyme (Axelrod, 1962a; Fuller and Hunt, 1965). Both noradrenaline and adrenaline, to a greater extent, are themselves inhibitors of PNMT *in vitro* (Fuller and Hunt, 1967a), a finding which may be of physiological importance in the control of adrenaline synthesis.

Regulation of catecholamine biosynthesis in sympathetic nerves and the adrenal medulla.

Under physiological conditions noradrenaline levels in sympathetically innervated organs remain quite constant despite wide variations in neuronal activity (von Euler, 1962). The rate of turnover of catecholamines in peripheral sympathetic nerves is much faster than that occurring in the adrenal gland (Burack and Draskóczy, 1964; Brodie, Costa, Dlabac, Neff and Smookler, 1966).

Several recent reports have indicated that increased activity of the sympathetic nervous system is associated with an accelerated synthesis of noradrenaline. Earlier work on the isolated adrenal gland of the cat (Holland and Schümann, 1956; Bygdeman and von Euler, 1958) had pointed to such a relationship since, on prolonged stimulation of the splanchnic nerve, the amount of adrenaline released into the adrenal vein plus that remaining in the gland after stimulation exceeded the quantity present in the gland before stimulation.

Increase in noradrenaline synthesis after nerve stimulation has been observed in the heart and brain (Oliverio and Stjärne, 1965; Gordon, Reid, Sjoerdsma and Udenfriend, 1966; Gordon, Spector, Sjoerdsma and Udenfriend, 1966) and in the submaxillary gland (Sedvall and Kopin, 1967b) of the intact animal; a similar finding has been noted in the guinea-pig hypogastric nerve-vas deferens preparation (Weiner and Alousi, 1966; Roth, Stjärne and von Euler, 1966; Austin, Livett and Chubb, 1967) and perfused cat spleen (Blakeley, Brown, Dearnaley and Harrison, 1969). Studies with radioactive tyrosine and DOPA indicate that the accelerated synthesis of noradrenaline arises from an increased rate of conversion of tyrosine to DOPA (Gordon, Reid, Sjoerdsma and Udenfriend, 1966; Sedvall and Kopin, 1967; Weiner and Rabadjija, 1968) although an increased synthesis of dopamine and noradrenaline from ^{14}C-DOPA has also been reported on electrical stimulation of the perfused guinea-pig hypogastric nerve-vas deferens preparation (Austin, Livett and Chubb, 1967).

Bovine splenic nerve trunk, unlike most tissues, does not respond to nerve stimulation with increased synthesis of noradrenaline from tyrosine (Roth, Stjärne and von Euler, 1967); the normal rate of synthesis in this organ is very high, however, being about 15 to 20 times that of the vas deferens. This finding suggests that depolarization <u>per se</u> does not control noradrenaline synthesis. As previously

mentioned, hydroxylation of tyrosine is thought to be the rate-limiting step in the biosynthesis of noradrenaline (Levitt, Spector, Sjoerdsma and Udenfriend, 1965) and since catecholamines and catechol derivatives are inhibitors of the enzyme (Udenfriend, Zaltzman-Nirenberg and Nagatsu, 1965; Ikeda, Fahien and Udenfriend, 1966), it has been suggested that noradrenaline synthesis may be regulated by a process of end-product inhibition at the tyrosine hydroxylase step (see Udenfriend, 1968). Studies on the vas deferens preparation led Alousi and Weiner (1966) to make a similar proposal. Both in vivo (Spector, Gordon, Sjoerdsma and Udenfriend, 1967; Neff and Costa, 1968) and in vitro (Stjärne, Lishajko and Roth, 1967; Weiner and Rabadjija, 1968) evidence is available to support this hypothesis.

Whilst dopamine-β-hydroxylase is not the rate-limiting step in noradrenaline synthesis under physiological conditions, low concentrations of noradrenaline can inhibit the formation of noradrenaline from dopamine in homogenates of bovine splenic nerve (Stjärne, Lishajko and Roth, 1967). This inhibition may be of importance under special circumstances such as those existing after treatment with reserpine (see page 116). Furthermore, an effective inhibitor of this enzyme, such as disulfiram, will lower endogenous noradrenaline levels. The tissue concentration of noradrenaline adjusts to a lower steady-state level where destruction is equal to synthesis. Thus in the

presence of disulfiram, dopamine-β-hydroxylase may become rate-limiting (Musacchio, Goldstein, Anagnoste, Poch and Kopin, 1966).

Recent observations on the isolated cat spleen indicate that newly synthesized noradrenaline is selectively released by rapid nerve stimulation (Kopin, Breese, Krauss and Weise, 1968) but these findings have not been confirmed by Blakeley, Brown, Dearnaley and Harrison, (1969).

Influence of steroids on the conversion of noradrenaline to adrenaline

In 1952 Hökfelt recognized that adrenocorticotrophi hormone (ACTH) will restore to normal the depleted adrenaline content of the adrenal glands of hypophysectomized rats. In the early 1950's Shepherd and West (1951, 1952) and Coupland (1953), on the basis of separate comparative studies, observed that the proximity of cortical tissue to chromaffin cells and the amount of cortical tissue relative to medullary tissue may not be unconnected with higher medullary cocentrations of adrenaline relative to its precursor, noradrenaline; the adrenal cortex may exert some trophic influence on the chromaffin cell thus facilitating the conversion of noradrenaline to adrenaline. Furthermore, the pattern of blood flow in the adrenal gland favours the delivery of high concentrations of steroid to the adrenal medulla (Harrison and Hoey, 1960; Coupland, 1965).

Wurtman and Axelrod (1965, 1966) investigated the

steroidal factors necessary for optimal activity of PNMT, the enzyme which converts noradrenaline to adrenaline. They showed that its activity declines in the hypophysectomized rat but can be restored to normal by administration of ACTH or large doses of the synthetic glucocorticoid, dexamethasone. Similar findings have been reported in the dog (Wurtman, Casper, Pohorecky and Bartter, 1968). On the other hand, low doses of dexamethasone depress PNMT activity and adrenaline levels in rat adrenals (Wurtman, Noble and Axelrod, 1967). Small amounts of ACTH reverse this effect. In man, physiological doses of dexamethasone depress plasma cortisol levels and bring about a progressive decrease in urinary adrenaline output suggesting a fall in PNMT activity (Noble, Silbergeld, Kopell, McKinney, Wittner and Butte, 1968). No significant effect on noradrenaline output is observed. Since the adrenal medulla is normally perfused with venous portal blood which has drained through the adrenal cortex (Coupland, 1965), it is thus likely to be richly supplied with steroid hormones. In the normal intact rat at least, it appears that the concentration of PNMT is maximal since the rate of adrenaline production is not increased by further stimulation (Fuller and Hunt, 1967a) However, in rats bearing a transplantable pituitary tumour a large increase in plasma corticosterone levels was associated with a 3.5 fold increase in adrenal PNMT and a 1.6 fold increase of the adrenaline content of the adrenal gland

(Vernikos-Danellis, Ciaranello and Barchas, 1968). Coupland (1968) has shown that corticosterone, but not deoxycorticosterone, induces methylation of noradrenaline in organ cultures of extra-adrenal chromaffin cells of the neonatal rabbit, indicating the importance of the 11-oxy group in steroid-induced methylation of catecholamines.

When animals are pretreated with compounds which inhibit protein synthesis (actinomycin D or puromycin) there is no increase in PNMT activity after dexamethasone (Wurtman and Axelrod, 1966). Corticoids therefore increase PNMT activity by increasing the rate of synthesis of enzyme protein. In the rat, removal of the pituitary is associated with small increases in noradrenaline levels (Wurtman, 1966), and hypertrophy of the rough endoplasmic reticulum and the Golgi complex in the medullary cells which store adrenaline (Pohorecky and Rust, 1968). As before, these changes can be reversed by the administration of ACTH or glucocorticoids. The normal developmental increase in PNMT activity and adrenaline accumulation in the foetal rat (Margolis, Roffi and Jost, 1966; Fuller and Hunt, 1967b; Roffi, 1968) and rabbit (Roffi, 1968) are also prevented by hypophysectomy (decapitation). The effects of foetal decapitation are reversed by ACTH or cortisol.

Alternative biosynthetic pathways

The lack of specificity of some of the enzymes

involved in the main pathway of catecholamine biosynthesis, particularly of aromatic L-amino acid decarboxylase and dopamine-β-hydroxylase, suggests that alternative routes of catecholamine synthesis might occur in the body. Some of the possible pathways are illustrated in Fig.2 and have been described in detail by Sandler and Ruthven (1969).

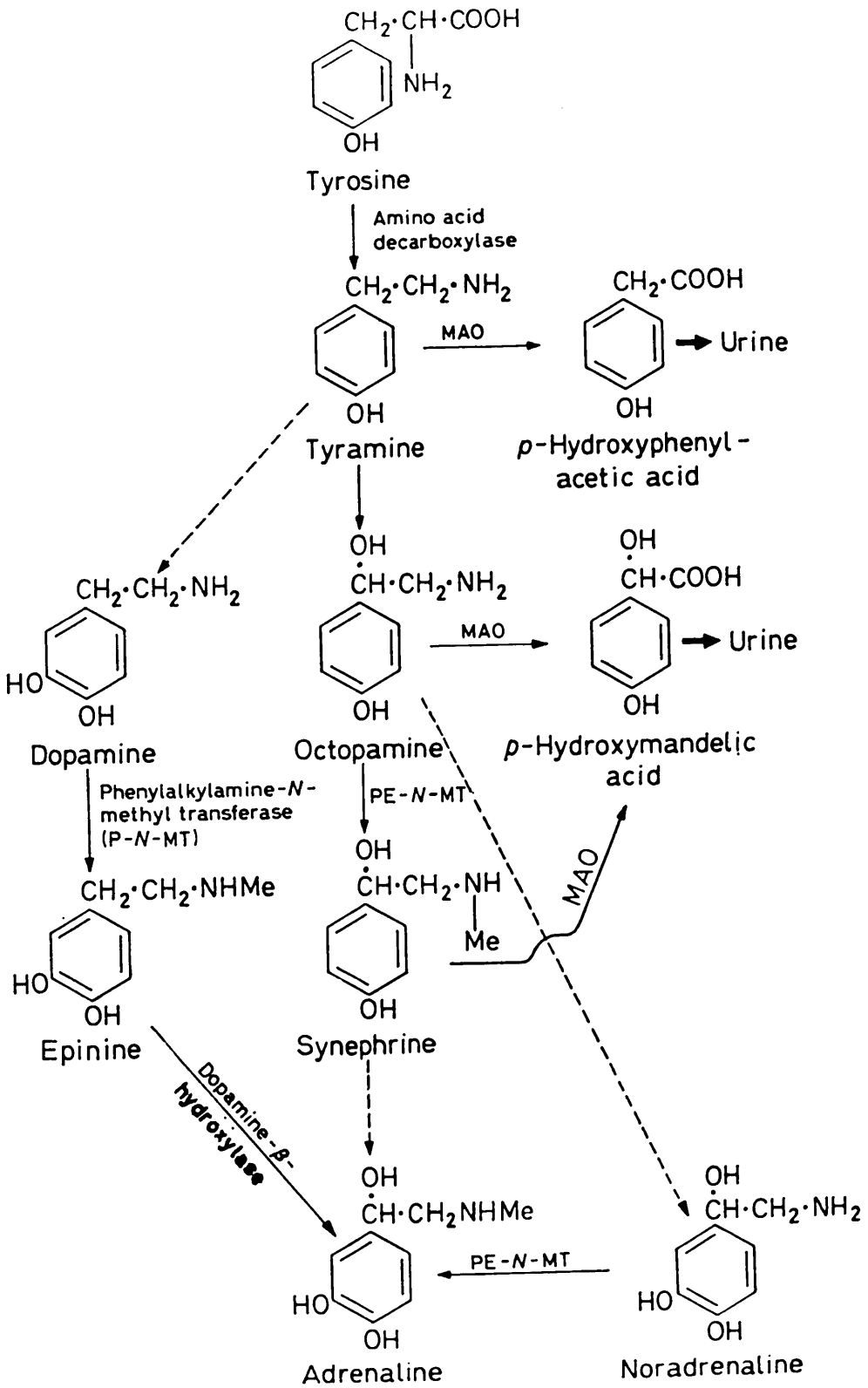


Fig.2 Alternative pathways of catecholamine biosynthesis.

Dopamine

Dopamine is a comparatively weak sympathomimetic amine (Barger and Dale, 1910; Holtz, Heise and Lüdtke, 1938) which may have a physiological function, in addition to being a precursor of noradrenaline (Wurtman, 1965; Hornykiewicz, 1966). In contrast to noradrenaline, dopamine may produce a vasodepressor response in animals (Burn and Rand, 1958; Hornykiewicz, 1958; Halushka and Hoffman, 1968), although large doses of the amine cause a pressor response in the dog (Eble, 1964; McNay, McDonald and Goldberg, 1965). Dopamine is the only endogenous catecholamine known to dilate the renal vasculature (Eble, 1964; McDonald, Goldberg, McNay and Tuttle, 1964; McNay, McDonald and Goldberg, 1965). In man, infusion of dopamine causes an increase in systolic blood pressure, with a minimal effect on diastolic pressure (Horwitz, Fox and Goldberg, 1962; Allwood and Ginsberg, 1964).

Dopamine has been detected in several mammalian tissues as well as in normal human urine (Holtz, Credner and Kroneberg, 1947; von Euler, Hamberg and Hellner, 1951) where it is present in amounts greater than adrenaline or noradrenaline. The major portion of urinary dopamine may be produced by direct renal decarboxylation of DOPA; large quantities of dopamine are present in the kidney and urine after parenteral administration of DOPA (Holtz, Credner and Koepp, 1942; Murphy and Sourkes, 1961), although Weil-Malherbe and Bone (1957a) failed to detect dopamine in normal human

plasma. Small quantities of dopamine have, however, recently been reported in cat plasma (Street and Roberts, 1969). Furthermore, after administration of ^{14}C-dopamine to guinea pigs, Halushka and Hoffman (1968) observed that the kidney contains a proportionately larger amount of dopamine than all other tissues investigated, 1.5-2 minutes after injection.

In the mammal, dopamine was first detected in the pancreas (Holtz and Credner, 1942); its presence in the heart was first demonstrated by Goodall (1951), and in the blood vessels by von Euler and Lishajko (1958). Dopamine is present in many tissue extracts including pancreas, parotid gland and spleen (von Euler and Lishajko, 1957; Schümann, 1958b, 1959). In certain tissues such as lung, liver and intestine, dopamine comprises up to 99% of total catecholamine. Very high concentrations are present in bovine and sheep liver and lung, where there are chromaffin cells peculiar to ruminants (Bertler, Falck, Hillarp, Rosengren and Torp, 1959; Falck, Nystedt, Rosengren and Stenflo, 1964). Dopamine has been identified in the carotid body of the rabbit (Dearnaley, Fillenz and Woods, 1969), and the horse (Knoche, Alfes and Möllman, 1969), and is present in adrenergic nerves (Schümann, 1956, 1958a,b; Laverty and Sharman, 1965a) and ganglia (Schumann, 1956; Laverty and Sharman, 1965a).

Dopamine represents more than 50% of the total catecholamine content in the brain and spinal cord of many

species, but its presence in the mammalian brain was not detected until 1957 (Montagu,1957; Weil-Malherbe and Bone, 1957b). These reports were soon confirmed (Carlsson, Lindqvist, Magnusson and Waldeck, 1958; Bertler and Rosengren, 1959 a,c; Sano, Gamo, Kakimoto, Taniguchi, Takesada and Nishinuma, 1959; Schümann, 1959; Ehringer and Hornykiewicz, 1960, Bertler, 1961a). The distribution of dopamine in the brain is irregular, the highest concentrations occurrig in the neostriatum (Bertler and Rosengren, 1959a,c; Sano, Gamo, Kakimoto, Taniguchi, Takesada and Nishinuma, 1959; Ehringer and Hornykiewicz, 1960; Bertler, 1961a; Laverty and Sharman, 1965a). Relatively large quantities of dopamine have also been found in the globus pallidus and substantia nigra (Sano, Gamo, Kakimoto, Taniguchi, Takesada and Nishinuma, 1959; Bertler, 1961a; Hornykiewicz, 1963). There is a marked difference in the dopamine to noradrenaline ratio of different brain regions (Bertler and Rosengren, 1959a,c; Hornykiewicz, 1966); for example in the noradrenaline-rich hypothalamus, dopamine levels are low. It has been shown that the caudate nucleus contains a dopamine nerve terminal system, originating from cell bodies in the substantia nigra (Andén, Carlsson, Dahlström, Fuxe, Hillarp and Larsson, 1964; Fuxe, Hökfelt and Nilsson, 1964; Andén, Dahlström, Fuxe and Larsson, 1965a,b). The release of dopamine from the caudate nucleus has been demonstrated on stimulation of the nucleus centrum medianum (McLennan, 1964), and after addition of tubocurarine to fluid perfusing the cat caudate nucleus

(Portig, Sharman and Vogt, 1968). Stimulation of the substantia nigra caused a release of homovanillic acid into the caudate nucleus perfusate (Portig and Vogt, 1968).

A finding of clinical significance was the discovery that the brains of Parkinsonian patients contain abnormally low levels of dopamine in the corpus striatrum and substantia nigra (Ehringer and Hornykiewicz, 1960; Bernheimer, Birkmayer and Hornykiewicz, 1963; Hornykiewicz, 1963; Bernheimer and Hornykiewicz, 1964, 1965).

Catecholamine metabolism

Until 1957, relatively little was known about the pathways of catecholamine metabolism in the body. On injection of adrenaline or noradrenaline, only about 1-5% is excreted unchanged in the urine, and the urinary output of endogenous catecholamines is extremely low (von Euler, 1956), suggesting either almost complete retention or breakdown in the body. For many years, the main route of metabolism was thought to be by means of the enzyme MAO (see Blaschko, 1954). However, although inhibition of the enzyme resulted in an increase of noradrenaline levels in many tissues (Shore, Mead, Kuntzman, Spector and Brodie, 1957), the actions of administered catecholamines were not potentiated (Griesemer, Barsky, Dragstedt, Wells and Zeller, 1953)

The metabolism of adrenaline and noradrenaline

In 1957, Armstrong, McMillan and Shaw found that large quantities of 4-hydroxy-3-methoxymandelic acid (VMA) were excreted in the urine of subjects with phaeochromocytoma and also after the administration of noradrenaline and adrenaline. Previously, Armstrong, Shaw and Wall (1956) had noted the widespread occurrence of the 4-hydroxy-3-methoxyphenyl ('vanillyl') group on chromatograms of urinary phenolic acids. The biological methylation of 3,4-dihydroxyphenylacetic acid (DOPAC) to homovanillic acid (HVA) (Shaw, McMillan and Armstrong, 1957) and the excretion of HVA after

DOPA administration (de Eds, Booth and Jones, 1957) had also been demonstrated.

These results prompted a search for the corresponding O-methylated amines of VMA, normetadrenaline (NMA) and metadrenaline (MA) which were soon shown to be present in the normal urine of rats (Axelrod, 1957; Axelrod, Inscoe, Senoh and Witkop, 1958; Axelrod, Senoh and Witkop, 1958), and in greater amount after administration of adrenaline and noradrenaline. In the rat metadrenalines are conjugated as glucuronides (Axelrod, Senoh and Witkop, 1958). In 1958, La Brosse, Axelrod and Kety noted O-methylation, of labelled adrenaline, in man also and the presence of metadrenaline and normetadrenaline in normal human urine was reported by La Brosse and Mann (1960). The O-methylated amines are excreted mainly as O-sulphates. After administration of ^{3}H-adrenaline, most of the ^{3}H-metadrenaline present in human urine is also conjugated, mainly as a sulphate ester (La Brosse, Axelrod, Kopin and Kety, 1961). The importance of O-methylation in catecholamine catabolism was emphasized by the identification of another major deaminated methylated metabolite of noradrenaline and adrenaline, 4-hydroxy-3-methoxyphenylglycol (HMPG), in the urine of the rat (Axelrod, Kopin and Mann, 1959; Kopin, Axelrod and Gordon, 1961), cat (Goldstein and Gertner, 1960; Kirshner, Terry and Pollard, 1961), guinea pig (Smith, 1961) and man (Axelrod, Kopin and Mann, 1959). In both man and rat, HMPG is conjugated as a sulphate ester (Axelrod, Kopin and Mann, 1959). Recently,

however, the glucuronic acid conjugate of HMPG has been identified as a major metabolite in human urine (Shimizu and La Brosse, 1969). VMA, on the other hand, is thought to be excreted largely in the free state in the human (Sandler and Ruthven, 1969). In a recent study, Goodall and Alton, (1958a) reported that about 25% of infused dopamine is converted to noradrenaline in the human, whilst the 4-O sulphate of VMA also appears to be present, although as a relatively small proportion of the total. The conjugation of VMA in rat urine is described in chapter 3.

Although O-methylation preceding deamination is the predominant route of metabolism of injected catecholamines (Axelrod, 1957; Axelrod, Inscoe, Senoh and Witkop, 1958; Axelrod, Senoh and Witkop, 1958; La Brosse, Axelrod and Kety, 1958) deaminated products are also excreted in the urine in smaller quantities. Thus, dihydroxymandelic acid (DHMA) has been detected in human urine (Kirshner, Goodall and Rosen, 1958) after intravenous infusion of adrenaline. Other studies confirm that DHMA is a metabolic product of both adrenaline and noradrenaline in man (Goodall, Kirshner and Rosen, 1959; La Brosse, Axelrod, Kopin and Kety, 1961; La Brosse, Mann and Kety, 1961; Goodall and Alton, 1965). Over 50% of the small amount of DHMA present in human urine appears to be conjugated (La Brosse, Axelrod, Kopin and Kety, 1961; La Brosse, Mann and Kety, 1961), but Weil-Malherbe (1967) could not detect any increase in total DHMA levels after acid hydrolysis. In the cat, following administration

of ^{14}C-adrenaline, about 1% of the radioactivity could be recovered as free DHMA but the proportion may be greatly increased after administration of the COMT inhibitor, pyrogallol (Kirshner, Terry and Pollard, 1961). Goodall and Alton (1969) have recently investigated the fate of ^{14}C-DHMA in man. About 18% of DHMA, inflused for 1 hour, could be recovered in 24 hour urine samples as VMA, 8% as 3,4-dihydroxybenzoic acid and 2% as vanillic acid. Kopin and Axelrod (1960) have demonstrated the presence of the corresponding alcohol, 3,4-dihydroxyphenylglycol (DHPG) in rat urine, but only after the formation of O-methylated metabolites had been inhibited by pyrogallol.

Minor metabolic pathways

Several minor pathways of adrenaline and noradrenaline metabolism have been demonstrated. Side-chain dehydroxylation of catecholamines has been shown to occur in vivo. In man, about 1% of an injected dose of labelled noradrenaline was excreted as HVA (Sandler, Ruthven and Contractor, 1963; Sandler, Ruthven and Wood, 1964); equivalent levels of HVA, plus about twice as much DOPAC, are excreted after injection of ^{14}C-adrenaline (Goodall and Alton, 1968b). The formation of HVA from noradrenaline may proceed via a route involving β-dehydroxylation to dopamine, which would then be metabolized to HVA (Sandler and Ruthven, 1969). A similar pathway, involving formation of DOPAC and HVA from adrenaline, would need prior N-demethy-

lation of the catecholamine. In vitro, noradrenaline can be β-dehydroxylated to DOPAC by liver slices (von Euler, 1955), and Fellman (1958) has described a chemical rearrangement of adrenaline and noradrenaline leading to the formation of dihydroxyphenylacetaldehyde, an intermediate in the formation of DOPAC.

Vanillic acid may be a final product of noradrenaline and adrenaline metabolism (Dirscherl and Thomas, 1966). The small endogenous excretion in man (0.15-0.7 mg/24 hour), after omission of vegetables from the diet (Dirscherl and Wirtzfeld, 1964), and the low levels of labelled vanillic acid detected after injection (Rosen and Goodall, 1962a; Sandler, Ruthven and Contractor, 1963; Sandler, Ruthven and Wood, 1964; Goodall and Alton, 1965), or oral administration of catecholamine (Resnick, 1963) suggests that endogenous production of vanillic acid by this pathway is of minor significance. The endogenous origin of vanillic acid from catecholamines has been difficult to prove, however, because of synthesis of the acid in the body from a variety of foods (Shaw and Trevarthen, 1958; Dirscherl and Wirtzfeld, 1964). However, vanillic acid was still present in the urine of a subject maintained solely on glucose for nine days (Coward, Smith, Wilson and Robinson, 1962). Furthermore, a high urinary output of vanillic acid has been reported in patients with malignant phaechromocytoma (Sturm, 1963).

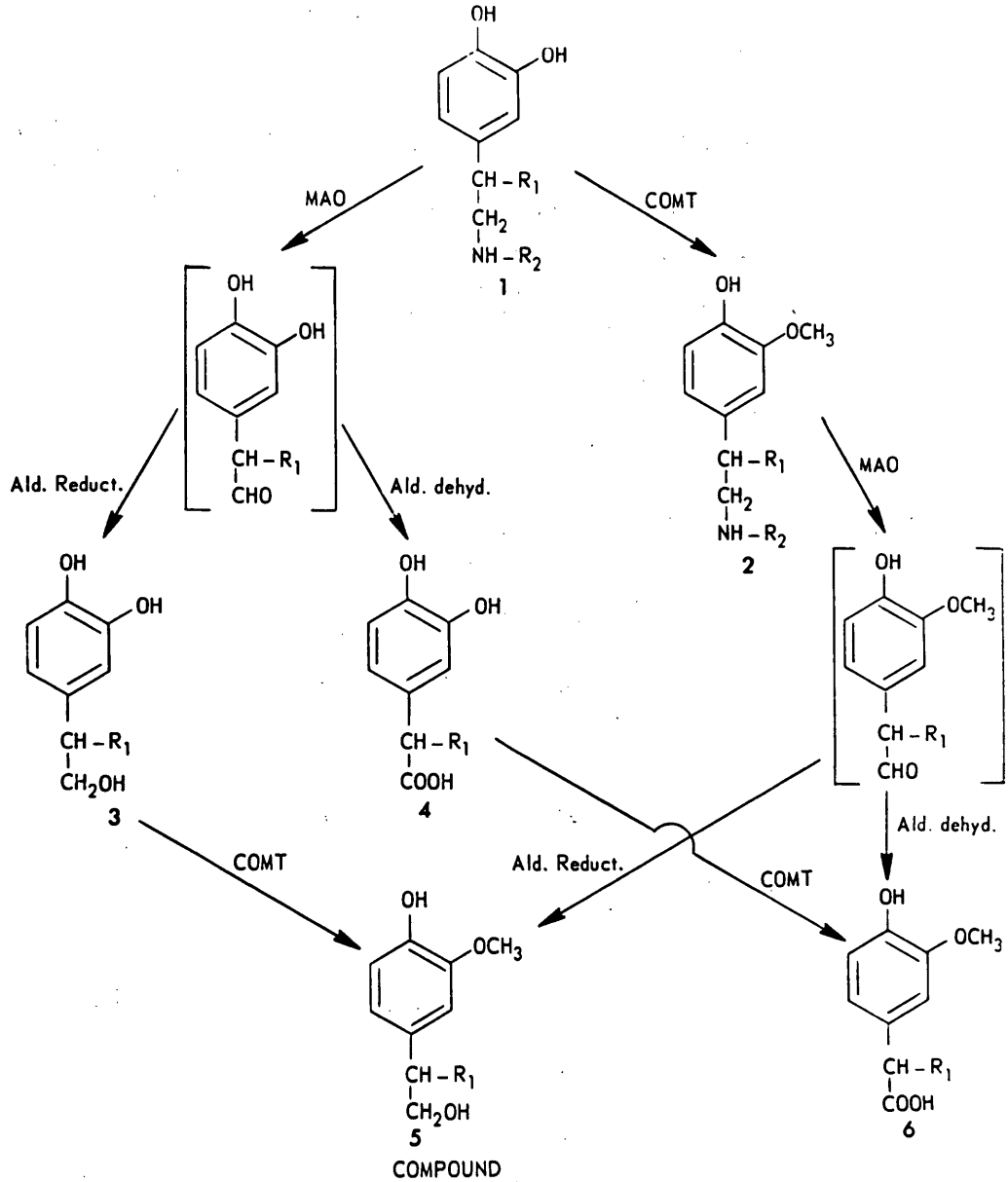
N-Methyl(meta)adrenaline resulting from N-methylation of adrenaline has been detected in normal human urine

(40 µg/24 hour) and in urine from patients with phaeochromocytoma (Itoh, Yoshinaga, Sato, Ishida and Wada, 1962). After its administration to rats, N-methyladrenaline is metabolized by COMT to yield N-methylmetadrenaline which can be detected in the urine (Axelrod, 1960).

The in vitro O-demethylation of metadrenaline to adrenaline by a microsomal enzyme preparation from rabbit liver has been reported (Axelrod and Szara, 1958), andaa similar preparation N-demethylates metadrenaline to normetadrenaline (Axelrod, 1960); in vivo N-demethylation, however, does not seem to occur in the rat (Axelrod, 1960; de Potter, Bacq, Criel, de Schaepdryver and Renson, 1963). O-Demethylation of metadrenaline appears to take place in vivo in the rat, but the adrenaline formed may be rapidly remethylated (Kopin, Axelrod and Gordon, 1961). Both O- and N-demethylation have been reported in the rabbit (de Potter, Bacq, Criel, de Schaepdryver and Renson, 1963) and the cat (Verly, Koch and Hunebelle, 1962).

The metabolism of dopamine

The major routes of dopamine metabolism are similar to those of noradrenaline and adrenaline (Fig.3). Thus oxidative deamination of dopamine to 3,4-dihydroxyphenylacetaldehyde may occur, followed by oxidation of the intermediate aldehyde to DOPAC or reduction to 3,4-dihydroxyphenylethanol. These products can be O-methylated to HVA



R_1 =	H	OH	OH
R_2 =	H	H	CH_3
1	Dopamine	Noradrenaline	Adrenaline
2	3-Methoxytyramine	Normetadrenaline	Metadrenaline
3	Dihyxroxyphenylethanol	Dihydroxyphenyl glycol	
4	Dihydroxyphenylacetic acid	Dihydroxymandelic acid	
5	4-Hydroxy-3-methoxyphenylethanol	4-Hydroxy-3-methoxy-phenylgylcol	
6	Homovanillic acid	4-Hydroxy-3-methoxymandelic acid	

Fig. 3. The metabolism of catecholamines.

and 4-hydroxy-3-methoxyphenylethanol respectively. Alternatively, dopamine can undergo initial O-methylation to 3-methoxytyramine which, in turn, is oxidatively deaminated to 4-hydroxy-3-methoxyphenylacetaldehyde; the latter is either reduced to 4-hydroxy-3-methoxyphenylethanol or oxidised to HVA (Goldstein, Friedhoff, Pomerantz and Simmons, 1960; Goldstein, Friedhoff, Pomerantz and Contrera, 1961; Goldstein and Gerber, 1963; Goodall and Alton, 1969).

The major metabolite of parenterally administered labelled dopamine in the rat (Goldstein, Friedhoff and Simmons, 1959; Williams, Babuscio and Watson, 1960) and man (Goodall and Alton, 1969) is HVA. This metabolite is excreted mainly in the free state in man (Shaw, McMillan and Armstrong, 1957; Goodall and Alton, 1969) and also in the rat, whether derived from exogenous sources (Williams, Babuscio and Watson, 1960), or endogenously (Shaw, McMillan and Armstrong, 1957). Only small quantities of 3-methoxytyramine are normally present in the urine. In rats this metabolite has been detected in the urine mostly (50-80%) as a glucuronide, after previous administration of dopamine (Axelrod, Senoh, and Witkop, 1958; Williams, Babuscio and Watson, 1960). Goodall and Alton (1969) have indicated that virtually all the urinary 3-methoxytyramine formed from ^{14}C-dopamine in the human is present as unspecified conjugates.

The presence of DOPAC in the urine was first demonstrated by von Euler, von Euler and Floding (1955)

and later confirmed by others (Armstrong, Shaw and Wall, 1956; Weil-Malherbe and Bone, 1957a). DOPAC was shown to be a metabolite of dopamine (Goldstein, Friedhoff and Simmons, 1959; Williams, Babuscio and Watson, 1960; Goodall and Alton, 1968a) although the urinary output of this metabolite is much lower than the corresponding excretion of HVA. Relatively more DOPAC arises from dopamine than DHMA from noradrenaline because dopamine is a better substrate than noradrenaline for MAO (Blaschko, 1952). Alton and Goodall (1969) have recently shown that a high proportion of infused ^{14}C-DOPAC is rapidaly O-methylated to HVA, mostly excreted in free form with a few per cent put out as conjugate. 3,4-Dihydroxybenzoic acid and trace amounts of vanillic acid were also found, together with several unidentified metabolites. A large proportion of urinary DOPAC is excreted in the conjugated form in man (von Euler, Floding and Lishajko, 1959; Goodall and Alton, 1968a). On the other hand, after injection of ^{14}C-DOPAC, only trace amounts of its conjugate, probably sulphate, could be detected in human urine (Alton and Goodall, 1969). DOPAC, formed from labelled dopamine, was present in the free state in rat urine (Williams, Babuscio and Watson, 1960).

The conversion of labelled dopamine to 4-hydroxy-3-methoxyphenylethanol (HMPE) in the rat was first demonstrated by Goldstein, Friedhoff, Pomerantz and Simmons (1960). The formation of this neutral compound was enhanced by

iproniazid treatment. In a subsequent publication, Goldstein, Friedhoff, Pomerantz and Contrera (1961) confirmed this finding, and also reported the presence of low concentrations of HMPE in normal human urine (about 50 µg per 1000 ml). Following administration of ^{14}C-dopamine, the deaminated product, 3,4-dihydroxyphenylethanol (DHPE) could only be detected in rat urine after inhibition of COMT with pyrogallol, or after aldehyde deydrogenase had been inhibited with calcium carbide. In a recent study, administration of large doses of DOPA in man resulted in the appearance of appreciable levels of HMPE in the urine (Calne, Karoum, Ruthven and Sandler, 1969). Goodall and Alton (1968a) have tentatively identified HMPE and DHPE as metabolites of infused ^{14}C-dopamine in man, mainly excreted in the form of (unspecified) conjugates.

N-Acetylation is a minor pathway of dopamine metabolism (Goldstein and Musacchio, 1962). On injection of radioactive amine in rats, both N-acetyldopamine and N-acetyl-3-methoxytyramine were excreted, with an increase in output after MAO inhibition. N-Acetylnormetadrenaline was not found in rat urine after injection of normetadrenaline, unless MAO had been blocked (Smith and Wortis, 1962), suggesting that N-acetylation is not a normal metabolic route for noradrenaline. The enzyme responsible for N-acetylation in vivo is probably the soluble rat liver enzyme isolated by Weissbach, Redfield and Axelrod (1961).

The metabolism of DOPA

Although the decarboxylation of DOPA to dopamine is an essential step in the major pathway of catecholamine biosynthesis, DOPA can be metabolized by other routes. Thus it may undergo transamination to form 3,4-dihydroxyphenylpyruvic acid (DHPPA) and 3,4-dihydroxyphenyllactic acid (DHPLA) which, in turn, may be 3-O-methylated to vanylpyruvic acid (VPA) and vanyllactic acid (VLA) respectively. Alternatively, these compounds are more likely to be formed by the transamination of β-(4-hydroxy-3-methoxyphenyl)alanine (Gjessing, 1963a; Gjessing and Borud, 1965); because, whilst DOPA is readily O-methylated to β-(4-hydroxy-3-methoxyphenyl)alanine (von Studnitz, 1960, 1961a; Gjessing, 1963b, 1964), the latter compound is not easily decarboxylated and is, therefore, more available for transamination (Gjessing and Borud, 1965). DOPA, VPA and VLA have all been detected in the urine of certain patients with catecholamine secreting tumours (Gjessing, 1963a; Gjessing and Borud, 1965; Smith, 1967).

Monoamine oxidase

A vast literature exists on monoamine oxidase (monoamine: O_2 oxidoreductase (deaminating) EC 1,4,3,4.) (MAO), but in the following account certain salient features only are considered. A number of reviews on various aspects of this enzyme are available (Blaschko,1952, 1963; Davison, 1958, Zeller, 1963; Gorkin, 1966; Pletscher, Gey and Burkard, 1966; Levy, 1968).

MAO promotes the biological inactivation of many amines, including the catecholamines and the indolealkylamines. The enzyme was initially described by Hare (1928) and Bernheim (1931) as tyramine oxidase, but was later shown to have a much broader spectrum of activity (Blaschko, Richter and Schlossmann, 1937a, b, c; Kohn, 1937; Pugh and Quastel, 1937a,b; Richter, 1937; Bhagvat, Blaschko and Richter, 1939). Under aerobic conditions, amine oxidases oxidatively deaminate amines with the production of an aldehyde, H_2O_2 and NH_3 or alkylamine. Since H_2O_2 is generally decomposed in tissues to H_2O and $\frac{1}{2}O_2$, e.g. by catalase, the overall reaction can be summarized thus:

$$R - CH_2 - NH_2 + \tfrac{1}{2}O_2 \rightarrow R - CHO + NH_3$$

The following discussion is limited, however, to enzymes classified as monoamine oxidases. As defined (Pletscher, Gey and Burkard, 1966), these enzymes cannot deaminate histamine and short-chain diamines, and are not inhibited by semicarbazide, thus distinguishing them from diamine oxidases.

MAO has been found in most vertebrates and occurs widely in mammalian tissues (Blaschko, 1952), where all organs appear to contain the enzyme apart from skeletal muscle. High MAO activity is invariably found in the liver (Bhagvat, Blaschko and Richter, 1939; Blaschko, 1952). With the exception of the rat, MAO activity is also high in the kidney; but the richest source of MAO yet encountered in mammalian tissue is the human salivary glands (Strömblad, 1959). MAO activity varies throughout the different anatomical regions of the brain; for example, Bogdanski Weissbach and Udenfriend (1957) observed highest activity in the hypothalamus of dog brain, using 5-hydroxytryptamine as substrate. Although the concentration of brain MAO, in terms of tissue weight, is moderate in comparison with that of tissues such as the liver, the presence of biologically active monoamines in the brain as potential substrates enhances the significance of the enzyme in that site. Similarly, MAO found in the peripheral sympathetic nervous sytem, particularly in the postganglionic nerve endings (Snyder, Fischer and Axelrod, 1965) may be associated with regulation of intraneuronal monoamine stores (Kopin, 1966).

The subcellular localization of MAO is mainly mitochondrial (Cotzias and Dole, 1951; Hawkins, 1952; Zile and Lardy, 1959; Weiner, 1960; Rodríguez de Lorez Arnaiz and de Robertis, 1962; Ostwald and Strittmatter, 1963; Baudhuin, Beaufay, Rahman-Li, Sellinger, Wattiaux, Jacques and de Duve, 1964). The enzyme shows great stability at physiological

pH if bound to mitochondria (Blaschko, Richter and Schlossmann, 1937b; Davison, 1958). MAO is located on the outer membrane of the mitochondrion (Schnaitman, Erwin and Greenawalt, 1967; Sottocasa, Kuylenstierna, Ernster and Bergstrand, 1967; Tipton, 1967, Boadle and Bloom, 1969), and although a soluble form of the enzyme is found in guinea pig liver supernatant (Blaschko and Duthie, 1945; Weissbach, Redfield and Udenfriend, 1957), MAO from other sources has proved difficult to solubilize and purify. Recently however, the use of sonication and/or detergents to solubilise the enzyme has led to samples of soluble MAO with activities several hundred fold greater than those of the original preparation (Youdim and Sourkes, 1966; Tipton, 1968a; Yasunobu, Igaue and Gomes, 1968). Estimates of the molecular weight of MAO vary from values of 102,000 for pig brain mitochondrial MAO (Tipton, 1968a) to 290,000 for the rat liver enzyme (Youdim and Sourkes, 1966). There is evidence for the presence of a flavin-like material in purified preparations of monoamine oxidase (Erwin and Hellerman, 1967; Sourkes, 1968; Tipton, 1968b, Yasunobu, Igaue and Gomes, 1968). Following analysis of the solubilized material the role of copper as a co-factor of MAO (Nara and Yasunobu, 1966; Nara, Gomes and Yasunobu, 1966) has recently been questioned (Erwin and Hellerman, 1967; Sourkes, 1968; Tipton, 1968b; Yasunobu, Igaue and Gomes, 1968).

There is now considerable evidence for the

existence of multiple forms of monoamine oxidase (Gorkin, 1966; Youdim and Sandler, 1967; Collins, Youdim and Sandler, 1968; Kim and D'Iorio, 1968; Ragland, 1968; Squires, 1968; Hartman, Kloepter and Yasunobu, 1968; Gomes, Igaue, Kloepfer and Yasunobu, 1969) but whether these are separate enzymes or different molecular forms of the same enzyme is still unclear. Solubilized preparations from a variety of sources have been separated electrophoretically into several bands (Youdim and Sandler, 1967; Collins, Youdim and Sandler, 1968; Kim and D'Iorio, 1968; Ragland, 1968; Gomes, Igaue, Kloepfer and Yasunobu, 1969; Youdim, Collins and Sandler, 1969) with sifferent substrate and inhibitor specificities, pH-activity curves and heat inactivation characterstics.

The affinity of MAO for its different substrates varies widely: it is relatively high for monophenolic or catecholic amines, but is lowered by the presence of a β-hydroxyl substituent in the side chain. Thus, of the physiological amines, β-phenylethylamine derivatives such as dopamine and tyramine are generally better substrates than noradrenaline and adrenaline (Blaschko, Richter and Schlossman, 1937a; Blaschko, 1952). Introduction of an α-methyl group into the β-phenylethylamine molecule results in competitive inhibition of the enzyme (Blaschko, Richter and Schlossmann, 1937a).

MAO has been reported to lack stereo-specificity towards the optical enantiomers of adrenaline and noradrenaline (Pratesi and Blaschko, 1959). Giachetti and Shore

(1966), however, reinvestigated the problem using a variety of MAO preparations and found that (-)-noradrenaline and adrenaline were deaminated at a faster rate than the (+)-isomers.

Numerous MAO inhibitors exist: they belong to various chemical classes and exhibit a variety of pharmacological actions. A number of reviews are available on this topic, (e.g. Zeller, 1963; Pletscher, Gey and Burkard, 1966). Most inhibotors act only <u>in vitro</u> (Pletscher, Gey and Burkard, 1966). The original discovery of iproniazid (1-isonicotinyl-2-isopropylhydrazine) as a potent MAO inhibitor (Zeller and Barsky, 1952) led to the introduction of other hydrazine derivatives, related to iproniazid, as inhibitors. These drugs and the chemically unrelated non-hydrazide pargyline (N-benzyl-N-methyl-propynylamine) (Taylor, Wykes, Gladish and Martin, 1960) are initially competitive inhibitors but rapidly form derivatives which inhibit in a non-competitive, irreversible and therefore, long lasting manner. (Taylor, Wykes, Gladish and Martin, 1960). Inhibition with drugs such as tranylcypromine (phenylcyclopropylamine) (Maass and Nimmo, 1959) can be either competitive or non-competitive according to the substrate (Sarkar, Banerjee, Ise and Zeller, 1960; Zeller and Sarkar, 1962). Other MAO inhibitors, as exemplified by the harmala alkaloids, harmine and harmaline, have a short-lasting revisible action on the enzyme (Udenfriend, Witkop, Redfield and Weissbach, 1958).

The role of oxidative deamination in the metabolism of catecholamines and 5-hydroxytryptamine is discussed elsewhere in this chapter. MAO is also involved in the metabolic degradation of other amines which may be endogenous or dietary in origin. Those arising as normal products of mammalian metabolism include phenylethylamine (Jepson, Lovenberg, Zaltzman, Oates, Sjoerdsma and Udenfriend, 1960) and tryptamine (Weissbach, King, Sjoerdsma and Udenfriend, 1959). The role of MAO in deaminating exogenous ammines is of some clinical importance. Patients treated with MAO inhibitors experience severe and occasionally fatal hypertensive crises after eating certain kinds of cheese (Blackwell, 1963). Horwitz, Lovenberg, Engelman and Sjoerdsma (1964) showed that the degree of the hypertension tends to be proportional to the tyramine content of the cheese. The association of MAO with its substates within the nerve cell has naturally attracted much attention; but in many tissues, particularly the liver (Klingman, 1966a), the greater proportion of enzyme activity is located in extraneuronal sites and probably fulfils some more general function such as detoxication. Changes in MAO activity in a variety of different tissues have been studied under various conditions; e.g. after the administration of the thyroid hormones (Novick, 1961) or sex steroids (Wurtman and Axelrod, 1963), or during the different phases of the menstrual cycle (Southgate, Grant, Pollard, Pryse-Davies, and Sandler, 1968). It is not known, however, whether

the observed fluctuations derive from activation/inhibition mechanisms or effects on enzyme synthesis.

Catechol-O-methyltransferase (COMT)

The 3-O-methylating enzyme, catechol-O-methyltransferase (COMT, EC.2.1.1.6) which catalyses the transfer of methyl groups from S-adenosylmethionine to the meta-hydroxyl group of catecholamines and other catechols, was first described by Axelrod (1957). Mg^{++} or other divalent cations, such as Mn^{++}, Co^{++}, Zn^{++} or Ni^{++}, are required for activation of the enzyme (Axelrod and Tomchick, 1958; Sehoh, Tokuyama and Witkop, 1962). A 200-fold purification of rat liver COMT has recently been reported (Anderson and D'Iorio, 1968). All catechols were substrates of the purified enzyme, which had an estimated molecular weight of 29,000. The presence of a sulphydryl group, as an active site on the enzyme surface is indicated, since SH-binding agents inhibit COMT activity (Axelrod and Tomchick, 1958). A potent and specific antibody to rat liver COMT has recently been produced by Assicot and Bohuon (1969).

COMT is distributed widely in mammalian tissues, with particularly high activity in the liver and the kidney (Axelrod and Tomchick, 1958; Axelrod, Albers and Clemente, 1959). Appreciable enzyme activity has been detected in the central nervous system and in peripheral nerves. COMT was associated with a nerve-ending fraction in rat brain

during density-gradient centrifugation studies (Alberici, Rodríguez de Lorez Arnaiz and de Robertis, 1965). An atypical COMT is present in the microsomal fraction of liver, with a pH optimum and species distribution unlike that of the more common enzyme (Inscoe, Daly and Axelrod, 1965). COMT is a highly soluble enzyme localized in the cell sap of most tissues (Axelrod and Tomchick, 1958). Traiger and Calvert (1969), however, have recently described particle bound COMT activity in rat epididymal adipose tissue. The enzyme is thought to be localized outside the neurone in both peripheral tissue (Kopin and Gordon, 1962; Iversen, Glowinski and Axelrod, 1966; Iversen, Jarrott and Langer, 1968) and brain (Carlsson and Hillarp, 1962).

COMT does not exhibit stereo-specificity, and methylates a variety of catecholamines including DOPA (von Studnitz, 1961a). The 3-hydroxyl group of 3,4-dihydroxy catechols (Masri, Booth and de Eds, 1962) is preferentially O-methylated by the enzyme. Partial interconversion of 4-hydroxy-3-methoxyphenylethylamine or 3-hydroxy-4-methoxy-phenylethylamine, however, in the presence of purified COMT and S-adenosylmethionine, has been reported <u>in vitro</u> (Daly, Axelrod and Witkop, 1960; Kopin, Axelrod and Gordon, 1961).

Polyphenols and catechols, such as pyrogallol (Axelrod and Laroche, 1959; Udenfriend, Creveling, Ozaki, Daly and Witkop, 1959), act as competitive inhibitors <u>in vitro</u> and <u>in vivo</u>, but more potent inhibitors including

tropolones, dopacetamide, dimethylpapaverine, gallic acid ester, and other catechol esters (Belleau and Burba, 1961; Carlsson, Lindqvist, Fila-Hromadko and Corrodi, 1962; Ross and Haljasmaa, 1964a,b; Burba and Murnaghan, 1965) have been described. Adnamine and noradnamine, which are acid degradation products of adrenaline and noradrenaline respectively (Roberts and Broadley, 1965), and potent inhibitors of COMT in vitro (Abbs, Broadley and Roberts, 1967), have been postulated as the biochemical cause of endogenous depression (Pare, 1965).

The metabolism of circulating catecholamines

The metabolic fate of circulating catecholamines has been extensively investigated by the use of radioisotopic precursors, both in man and the experimental animal. Early studies on the metabolism of labelled adrenaline (La Brosse, Axelrod and Kety, 1958; Kirshner, Goodall and Rosen, 1958, 1959; La Brosse, Axelrod, Kopin and Kety, 1961; La Brosse, Mann and Kety, 1961) and noradrenaline (Goodall, Kirshner and Rosen, 1959) revealed that O-methylation is of great importance as the initial step in the metabolism of circulating catecholamines in man. The relative magnitudes of O-methylation and deamination in adrenaline catabolism has been estimated by the use of a double-labelling technique (Kopin, 1960) involving simultaneous administration of ^{3}H-adrenaline and ^{14}C-metadrenaline. From the ratios of ^{3}H: ^{14}C and the total radioactivity of each metabolite isolated in the urine, the extent of O-methylation and deamination were determined. Thus, it was calculated that about 70% of injected adrenaline is initially O-methylated to metadrenaline and 25% deaminated. In the rat where the proportions are similar (Kopin, Axelrod and Gordon, 1961), intrajugular infusion of ^{3}H-adrenaline resulted in about 10% of the injected catecholamine being excreted as conjugated metabolites into the bile, most of which is lost in the faeces (Hertting and LaBrosse, 1962). Although initial

O-methylation is the predominant route of metabolism of circulating ^{3}H-noradrenaline in the rat (Kopin and Gordon, 1963), less than 10% of the injected dose is excreted as free normetadrenaline with approximately the same amount in conjugated form.

Quantitative differences exist in the ratio of metadrenaline or normetadrenaline:VMA found in the urine after administration of adrenaline or noradrenaline in man. Following infusion of adrenaline-2-^{14}C for 1 hour, 36-57% of the radioactivity recovered in the first 24 hours was present as metadrenaline (free and conjugated) and 22-27% as VMA (Kirshner, Goodall and Rosen, 1958, 1959), although in a similar study involving infusion of adrenaline-7-^{3}H for 30 minutes, La Brosse, Axelrod, Kopin and Kety (1961) detected approximately 40% of each metabolite in a 54 hour urine sample. VMA (32-36%) was present in greater quantity than normetadrenaline (23-24%), however, 24 hours after infusion with noradrenaline-2-^{14}C for 1 hour (Goodall, Kirshner and Rosen, 1959; Rosen and Goodall, 1962b). Noradrenaline is more readily inactivated by tissue binding (Whitby, Axelrod and Weil-Malherbe, 1961) than adrenaline (Axelrod, Weil-Malherbe and Tomchick, 1959), and bound amine, when released, is preferentially deaminated rather than O-methylated (Kopin and Gordon, 1963), so that a greater proportion of VMA derived from noradrenaline might be

expected to be present at 24 hours in comparison with that derived from adrenaline. Furthermore, noradrenaline is a slightly better substrate for MAO than adrenaline and normetadrenaline undergoes oxidative deamination more readily than metadrenaline (Leeper, Weissbach and Udenfriend, 1958).

The excretion pattern of the metabolites of intravenously injected adrenaline (Goodall and Alton, 1965) and noradrenaline (Goodall and Rosen, 1963; Goodall, Harlan and Alton, 1967) in man have been measured at 10 minute intervals up to 60 or 70 minutes and, thereafter, at 6 and 24 hours after injection. After both, there was an initial rapid excretion of injected amine and free metadrenalines, followed closely by a sharp rise in VMA levels. The amount of conjugated metadrenalines rose more slowly, approaching VMA levels after 24 hours when noradrenaline was injected, and exceeding them after adrenaline. The metabolic fate of injected noradrenaline-7-^{3}H followed a different pattern in the rat (Kopin and Gordon, 1963). The quantities of both free and conjugated normetadrenaline in the initial 3 hour period after injection exceeded those found at 10-13 hours, whereas the levels of VMA and HMPG increased at the later time. A much greater amount of noradrenaline, however, was administered to the rats on a weight basis than to humans in the experiments described above, so that a greater proportion of free amine was probably available for preferential O-methylation.

Alton and Goodall (1968) reported that the metabolism of smaller doses of adrenaline-2-^{14}C infused over 9 hours in the human was similar to the pattern reported previously for larger doses of rapidly injected adrenaline (Goodall and Alton, 1965), suggesting that the large amounts of adrenaline released into the circulation in response to transient stress are metabolized in the same way as smaller quantities of the amine which may be released in normal quiescent states.

Dopamine is a much better substrate for MAO than either noradrenaline or adrenaline (Leeper, Weissbach and Udenfriend, 1958). The percentage of total DOPAC recovered in 24 hour urine samples after administration of dopamine to rats (Goldstein, Friedhoff and Simmons, 1959; Williams, Babuscio and Watson, 1960) and man (Alton and Goodall, 1969) exceeds the percentage of urinary DHMA derived from circulating adrenaline and noradrenaline (La Brosse, Axelrod, Kopin and Kety, 1961; Goodall, Harlan and Alton, 1967). Conversely, although dopamine is a good substrate for COMT (Axelrod, 1957), the relative proportion of the O-methylated metabolite of dopamine, 3-methoxytyramine, in the urine is considerably less than the equivalent proportions of urinary metadrenalines following injection of noradrenaline and adrenaline (Williams, Babuscio and Watson, 1960; La Brosse, Axelrod, Kopin and Kety, 1961; Goodall, Harlan and Alton, 1967; Alton and Goodall, 1969). A significant decrease in the rate of dopamine-^{14}C

metabolism in the rat and mouse spleen and heart has been reported after pretreatment of the animals with the MAO inhibitor, iproniazid (Symchowicz and Korduba, 1967).

Extensive investigation of the metabolism of circulating dopamine-2-^{14}C in man has recently been undertaken (Goodall and Alton, 1968a). At the end of 5 days, approximately 75% of the infused dopamine has been directly metabolized into the non-β- hydroxylated dopamine series of metabolic products; of these, HVA was present in by far the largest quantities. The remaining 25% of the infused dopamine was synthesized into noradrenaline or its metabolic products. Conversion to HVA occurred very rapidly during the initial 4 hour infusion period and the major portion of the acid was present in the free form in the urine. Detailed monitoring of metabolites at short intervals (10 minutes) following infusion of ^{14}C-DOPAC indicated that this compound is rapidly O-methylated to HVA (Alton and Goodall, 1969). The results of these human studies confirm previous findings in animals (Goldstein, Friedhoff and Simmons, 1959; Williams, Babuscio and Watson, 1960) showing that HVA is the major urinary metabolite of injected dopamine.

Distinct differences exist between patterns of urinary metabolites derived from endogenous and administered catecholamines, so that results obtained from studies of the metabolism of injected labelled catecholamines are necessarily limited in value; whereas for example, parenteral

radioactive adrenaline or noradrenaline in man show recoveries of between 20 and 40% of metadrenaline or normetadrenaline and a similar amount of VMA, the total quantity of endogenous metadrenalines excreted in normal human urine, is only one-tenth to one-twentieth of the VMA output (Sandler and Ruthven, 1969).

Such variations can be explained in terms of differences in mode of inactivation. On intravenous injection noradrenaline enters the general circulation where a proportion is likely rapidly to reach the liver, a rich source of both COMT and MAO (Blaschko, 1952; Davison, 1958; Axelrod, 1959, 1966); here O-methylation and deamination with O-methylation will occur, followed by hepatic conjugation (Hertting and La Brosse, 1962; Carlsson and Waldeck, 1963; Goodall, Alton and Henry, 1964; Hazelrig, Zimon, Sheps, Schirger and Owen, 1967; Zimon, Tyce, Flock, Sheps and Owen, 1967). Another portion of circulating catecholamine is rapidly taken up and bound in the tissues, particularly within sympathetic nerves (Axelrod, Weil-Malherbe and Tomchick 1959; Whitby, Axelrod and Weil-Malherbe, 1961; Iversen and Whitby, 1962; Wolfe, Potter, Axelrod and Richardson, 1962; Chidsey and Harrison, 1963) where it is likely to equilibrate to a greater or lesser extent with endogenous noradrenaline; the mixed amine is then slowly released and metabolized. Oxidative deamination appears to be the predominant route of metabolism for catecholamines

within the neurone (Kopin and Gordon, 1962; 1963; Kopin, 1966) and some of the amine taken up will be deaminated before re-release into the circulation and further metabolism. A greater involvement of intraneuronal MAO in the metabolism of endogenous, as opposed to administered, noradrenaline may be responsible for the larger proportion of deaminated metabolites detected in normal urine. In contrast, the physiologically active amine released by sympathetic nerve stimulation, and overflowing into the circulation, will be metabolized primarily by COMT and is presumably a source of urinary normetadrenaline (Kopin and Gordon, 1962, 1963; Kopin, 1966).

The metabolism of parenterally-administered adrenaline should more closely resemble the catabolism of the high concentrations of endogenous amine released into the circulation on stimulation of the adrenal medulla than that derived from other neural sites. Furthermore, tissue binding appears to be less important for the inactivation of adrenaline than it is for noradrenaline (Axelrod, Weil-Malherbe and Tomchick, 1959; Whitby, Axelrod and Weil-Malherbe, 1961). Similar considerations apply to the metabolism of catecholamines secreted into the circulation from a catecholamine-secreting tumour (Crout, 1966; von Studnitz, 1966).

Much of the work on the metabolism of circulating catecholamines has involved the administration of racemic mixtures of amines, whereas the endogenous compounds are

laevo-rotatory. Whilst in vitro findings (Axelrod and Tomchick, 1958) suggest that the two isomers are O-methylated at similar velocities, their rates of oxidative deamination may differ (see page 55).

Catecholamine metabolism in tissues

Although there have been few studies of normal tissue catecholamine metabolism in man, details of the catabolic processes which occur in individual animal tissues have steadily accumulated. Some of the salient features are briefly described below.

The finding that O-methylated compounds are the principal metabolites of noradrenaline-^{14}C injected into the lateral ventricle of cat brain (Mannarino, Kirshner and Nashold, 1963) indicates that O-methylation is important in the metabolism of noradrenaline in the brain. Following the injection of small amounts of noradrenaline-^{3}H into the lateral ventricle of rat brain (Glowinski, Kopin and Axelrod, 1965), analysis of radioactivity remaining two hours after administration showed that unchanged noradrenaline-^{3}H accounted for nearly 50%, normetadrenaline-^{3}H for 14%, deaminated catechol metabolites for 3% and O-methylated deaminated metabolites for 30%.HMPG, rather than VMA, was the predominant O-methylated oxidatively deaminated metabolite of noradrenaline. Schanberg, Schildkraut, Breese and Kopin (1968) reported that the major metabolite of intracisternally-injected normetadrenaline-^{3}H and noradrenaline-^{3}H in rat

brain is the sulphate conjugate of HMPG. The very slow rate of disappearance of normetadrenaline-^{3}H from the brain, after inhibition of MAO, suggested that unaltered normetadrenaline does not readily pass out of the brain. This conclusion was also indirectly supported by the findings of Glowinski, Kopin and Axelrod (1965). In an investigation utilising a double-isotope technique, Maas and Landis (1968) estimated that 22-27% of HMPG, up to 1% of VMA, and less than 11% of normetadrenaline excreted in dog urine had its origin in the brain pool of noradrenaline. The results of the in vivo brain studies are supported by in vitro findings. On incubation with rabbit brain cerebral cortex slices (Rutledge and Jonason, 1967; Jonason, 1969), noradrenaline was primarily deaminated to the two phenolic glycols, HMPG and DHPG. DHMA and normetadrenaline constituted about 20% of total catabolites, whereas VMA occurred in the smallest quantity (7%).

Recently, observations of endogenous levels of HMPG and HMPG sulphate in the brains of several mammalian species have been reported (Schanberg, Breese, Schildkraut, Gordon and Kopin, 1968; Sharman, 1969a,b). Free HMPG has been detected in the whole brain and hypothalamus of the cat, in the whole brain of the rhesus monkey, and in the hypothalamus of the mouse and the rabbit; conjugated HMPG was present in the brain of the rat, guinea pig and green monkey, and in human cerebro-spinal fluid. Free DHPG has also been

found in the hypothalamus of cat, mouse and rabbit (Sharman, 1969a,b). Attempts to isolate endogenous VMA from brain tissue have been negative (Andén, Roos and Werdinius, 1964; Laverty and Sharman, 1965b; Juorio, Sharman and Trajkov, 1966). The occurrence of DHMA in brain has been reported (Matsuoka, 1964; Matsuoka, Yoshida and Imaizumi, 1964), but this finding has not been confirmed (Andén, Roos and Werdinius, 1969).

The only deaminated metabolites found endogenously are DOPAC (Rosengren, 1960; Andén, Roos and Werdinius, 1963a) and HVA (Andén, Roos and Werdinius, 1963b; Sharman, 1963; Bernheimer, 1964). Conjugates of DHPE and HMPE have been found in rat brain after intravenous injection of DOPA-^{14}C (Goldstein and Gerber, 1963). On incubation with slices of rabbit cerebral cortex, dopamine was primarily deaminated to DOPAC and HVA (Rutledge and Jonason, 1967; Jonason, 1969), whilst smaller quantities of 3-methoxytyramine (8%) and negligible amounts of DMPE and HMPE were detected. On incubation of dopamine-^{14}C with slices of rabbit caudate nucleus (Jonason and Rutledge, 1968; Jonason, 1969) however, the levels of HVA formed were found to be low in comparison with those of DOPAC. DHPE, although a minor metabolite, was detected in quantities five times higher than the levels formed in rabbit cortex <u>in vitro</u>, whereas the amount of HMPE formed was negligible. In rabbit caudate nucleus, noradrenaline was predominantly metabolized to

DHPG and DHMA (Jonason and Rutledge, 1968).

An HVA transport system which can be inhibited by probenecid is present in the rat (Werdinius, 1966) and mouse (Sharman, 1966). The concentration of DOPAC in the brain is unaffected, however, when probenecid is administered to mice (Sharman, 1968).

Catecholamine metabolism has also been studied in severl peripheral tissues, using <u>in vitro</u> techniques. Isolated rabbit atria incubated with noradrenaline-^{3}H metabolized it predominantly to DHPG, and to a lesser extent, HMPG (Rutledge and Weiner, 1967). Smaller quantities of DHMA and VMA were also formed. Labelled dopamine was converted to its acid metabolites, DOPAC and HVA, but its neutral metabolites were not assayed. Similarly, the major deaminated metabolic products of noradrenaline in the isolated guinea-pig vas deferens are the glycols, notably DHPG (Weiner and Rabadjija, 1968). The proportion of O-methylated acid and neutral metabolites detected, was dependent on the quantity of noradrenaline present in the incubation mixture, COMT being more active at lower amine concentration. Normetadrenaline was not assayed. In slices of rat submaxillary and sublingual glands, however, noradrenaline was primarily 3-O-methylated by COMT to normetadrenaline which constituted about 60% of the total metabolites (Jonason, 1969). The salivary glands are known to have a high activity of COMT (Axelrod, Albers and Clemente, 1959).

DHPG and HMPG together constituted 26%, and VMA plus DHMA, 14%. On incubation of the slices with dopamine, the noradrenaline formed was predominantly metabolized to the glycols, an indication that intraneuronal noradrenaline is initially deaminated by MAO, prior to O-methylation by extraneuronal COMT (Kopin and Gordon, 1962). As in the brain, dopamine was primarily converted to the phenolic acids, DOPAC and HVA.

The main feature of the studies reported above is the qualitative difference between the major end-products of dopamine and noradrenaline metabolism in the tissues, particularly in the brain; the aldehyde formed from dopamine by MAO is mainly oxidized by aldehyde dehydrogenase to phenolic acids, whereas that of noradrenaline is predominantly reduced by an aldehyde reductase. In a recent comprehensive study, Breese, Chase and Kopin (1969a) showed that in the rat brain at least, the present of a β-hydroxyl group favours intermediate aldehyde reduction. A hydroxyl group on the benzene ring also appears to enhance reduction in the brain, since phenylethanolamine was not converted primarily to glycol, as were other β-hydroxylated compounds with a free phenolic group. The observation that 4-hydroxyphenylglycol, or its conjugate, was the major metabolite formed after intracisternal injection of octopamine-^{3}H in rat brain, whereas the principal metabolite formed from tyramine-^{3}H was 4-hydroxyphenylacetic acid (Breese, Chase and Kopin, 1969b), support these conclusions.

Termination of the actions of noradrenaline after release from sympathetic nerves

In theory, at least three different mechanisms can be postulated for elimination of the action of the adrenergic transmitter: 1. by local enzymatic destruction, 2. by diffusion into the blood stream, 3. by reabsorption into the nerve fibres.

There is a substantial body of evidence suggesting that at normal physiological stimulation frequencies only a small proportion of transmitter escapes into the circulation or tissue spaces (Celander, 1954; Brown and Gillespie, 1957; Eakins and Eakins, 1964; Brown, 1965; Folkow, Häggendal and Lisander, 1967). von Euler and Luft (1951) calculated that 0.5-1µg of noradrenaline per minute overflows into the circulation in normal man but, in general, this overflow only increases markedly when adrenergic fibres are stimulated at supraphysiological rates (Celander, 1954) or during skeletal muscle exercise (Carlsson, Folkow and Häggendal, 1964; Folkow, Häggendal and Lisander, 1967) where an increased blood supply may contribute to the overflow.

Regarding the two possible local inactivation mechanisms, that of enzymic inactivation by COMT and MAO appears to be of minor importance for local transmitter inactivation under physiological conditions. Pharmacological

evidence shows that even when both enzymes are inhibited simultaneously, the pressor effects of intravenously administered catecholamines are not significantly potentiated or prolonged (Crout, 1961a). The inactivation of noradrenaline released by nerve stimulation is not prevented when MAO or COMT, or both enzymes, are inhibited (Brown and Gillespie, 1957; Bacq, Brown and Perry, 1960; Stinson, 1961; Folkow, Häggendal and Lisander, 1967). Furthermore, there is no net change in the outflow of noradrenaline metabolites after sympathetic stimulation of skeletal muscle in dogs which had previously received labelled noradrenaline (Rosell, Kopin and Axelrod, 1963).

There is, however, considerable evidence that re-uptake of noradrenaline via a 'membrane pump' may be the main inactivation mechanism of transmitter released at the sympathetic nerve endings (see Iversen, 1968). Early tissue distribution studies, using labelled catecholamines of high specific activity, have indicated that sympathetic nerves might be involved in the selective uptake and retention of catecholamines (Axelrod, Weil-Malherbe and Tomchick, 1959; Whitby, Axelrod and Weil-Malherbe, 1961), and in 1960 Paton suggested that sympathetic nerves might take up and re-use most of the transmitter which they liberate. In the ensuing years many biochemical and pharmacological studies of the uptake of low concentrations of catecholamine into sympathetic nerves have provided some

insight into the processes which may occur under physiological conditions.

Uptake of radioactive noradrenaline is greatest in tissues with a rich sympathetic innervation (Whitby, Axelrod and Weil-Malherbe, 1961). Thus tissues which lack a normal sympathetic innervation, either because of surgical sympathectomy (Hertting, Axelrod, Kopin and Whitby, 1961; Strömblad and Nickerson, 1961; Fischer, Kopin and Axelrod, 1965; Van Orden, Bensch, Langer and Trendelenburg, 1967), immunosympathectomy (Iversen, Glowinski and Axelrod, 1965a, 1966; Sjöqvist, Titus, Michaelson, Taylor and Richardson, 1965; Zaimis, Berk and Callingham, 1965), or chemical sympathectomy (Thoenen and Tranzer, 1968) have a greatly reduced uptake capacity. Furthermore, sympathetic nerves have been shown to be the sites of noradrenaline uptake by radioautographic (Wolfe, Potter, Richardson and Axelrod, 1962) and histochemical (Malmfors, 1965) techniques, and in a study combining the two techniques (Gillespie and Kirpekar, 1965a).

Several observations show that the uptake of catecholamines occurs against a considerable concentration gradient (Axelrod, Weil-Malherbe and Tomchick, 1959; Dengler, Spiegel and Titus, 1961a; Whitby, Axelrod and Weil-Malherbe, 1961; Iversen, 1963; Lindmar and Muscholl, 1964). In studies using low doses of catecholamine (Muscholl, 1961; Iversen, 1963; Crout, 1964), it has been demonstrated that

the endogenous noradrenaline content of tissues can be approximately doubled by accumulation of the exogenous amine. The uptake process at low concentrations of amine shows structural (Axelrod, Weil-Malherbe and Tomchick, 1959; Strömblad and Nickersen, 1961; Whitby, Axelrod and Weil-Malherbe, 1961; Iversen and Whitby, 1962; Ross, 1963; Burgen and Iversen, 1965) and stereochemical (Iversen, 1963, 1967; Maickel, Beaven and Brodie, 1963; Beaven and Maickel, 1964) specificity, so that noradrenaline is taken up more rapidly than its N-substituted derivatives and the (-)-isomers of noradrenaline and adrenaline are accumulated more quickly than the corresponding (+)-isomers. In studies on the perfused rat heart, Iversen (1963, 1967) has shown that the initial rates of noradrenaline and adrenaline uptake at various external concentrations of the two amines can be described by classical enzyme kinetics, indicating that the 'membrane pump' can be saturated. Green and Miller (1966) have also shown that catecholamine uptake by strips of rat uterus follows similar kinetics. The uptake process is temperature dependent (Iversen, 1963) and requires the presence of extracellular Na^+ ions (Iversen and Kravitz, 1966; Kirpekar and Misu, 1967). Wakade and Furchgott (1968) have shown that energy is required for uptake of noradrenaline by isolated guinea pig atria and that either glycolysis of exogenous glucose or oxidation of non-carbohydrate endogenous substrates can be used as the source of energy.

The list of drugs which can inhibit this uptake process is extensive (Iversen, 1967). It includes compounds such as cocaine (Whitby, Hertting and Axelrod, 1960; Muscholl, 1961), phenoxybenzamine (Axelrod, Hertting and Potter, 1962), imipramine and desmethylimipramine (Axelrod, Whitby and Hertting, 1961; Dengler, Spiegel and Titus, 1961b; Hertting, Axelrod and Whitby, 1961; Axelrod, Hertting and Potter, 1962; Titus and Spiegel, 1962) as well as structural analogues of noradrenaline (Burgen and Iversen, 1965). Inhibitors of uptake should potentiate the actions of noradrenaline released in response to sympathetic nerve stimulation. Such a potentiation has been demonstrated for cocaine (Huković, 1959; Trendelenburg, 1959; Haefely, Hürlimann and Thoenen, 1964), phenoxybenzamine (Huković, 1959) and imipramine (Thoenen, Hürlimann and Haefely, 1964a). These drugs might also be expected to promote the overflow of transmitter on stimulation of sympathetic nerves. However, the results of such studies have been equivocal and whilst most investigations have indicated that phenoxybenzamine causes a large increase in transmitter overflow (Brown and Gillespie, 1957; Thoenen, Hürlimann and Haefely, 1964b; Brown, 1965), both positive and negative results have been obtained with cocaine and imipramine (Trendelenburg, 1959; Blakeley, Brown and Ferry, 1963; Kirpekar and Cervoni, 1963; Eakins and Eakins, 1964; Haefely, Hürlimann and Thoenen, 1964; Thoenen, Hürlimann and Haefely, 1964a,c:

Boullin, Costa and Brodie, 1967). These discrepancies have been discussed by Iversen (1968). Blakeley, Brown and Geffen (1964) have shown that the amount of noradrenaline released in response to repeated stimulation of the cat spleen rapidly falls off in the presence of phenoxybenzamine, thus providing further evidence for the normal reuptake of transmitter. Finally, in an electro-physiological study, Bell (1966) showed that cocaine significantly increases the time course of excitatory junctional potentials in the smooth muscle cells of guinea-pig vas deferens.

Catecholamine metabolism in neuroblastoma

The neuroblastoma (sympathoblastoma or sympathogonioma) is a malignant neoplasm of sympathetic origin, and can arise in any site where tissues of this type exists (Tefft and Wittenborg, 1968). Embryologically, the nerve cells of the sympathetic system develop from primitive elements of the neural crest, the sympathogonia, which can differentiate either along the chromaffin line to yield the phaeochromoblast and the phaeochromocyte, or along the neurogenic line forming the sympathoblast, also called the neuroblast (Käser, 1966). The latter finally gives rise to the mature ganglion cell, tumours of which are called ganglioneuroma (Willis, 1962). Tumours of sympathogonia and sympathoblasts have been referred to as sympathogonioma and sympathoblastoma respectively, both of which are commonly

known as neuroblastoma (Gjessing, 1968). Neuroblastoma and ganglioneuroma, however, are not distinct species of tumours (Willis, 1962); growths of transitional or mixed structure ("ganglioneuroblastomas") often occur. The more malignant neuroblastoma, which tends to arise in the infant and younger child, is the sympathogonioma (Rubin, 1968); the sympathoblastoma is more highly differentiated and less malignant when it occurs in later life.

The report describing increased excretion of pressor amines in the urine of an infant with neuroblastoma (Mason, Hart-Mercer, Millar, Strang and Wynne, 1957), was the forerunner of numerous studies in which a broad range of catecholamine precursors and metabolites has been investigated.

In contrast with phaeochromocytoma tumour tissue, which originates from the chomaffin cell system and often contains large quantities of both adrenaline and noradrenaline (Kennedy, Symington and Woodger, 1961; Robinson, 1963; Crout and Sjoerdsma, 1964; Page and Jacoby, 1964; Gjessing, 1964; Ruthven, 1965) relatively low tissue concentrations of either amine are found in neuroblastoma (Mason, Hart-Mercer, Millar, Strang and Wynne, 1957; Bell, 1963; Crout and Sjoerdsma, 1964; Page and Jacoby, 1964; Gjessing, 1964; Greer, Anton, Williams and Schevarria, 1965; Käser, 1966). An excess of noradrenaline is, however, frequently (Greenberg and Gardner, 1960; von Studnitz, Käser and Sjoerdsma 1963; Page and Jacoby, 1964; Ruthven, 1965), though not

invariably (Voorhess and Gardner, 1960; Bell, 1963; Sourkes, Denton, Murphy, Chavez and St. Cyr, 1963), detected in the urine of neuroblastoma patients. Adrenaline excretion has been reported to be normal in neuroblastoma.

A raised urinary output of β-hydroxylated metabolites, deriving from noradrenaline, is generally found in neuroblastoma. The original observations of an elevated excretion of VMA in patients with neuroblastoma (Sandler and Ruthven, 1959) and the related neural tumour of ganglionic cells, ganglioneuroma (Greenberg and Gardner, 1959), have been confirmed many times (see Käser, 1966). VMA excretion has, however, been reported to be normal in some cases of neuroblastoma (Bell and Steward, 1961; Voorhess and Gardner, 1960, 1961a; Kontras, 1962; Greer and Williams, 1963a; Käser, Bettex and von Studnitz, 1964; Sunderman, 1964; Ruthven, 1965; Käser, 1966). In these cases, however, normetadrenaline or noradrenaline excretion may be significantly increased (Voorhess and Gardner, 1960, 1961b, 1962; Bell, 1963, 1968; Käser, 1966). A significantly increased output of HMPG has also been reported in the urine of neuroblastoma patients (La Brosse and Karon, 1962; Gjessing, 1963b; Ruthven and Sandler, 1965). Another minor metabolite of noradrenaline, N-acetylnoradrenaline, has been detected in several cases of neuroblastoma (Herrlich and Sekeris, 1964). Gjessing (1963b) detected considerable quantities of vanillic acid in the urine of neuroblastoma

patients, but expressed doubts about its endogenous origin.

The most characteristic biochemical feature of neuroblastoma, however, is the predominant secretion of noradrenaline precursors, leading to an elevated urinary output of dopamine (Greenberg and Gardner, 1960; von Studnitz, 1960) and DOPA (von Studnitz, 1960). These observations have been frequently confirmed (see Kaser, 1966). The occurrence of DOPA in neuroblastoma tissue has also been described (Smellie and Sandler, 1961). The 3-methoxy-derivative of DOPA, β -(4-hydroxy-3-methoxyphenyl) alanine has been detected in the urine of some cases of neuroblastoma (von Studnitz, 1960, 1961a; Gjessing, 1963b, 1964). Excess DOPA is also the likely precursor of the large quantities of vanylpyruvic and vanyllactic acids, and the smaller amounts of dihydroxyphenylpyruvic acids, detected in the urine of neuroblastoma patients (Gjessing, 1963a; Gjessing and Borud, 1965).

The increased dopamine excretion in neuroblastoma is accompanied by a significantly raised output of its main metabolites, 3-methoxytyramine (von Studnitz, 1960; Gjessing, 1963b, 1964; Ruthven, 1965), DOPAC (von Studnitz, 1960; Sourkes, Denton, Murphy, Chavez and St. Cyr, 1963) and HVA (Greenberg and Gardner, 1960; von Studnitz, 1960). The minor metabolite of dopamine, HMPE, which is normally barely detectable in human urine, is present in increased amounts in the urine of neuroblastoma patients (La Brosse and Karon, 1962; Gjessing, 1963b; Karoum, 1969). The wide spectrum of

metabolites which may be present in neuroblastoma is emphasized by the detection in the urine of another minor metabolite of dopamine, N-acetyldopamine (Hanson and von Studnitz, 1965).

Only a small proportion of patients with neuroblastoma show a significant rise in blood pressure (Voorhess and Gardner, 1962; Sourkes, Denton, Murphy, Chavez and St. Cyr, 1963; von Studnitz, Käser and Sjoerdsma, 1963), whereas a severe degree of hypertension is generally found in phaeochromocytoma (Hermann and Mornex, 1964). A possible explanation for these findings has been postulated by Sandler and Ruthven (1966); a relatively small proportion, only, of free noradrenaline may enter the circulation of the patient with neuroblastoma, metabolic degradation occurring either in the tumour tissue, or at peripheral sites from the immediate further catabolism of noradrenaline formed there from circulating dopamine.

5-Hydroxytryptamine

Origin and distribution

In the 1930's Erspamer and his colleagues began to study a substance which they called enteramine, responsible for the peculiar histochemical properties of the enterochromaffin cells of the gastro-intentinal mucosa (Vialli, 1966). By the late 1940's another, independent group of workers were seeking to identify the principle responsible for the vasoconstrictor properties of serum and defibrinated blood (Battelli, 1905a,b; Hirose, 1918) which they termed serotonin. This problem was solved in 1948, when the active substance was isolated from bovine serum (Rapport, Green and Page, 1948) and identified chemically as 5-hydroxytryptamine (5-HT). Meanwhile, the indolic nature of enteramine had been pointed out provisionally by Erspamer as early as 1946, (Erspamer, 1954a), but, only after the successful chemical synthesis of 5-HT (Hamlin and Fischer, 1951; Speeter, Heinzelmann and Weisblat, 1951), were Erspamer and Asero (1952) able to identify enteramine as 5-hydroxytryptamine - creatinine - sulphate. The two apparently separate problems thus had a single solution.

Large quantities of 5-HT are stored in the mammalian gastrointestinal tract (Erspamer, 1954a), mainly in the mucosa (Feldberg and Toh, 1953; Thompson and Campbell, 1966a). The concentration parallels the distribution of the enterochromaffin cells (Benditt and Wong, 1957)

which are known to produce the monoamine (Erspamer and Asero 1952; Benditt and Wong, 1957). Thompson and Campbell (1966a) have made a comprehensive survey of the literature on gastrointestinal 5-HT levels in man, rat and other animals. Malignant carcinoid tumour (argentaffinoma), in which there is a neoplastic proliferation of the enterochromaffin cells and a high concentration of 5-HT (Lembeck, 1953) and, occasionally, appreciable levels of its precursor, 5-HTP (Sandler and Snow, 1958), provides evidence in favour of the suggestion that these cells can synthesize, store and liberate 5-HT (Dalgliesh and Dutton, 1957). Partial resection of the gastrointestinal tract of the rat reduces the 5-HT content of serum, spleen and lung, though not brain, and decreases the excretion of the 5-HT metabolite 5-hydroxyindoleacetic acid (Bertaccini, 1960); thus at least some of the 5-HT in organs other than brain is likely to be derived from enterochromaffin cells.

Other major storage sites of 5-HT are the blood platelets (Rand and Reid, 1951; Zucker, Friedman and Rapport, 1954) and the spleen (Erspamer, 1954a). The amine present in the spleen may derive from its platelet content. 5-HT is also found in the mast cells of certain rodents (Benditt, Wong, Arase and Roeper, 1955; Furth, Hagen and Hirsch, 1957) but not of other species investigated (Parratt and West, 1957a; Sturm and Stüttgen, 1962).

In the brain, 5-HT has been extensively studied

Relatively high concentrations of 5-HT are known to be present in the pineal gland (Giarman and Day, 1959; Giarman, Freedman and Picard-Ami, 1960), and Weissbach, Redfield and Axelrod (1960) have described an enzyme which acetylates 5-HT in the non-particulate cytoplasm of rat liver, rat brain and bovine pineal gland. This enzyme is not specific for 5-HT and can acetylate other amines. N-Acetyl-5-HT is O-methylated by the enzyme hydroxyindole-O-methyltransferase (HIOMT) which can transfer a methyl group from S-adenosylmethionine to the 5-hydroxy side chain of a variety of indoles, including N-acetyl-5HT (Axelrod and Weissbach, 1960; 1961) and, less effectively, to other indoles such as 5-HTOH (McIsaac, Farrell, Taborsky and Taylor, 1965), 5-HT and 5-HIAA (Axelrod and Weissbach, 1961). 5-Methoxyindoleacetic acid has been detected in rat urine after 5-methoxytryptamine (Erspamer, 1955; Kveder and McIsaac, 1961) and is also present in bovine pineal gland (Lerner and Case, 1960).

(iv) N-Methylation of 5-HT

N-Methylation of 5-HT has been effected *in vitro* using enzyme systems present in the rabbit lung (Axelrod, 1962b) and in the 'parotid gland' of the toad, *Bufo marinus* (Marki, Axelrod and Witkop, 1962). Bumpus and Page (1955) claimed to find traces of N-methyl-5-HT in human urine.

(v) Other reaction of 5-HT

Oxidation of 5-HT by caeruloplasmin, the copper-

containing oxidase of mammalian blood plasma, was reported by Porter, Titus, Sanders and Smith (1957), an observation which has been confirmed by several authors including Martin, Eriksen and Benditt (1958) and Curzon and Vallet (1960). The physiological significance of the reaction, if any, is unknown. 5-HT is destroyed by cytochrome C and cytochrome oxidase (Udenfriend, 1958) yielding unknown products, and can form complexes with NAD (Alivisatos, Mourkides and Jibril, 1960a; Remily and Wolfe, 1960) and N-methylnicotinamide (Alvisatos, Mourkides and Jibril, 1960b). Ultraviolet light transforms 5-HT into kynuramine (Hakim and Thiele, 1960).

Nerve growth factor

In 1948, Bueker described the profuse sensory innervation of fragments of mouse sarcoma 180 implanted into the body wall of a 3-day-old chick. The sensory ganglia providing fibres to the neoplastic tissue were also enlarged. The significance of these findings was enhanced by the discovery, three years later, of an even greater increase in size of sympathetic ganglia of chick embryos bearing transplants of mouse sarcomas 180 and 37 (Levi-Montalcini and Hamburger, 1951). Furthermore, the effect was quite evident in sympathetic ganglia remote from the implant which did not directly innervate the neoplastic tissue. The sarcomas were later shown to release a diffusible growth factor into the host circulation, responsible for the observed hypertrophic and hyperplastic effects (Levi-Montalcini, 1952; Levi-Montalcini and Hamburger, 1953). The factor was localized in nucleoprotein particles present in the microsomal fraction of the neoplastic cells, and termed 'nerve growth promoting factor' by Cohen, Levi-Montalcini and Hamburger (1954). In 1956, a more potent source of the factor was discovered in snake venom (Cohen and Levi-Montalcini, 1956; Levi-Montalcini and Cohen, 1956), whilst, two years later, a third, and even more active source, was discovered in mouse submaxillary glands (Levi-Montalcini, 1958). Since 1956 (Cohen and Levi-Montalcini, 1956) the factor has been identified as a protein rather than a nucleoprotein and has become known as the 'nerve growth

factor' (NGF) (Levi-Montalcini and Cohen, 1960). The range of action of NGF on target cells, and its sources, metabolic effects, mechanism of action and biological significance have been extensively reviewed by Levi-Montalcini and Angeletti (1968a,b).

NGF is localized in the convoluted tubules of its most important source, the mouse submaxillary glands (Cohen, 1960; Levi-Montalcini and Angeletti, 1961), appearing at puberty, and attaining maximum concentration in the adult male. The concentration of NGF is ten times higher in male than in female glands. Levi-Montalcini and Angeletti (1968b) have shown that the protein is synthesized *in situ* and is largely excreted with saliva. Small quantities of NGF have been detected in a number of organs and tissue extracts of mice and rats (Levi-Montalcini and Angeletti, 1961), as well as in mouse urine (Levi-Montalcini and Booker, 1960a), and the factor is present in the serum of all vertebrates assayed, including man (Levi-Montalcini and Angeletti, 1968a). Although neuroblastoma tumour extract has no nerve growth promoting activity, the sera from children with neuroblastoma contains increased amounts of NGF (Burdman and Goldstein, 1964).

On injection of mouse submaxillary gland NGF into newborn and adult mice there occurs a massive enlargement of the sympathetic ganglia of treated animals (Levi-Montalcini and Booker, 1960a), but no appreciable effect

on the sensory ganglia, showing that the latter cells are receptive to NGF only during a restricted period of their early embryonic life. The superior cervical ganglia of mice treated with a purified preparation of NGF from birth to the ninth day showed a 12-fold volume increase in comparison with controls (Levi-Montalcini and Angeletti, 1968a). The increase in volume of the sympathetic ganglia is due to an increase in nerve cell number, and an increase in size of the individual neurones. Only the latter effect is present in adult mice injected with NGF (Levi-Montalcini and Angeletti, 1968b). Electron microscopic evidence revealed that NGF produces striking and rapid changes in the fine strucutre of sensory nerve cells (Levi-Montalcini, Caramia, Luse and Angeletti, 1968).

The noradrenaline content of NGF-treated ganglia increases proportionally to the cell volume increase, whereas the amount of adrenaline present is barely detectable and similar to controls (Crain and Wiegand, 1961). In NGF-treated mice, there is a greatly increased density of adrenergic fibres in many organs (Hammer, Norberg, Olson and Sjöqvist, 1967; Olson, 1967), and even some normally non-innervated tissues, such as the parasympathetic ganglia of the submaxillary gland hilum contain adrenergic terminals (Olson, 1967). No effects were observed in the vas deferens. Edwards, Fenton, Kakari, Large, Papadaki and Zaimis (1966) showed that the daily administration of NGF to newborn mice, rats

and kittens causes an increase in size of sympathetic ganglia of treated animals; the overall increase in size is mainly due to cellular hypertropy, although an increase in cell numbers may also be observed. The catecholamine content of the tissues innervated by the ganglia increases approximately in proportion to the increase in ganglion size. In addition to its effect on sympathetic nerves, administration of NGF appears to influence the regeneration of crushed fibres of the spinal cord of kittens (Scott and Liu, 1963, 1964). Evidence has been produced to show that the number of fibres regenerating is proportional to the dosage.

There is general agreement that nerve growth factors present in snake venoms, mouse submaxillary gland and certain mouse sarcomas are protein in nature. Cohen (1959, 1960) reported molecular weights of 20,000 and 44,000 for NGF isolated from snake venom and mouse submaxillary gland respectively. More recent work on NGF isolated from the latter source has pointed to the molecular weight of the active molecule being about 140,000 (Varon, Nomura and Shooter, 1967a, 1967b); the high molecular weight protein dissociates readily into three subunits with molecular weights of approximately 30,000, one of which appears to carry the specific nerve growth promoting activity (Varon, Nomura and Shooter, 1968), whilst another subunit has potent esterase activity (Greene, Shooter and Varon, 1968). All

three types of subunit are required to reconstitute the original highly active protein (Varon, Nomura and Shooter, 1968). NGF of very high yield and specific activity, isolated from male mouse submaxillary glands, has recently been described by Schenkein, Levy, Bueker and Tokarsky (1968), whilst a highly active NGF with molecular weight of about 40,000, prepared from snake venom, has also been reported (Banks, Banthorpe, Berry, Davies, Doonan, Lamont, Shipolini and Vernon, 1968).

The primary site and mechanism of action of NGF on target nerve cells is, as yet, unknown. In an attempt to elucidate its mechanism of action, the effect of NGF on various aspects of the metabolism of sympathetic and sensory ganglia has been investigated in vitro. These experiments indicate that a marked enhancement occurs of oxidative and synthetic processes in NGF-treated cells. Thus the synthesis of proteins (Angeletti, Gandini-Attardi, Toschi, Salvi and Levi-Montalcini, 1965) and total lipids (Liuzzi, Angeletti and Levi-Montalcini, 1965; Liuzzi and Foppen, 1968) is increased in NGF-treated ganglia. Glucose metabolism is stimulated mainly through the hexose monophosphate pathway (Angeletti, Liuzzi, Levi-Montalcini and Gandini-Attardi, 1964). An increased rate of RNA synthesis precedes in time all other observed metabolic effects (Angeletti, Gandini-Attardi, Toschi, Salvi and Levi-Montalcini, 1965). Recently, however, it has been shown that the synthesis of a sterol-like compound accounting for as much as 5-10% of total

lipids of sensory ganglia is decreased in the presence of NGF (Liuzzi and Foppen, 1968). Other studies, exploring the possibility that NGF might affect the permeability of the cell membrane target cells to amino acids, have shown that the accumulation of acidic, rather than neutral and basic amino acids, is preferentially increased in embryonic sensory ganglia (Levi and Lattes, 1968, 1969).

Immunosympathectomy

The selective and permanent destruction of up to 95% of sympathetic nerve cells in newborn mice and other animals, injected with a specific antiserum to NGF (Cohen, 1960), was first reported by Levi-Montalcini and Booker (1960b). No effects are apparent in other parts of the nervous system (Levi-Monalcini and Angeletti, 1966). In general, the cell population of the superior cervical ganglion in mice is reduced to 2 to 5% of control values, while in rats the residual cell population amounts to 10 to 15% (Levi-Montalcini and Angeletti, 1966). A similar degree of atrophy occurs in other paravertebral ganglia. Prevertebral ganglia, however, appear to exhibit a differential response to NGF antiserum; whilst a severe atrophy of the coeliac ganglion is observed in immunosympathectomized rats (Zaimis, Berk and Callingham, 1965), the effect on the mesenteric ganglia is very limited (Vogt, 1964; Zaimis, Berk and Callingham, 1965). The peripherally located

ganglia, supplying short adrenergic neurones to the vas deferens and the uterus, appear to be resistant to the antiserum (Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965; Levi-Montalcini and Angeletti, 1966). Electron microscopic studies on newborn mice revealed that within a few hours after injection of the antiserum there is considerable damage of most sympathetic neurones, including folding of the nuclear membrane and condensation of chromatin (Sabatini, Pellegrino de Iraldi and de Robertis, 1965). The overall findings were interpreted by the authors as indicative of a cytotoxic antigen-antibody reaction. On histological examination, the affected ganglia comprise a small population of glial cells and sparsely scattered nerve cells, which are considerably reduced in size compared with controls (Berk, Filipe and Zaimis, 1965; Levi-Montalcini and Angeletti, 1966). In pregnant mice the antiserum can cross the placenta and produce a partial immunosympathectomy in the foetus (Klingman, 1966b). Postnatal administration of NGF antiserum is more effective, however, in causing marked reduction of tissue noradrenaline levels and ganglionic cell populations than prenatal exposure to the antiserum (Klingman and Klingman, 1967).

On gross inspection, enophthalmos in immunosymathectomized rats is the only feature which distinguishes them from controls (Zaimis, 1965). The average resting blood pressure, however, is about 70 mm Hg in the treated rat

while that of normal rats is 100 mm Hg.

The disposition of noradrenaline in immunosympathectomized animals

A reduction in the noradrenaline content of animal tissues affected by NGF antiserum has been described by several groups of workers (Levi-Montalcini and Angeletti, 1962; Klingman, 1965; Visscher, Lee and Azuma, 1965; Zaimis, Berk and Callingham, 1965). In general, the decrease in noradrenaline levels parallels the degree of immunosympathectomy observed in the tissue. The noradrenaline content of some tissues such as heart, spleen and submaxillary glands, is drastically reduced, whereas in other organs, such as small intestine and liver, an appreciable content of noradrenaline remains. In rats, the noradrenaline content of the superior cervical ganglia, stellate ganglia, adrenergic chain, splanchnic nerves and coeliac ganglia is significantly reduced (Klingman, 1965). The noradrenaline content of the vas deferens in the immunosympathectomized rat was 65% of the control value (Zaimis, Berk and Callingham, 1965). No changes were detected in brain noradrenaline levels in treated animals (Levi-Montalcini and Angeletti, 1962; Klingman, 1965; Visscher, Lee and Azuma, 1965). An increase in the noradrenaline content of the rat adrenal medulla (Carpi and Oliverio, 1964), or whole gland in the mouse (Visscher, Lee and Azuma, 1965),

after immunosympathectomy has been reported, although no significant change in rat adrenal noradrenaline could be detected by Klingman (1965). Berk, Filipe and Zaimis (1965), however, noted an increase in noradrenaline content of the adrenals of immunosympathectomized rats and Iversen, Glowinski and Axelrod (1966) showed the rate of turnover of adrenal medullary catecholamines to be more than doubled in treated mice compared with normal controls. The results obtained from biochemical studies in immunosympathectomized rats have been confirmed by fluorescence microscopy (Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965). Thus a complete loss of fluorescent axons and terminals was observed in the iris, submaxillary gland, heart and skeletal muscles, whereas adrenergic nerve terminals resistant to immunosympathectomy were found in the vas deferens. Essentially normal fluorescence was observed in the hypothalamus and the adrenal medulla. Other evidence for noradrenaline depletion in the tissues of immunosympathectomized animals comes from the observations of Sjöqvist, Titus, Michaelson, Taylor and Richardson (1965), who showed that dense-core vesicles, characteristic of normal adrenergic nerve endings, are absent in the submaxillary glands of immunosympathectomized mice and rats; in the vas deferens the vesicles appear to be present in normal abundance. Disappearance of catecholamine storage sites from the tissues of treated rats has been demonstrated during studies on binding and release of metaraminol (Shore, Busfield and Alpers, 1964).

Some data are available on the urinary output of free noradrenaline and adrenaline in the immunosympathectomized animal. Thus under resting conditions noradrenaline excretion is drastically reduced in rats (Brody, 1964; Carpi and Oliverio, 1964; Schönbaum, Johnson and Sellers, 1965, 1966), whilst that of adrenaline is normal (Carpi and Oliverio, 1964). However, under such conditions as amphetamine treatment or adrenal demedullation (Carpi and Oliverio, 1964), or on acclimation to cold (Schönbaum, Johnson, and Sellers, 1966), urinary noradrenaline output is increased in treated rats.

In common with its ability to deplete endogenous noradrenaline levels, immunosympathectomy has a differential effect on the uptake and retention of noradrenaline-^{3}H by adrenergic neurones located in different organs (Sjöqvist, Titus, Michaelson, Taylor and Richardson, 1965; Iversen, Glowinski, and Axelrod, 1965a, 1966; Zaimis, Berk and Callingham 1965; Sjöqvist, Taylor and Titus, 1967). In general, there is a striking decrease in the capacity to take up and retain labelled noradrenaline by organs such as rat heart and spleen, but a partial reduction, only, in uptake and retention of the amine in tissues such as small intestine and liver. Uptake is normal in uterus, brain and bladder and is significantly increased in the vas deferens. Accompanying these findings is a relative increase in accumulation of ^{3}H-metabolites of noradrenaline, particularly

O-methylated derivatives (Sjöqvist, Titus, Michaelson, Taylor and Richardson, 1965; Iversen, Glowinski and Axelrod, 1966; Sjöqvist, Taylor and Titus, 1967) in affected tissues. The retained metabolites in the immunosympathectomized vas deferens are qualitatively similar to those of the normal organ (Sjöqvist, Taylor and Titus, 1967). Despite the reported increase in the proportion of O-methylated metabolites in treated animal tissues, no change could be detected in the COMT activity of immunosympathectomized mouse tissues. Levi-Montalcini and Angeletti (1962) described large decreases (up to 70% of control values) in the MAO activity of spleen, heart and submaxillary glands after administration of NGF antiserum. In an extensive study of MAO activity in the peripheral organs and sympathetic ganglia of the immunosympathectomized rat, however, Klingman (1966a) detected significant, although smaller, decreases in the MAO activity of spleen and submaxillary glands, but did not find a significant decrease in enzymic activity of atria and ventricles. The only other tissues in which there was a significant decrease in MAO activity were the superior cervical, stellate and thoracic chain ganglia, the kidneys and the liver. No change in MAO activity of heart, jejenum, liver or brain of immunosympathectomized rats was found by Iversen, Glowinski and Axelrod (1966), although salivary gland MAO activity was significantly reduced. From a perusal of the results obtained in all these studies, it

is clear that the status of MAO inthe immunosympathectomized animal remains unclear.

A few reports are also available on the activity of certain enzymes of catecholamine biosynthesis in the immunosympathectomized animal. Thus Klingman (1965) reported that DOPA-decarboxylase activity decreases significantly in the atria, spleen, submaxillary glands, uterus and certain ganglia of treated rats, whereas no decrease could be detected in the ventricles, kidney, lung, liver or small intestine. Iversen, Glowinski and Axelrod (1966), however, have described a decrease in aromatic L-amino acid decarboxylase (DOPA-decarboxylase) activity of intestine, heart and salivary glands of the immunosympathectomized rat, but no change was reported in liver or brain. The β-hydroxylation of β-methyltyramine-^{3}H was impaired in immunosympathectomized tissues, suggesting a decreased dopamine-β-hydroxylase activity (Iversen, Glowinski and Axelrod, 1966).

The effect of immunosympathectomy on tissue 5-HT levels

Cells exhibiting a yellow fluorescence typical of 5-HT were found in increased numbers in the intestines of immunosympathectomized rats compared with controls (Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965). Later, Iversen, Glowinski and Axelrod (1966) were able to detect increased levels of 5-HT in the bowel of immunosympathectomized mice,

a finding confirmed and extended by Thompson and Campbell (1966b), who found increased concentrations of mucosal 5-HT in the proximal gastrointestinal tract of treated mice, including the stomach, but no change in the large bowel content. These elevated 5-HT levels appear to be the result of an increased oncentration of 5-HT per argentaffin cell rather than a larger population of cells (Thompson, 1967), and are due to an increase in particulate-bound amine rather than free 5-HT (Thompson and Campbell, 1968). The reason for the increase of intestinal 5-HT levels in the immunosympathectomized animal is at present unknown.

The above findings have recently been extended by Klingman (1969), who has reported significantly increased levels of 5-HT in the hearts, submaxillary glands and caecum of immunosympathectomized rats.

The effect of reserpine on monoamine metabolism

Reserpine (3,4,5-trimethoxybenzoyl methyl reserpate) is an alkaloid found in the tropical plant Rauwolfia serpentina and related species. It has pronounced central and peripheral pharmacological actions, including a characteristic sedative effect, and produces a long lasting inhibition of adrenergic transmission. For an account of the pharmacology of reserpine, see Goodman and Gilman (1965) and Carlsson (1966).

Effects on monoamine levels in tissues

Reserpine causes a decrease in catecholamine and 5-HT concentration in various tissues. Although a major fraction of administered drug is metabolized in the body within a few hours (Numerof, Gordon and Kelly, 1955), studies with labelled reserpine indicate that small amounts of radioactivity remain in the brain (Plummer, Sheppard and Schulert, 1957; Sheppard, Tsien, Plummer, Peets, Giletti and Schulert, 1958; Maggiolo and Haley, 1964; Mueller and Shideman, 1968) and other organs (Alpers and Shore, 1969) for relatively longer periods. Alpers and Shore (1969) have concluded that the persistent depletion of noradrenaline by small doses of reserpine is induced and maintained by the physical presence of minute amounts of very tightly bound drug. They have also calculated that each molecule of reserpine persisting in the heart is associated with a deficit of about 500 noradrenaline molecules.

Reserpine depletes catecholamine stores in the heart and other tissues innervated by the sympathetic nerves (Bertler, Carlsson and Rosengren, 1956; Burn and Rand, 1957; Muscholl and Vogt, 1958: Higuchi, 1962), in the brain (Holzbauer and Vogt, 1956; Carlsson, Rosengren, Bertler and Nilsson, 1957; Brodie, Olin, Kuntzman and Shore, 1957) and in the adrenals (Holzbauer and Vogt, 1956; Callingham and Mann, 1958). Similarly, brain dopamine levels are considerably reduced (Bertler, 1961b). In tissues from reserpine-

treated animals, adrenergic transmission is blocked.

The original observations of Brodie and co-workers on reserpine-induced reduction of tissue 5-HT levels (Pletscher, Shore and Brodie, 1955; Shore, Silver and Brodie, 1955) have been confirmed and extended by many others. Thus, depletion of brain 5-HT concentrations has been described in many species including rabbit (Hess, Shore and Brodie, 1956; Pletscher, Shore and Brodie, 1956), dog (Paasonen and Vogt, 1956), rat and mouse (Erspamer, 1956b; Bartlet, 1960) and monkey (Pscheidt and Himwich, 1963). Similar effects have been recorded for whole blood (Shore, Pletscher and Brodie, 1956) and platelets of various species (Erspamer, 1956b; Haddisty, Ingram and Stacey, 1956; Shore, Pletscher and Brodie, 1956). 5-HT levels in the small intestine are reduced in the reserpinized animal but relatively large doses of drug are required to produce a maximum response (Pletscher, Shore and Brodie, 1955; Waalkes, Bosicevich and Udenfriend, 1957). Mast cell 5-HT is almost completely resistant to the action of reserpine (Bhattacharya and Lewis, 1956; Parratt and West, 1957b; Moran and Westerholm, 1963).

Catecholamines and 5-HT in different locations show considerable variation in sensitivity towards the depleting action of reserpine, whilst species differences may also be marked (see Carlsson, 1966). The time course of the depletion depends on anatomical site, drug dosage and route of administration. In general, amine depletion occurs slowly in response to reserpine. Tissue amine levels remain depleted for several days and do not return to

normal levels until at least 7 days after the drug (Carlsson, Rosengren, Bertler and Nilsson 1957; Carlsson, 1966), the actual time varying with location. The action of reserpine appears to be strongly cumulative, repeated small doses over a period of time leading to extensive depletion of tissue 5-HT (Haverback, Dutscher, Shore, Tomich, Terry and Brodie, 1957; Häggendal and Lindqvist, 1963) or catecholamine (Schaepdryver, 1960; Häggendal and Lindqvist, 1963) concentrations.

Reserpine-induced depletion of histamine has been described in a variety of tissues (Waalkes, Coburn and Terry, 1956; Waalkes and Weissbach, 1956).

Monoamine metabolites in the urine after reserpine

Depletion of tissue 5-HT and catecholamine levels after reserpine appears to arise from increased access of the amines to sites of metabolic degradation by MAO rather than from their decreased formation. After drug administration, an increase in urinary 5-HIAA (Shore, Silver and Brodie, 1955; Erspamer, 1956b; Erspamer and Ciceri, 1957; Fischer and Lecomte, 1956; Todrick, Dick and Tait, 1958; Valcourt, 1959), VMA (McDonald and Weise, 1968; Anton and Greer, 1966; Sandler and Youdim, 1968a,b), HMPG (Sandler and Youdim, 1968a,b) and HVA (Williams, 1962; Greer and Williams, 1963b; Kopin and Weise, 1968) has been reported in a variety of species. The time at which the urine samples are collected

appears to be of importance, however, for after the initial rise in urinary deaminated metabolites, which may correspond to amine liberation, the levels in the urine may return to normal or decrease. Thus, for example, continued treatment with reserpine leaves urinary 5-HIAA excretion unchanged (Erspamer and Ciceri, 1957; Stacey, 1959) whilst the amount of VMA in the urine decreases (McDonald and Weise, 1968). Reports that reserpine causes a decrease (Allegranza, Bozzi and Bruno, 1965), or no change (Brunjes, Haywood and Maronde, 1963), in VMA levels in human urine may be consistent with this time-course of the drug's action. Similarly, after chronic treatment with reserpine, HMPG levels in rat urine are significantly decreased (Kopin and Weise, 1968), presumably a reflection of depleted noradrenaline tissue stores. Sandler and Youdim (1968a,b) have reported that reserpine increases the ratio of HMPG to VMA in human urine.

The urinary excretion of noradrenaline itself is reduced after reserpine treatment in animals (Carlsson, Rosengren, Bertler and Nilsson, 1957; Bickel, Carpi and Bovet, 1961; Leduc, 1961; Johnson, 1963; Biscardi, Carpi and Orsingher, 1964; Henning, 1966; Andén and Henning, 1968; Wennmalm, 1968) and in man (Gaddum, Krivoy and Laverty, 1958; Carlsson, Boje Rasmussen, and Kristjansen, 1959; Anton and Greer, 1966). In contrast, an increase in urinary adrenaline has been reported after a single dose of reserpine (Pscheidt, Brune and Himwich, 1961; Bickel, Carpi

and Bovet, 1961; Biscardi, Carpi and Orsingher, 1964; Anton and Greer, 1966). These changes are paralleled by a decrease in normetadrenaline but an increase in metadrenaline excretion after reserpine (Anton and Greer, 1966). Thus adrenaline may be released directly into the circulation from the adrenal by reserpine to be inactivated by COMT, though the drug may also release the amine indirectly from the adrenal medulla via the splanchnic nerves (Kroneberg and Schümann, 1958).

The mechanism of action of reserpine

The precise mechanism of amine depletion caused by reserpine both in peripheral and central sites is still unkown. The drug appears to interfere with the processes involved in the intraneuronal storage of amines. This aspect of reserpine action has been extensively studied in the sympathetic nervous system. Here, the uptake of catecholamines across the axonal membrane can occur at a normal rate after reserpine administration, but since intraneuronal storage mechanisms are impaired, the catecholamines are rapidly metabolized and leave the tissue. The studies of Kopin and Gordon (1962, 1963) in the intact animal showed that reserpine caused a release of deaminated metabolites, rather than of unchanged noradrenaline, into the circulation. Similar findings were reported in isolated rat hearts pretreated with reserpine (Kopin, Hertting and

Gordon, 1962). There is now abundant direct evidence for the hypothesis of unimpaired uptake and subsequent facilitated deamination of catecholamines in reserpinized tissues (Hamberger, Malmfors, Norberg and Sachs, 1964; Lindmar and Muscholl, 1964; Gillespie and Kirpekar, 1965b; Iversen, Glowinski and Axelrod, 1965b Carlsson and Waldeck, 1967). Likewise, studies with amines resistant to MAO such as metaraminol (Lundborg and Stitzel, 1967a) and α-methyl-noradrenaline (Carlsson, Lundborg, Stitzel and Waldeck, 1967) show that initial uptake into the neuronal cytoplasm is not affected by reserpine.

The effects of reserpine on brain catecholamines appear to resemble those observed in peripheral sites. Thus in the reserpinized brain, exogenous noradrenaline does not accumulate in catecholamine neurones unless MAO is inhibited (Fuxe and Hillarp, 1964; Glowinski, Iversen and Axelrod, 1966). Similarly, reserpine treatment causes an increase in the level of 5-HIAA concomitantly with 5-HT depletion (Ashcroft and Sharman, 1962; Roos and Werdinius, 1962), and also in the dopamine metabolites DOPAC and HVA (Andén, Roos and Werdinius, 1964). Neff, Tozer and Brodie (1967), using probenecid to block the active transport of 5-HIAA from the brain, have reported that reserpine causes an increase in 5-HT turnover in the rat brain.

Since reserpine appears to act only on intraneuronal storage mechanisms, studies on isolated storage particles

from the adrenal medulla or from adrenergic nerves have been undertaken in an effort to elucidate the mechanism involved in amine depletion. The results obtained from these investigations, however, do not necessarily apply to the intact animal; furthermore, several differences exist between medullary and nerve granules, so that the two systems are not always complementary (Stjärne, 1966).

The presence of low concentrations of reserpine (10^{-5}-10^{-6} M) inhibited the spontaneous release of catecholamines from isolated bovine splenic nerve granules (von Euler and Lishajko, 1961b; Stjärne, 1964) and from rabbit adrenomedullary granules (Weil-Malherbe and Posner, 1963), though not from bovine adrenal particles (Stjärne, 1964). Nor was this effect on release rate obtained in rat heart granules (Potter and Axelrod, 1963). At higher concentration (10^{-4}M), reserpine caused practically complete release of adrenaline from rabbit adrenal particles (Weil-Malherbe and Posner, 1963).

At low concentrations reserpine also inhibits the uptake of exogenous catecholamines in adrenal medullary particles (Kirshner, 1962a; Carlsson, Hillarp and Waldeck, 1963; von Euler, Stjärne and Lishajko, 1964; Jonasson, Rosengren and Waldeck, 1964; Stjärne, 1964) and in adrenergic nerve granules (von Euler and Lishajko, 1963c; von Euler and Lishajko, 1964; Stjärne, 1964). A higher reserpine concentration (10^{-3}M) was, however, required to cause uptake

inhibition in rat heart particles (Potter and Axelrod, 1963). Reserpine also inhibits noradrenaline uptake by subcellular particles in homogenates of rat brain (Mirkin, Giarman and Friedman, 1963).

These *in vitro* studies suggest that the low concentrations of reserpine which might be present *in vivo* after administration of a moderate dose of the drug are unlikely to exert their depleting action by a direct releasing effect, since low concentrations of reserpine actually inhibit the spontaneous release of intraparticulate amines. The latter effect combined with uptake inhibition and increased exposure to intraceullular MAO could lead to catecholamine depletion in the tissue (see von Euler and Lishajko, 1967b). On the basis of *in vivo* studies with α-methyl-noradrenaline, however, Lundborg (1969) could find no evidence for the hypothesis that reserpine acts by inhibition of release of amines from the nerve granules.

Impaired synthesis of noradrenaline may contribute to reserpine-induced amine depletion, brought about by inhibition of dopamine uptake into the storage vesicles which contain dopamine-β-hydroxylase (Kirshner, 1957; Potter and Axelrod, 1963; Musacchio, Kopin and Snyder, 1964; Oka, Kajikawa, Ohuchi, Yoshida and Imaizumi, 1967). This has been demonstrated directly and indirectly in a number of studies using intact adrenal storage granules (Kirshner, 1962a), adrenal gland homogenates after *in vivo* administration of reserpine (Kirshner, Rorie and Kamin, 1963),

isolated bovine splenic nerve vesicles (Stjärne and Lishajko, 1966; Stjärne, Roth and Lishajko, 1967), isolated rabbit heart (Rutledge and Weiner, 1967) and intact sympathetic nerve tissue (Roth and Stone, 1968). In the two latter studies, unchanged dopamine did not accumulate after reserpine pretreatment but, because of exposure of the amine to MAO, an increase in the acid metabolites DOPAC and HVA was demonstrated. Glowinski, Iversen and Axelrod (1966) did not observe any significant inhibition of the conversion of dopamine to noradrenaline in the reserpinized rat brain. However, in these experiments an MAO inhibitor was used which may allow an abnormal accumulation of dopamine in the brain, and thereby reverse the inhibitory effect of reserpine on noradrenaline synthesis (Stjärne, Roth and Lishajko, 1967).

Support for the above hypothesis is provided by the findings of Kopin and Weise (1968). After chronic administration of large doses of reserpine to rats, HVA excretion was markedly increased whereas HMPG levels were significantly reduced. The total amount of HVA and HMPG was not, however, greatly changed, suggesting that tyrosine hydroxylation proceeds at a normal rate and that dopamine is deaminated rather than converted to noradrenaline. Reserpine has, however, been reported to increase the activity of adrenal and superior cervical ganglion tyrosine hydroxylase (Thoenen, Mueller and Axelrod, 1969).

Isolated storage granules can take up and concentrate monoamines *in vitro* by an ATP-Mg^{++} dependent mechanism (Kirshner, 1962a,b; Carlsson, Hillarp and Waldeck, 1962; von Euler and Lishajko, 1963b). Even after pretreatment with reserpine, however, a small but significant proportion of exogenous noradrenaline appears to accumulate in the amine storage granules by a mechanism which is not dependent on ATP-Mg^{++}. This has been shown to occur both in mouse heart *in vivo* and in isolated bovine adrenal medullary particles (Lundborg, 1967; Lundborg and Stitzel, 1967b; Stitzel and Lundborg, 1967). A reserpine-resistant uptake of noradrenaline in isolated bovine splenic nerve granules has been reported by von Euler and Lishajko (1967b), and a similar finding has been reported in rat brain (Glowinski, Iversen and Axelrod, 1966).

The ability of tissues to respond to sympathetic nerve stimulation has returned to normal at a time when noradrenaline stores are still severely depleted. At the same time, the tissues recover their ability to accumulate small amounts of exogenous noradrenaline (Andén, Magnusson and Waldeck, 1964, Iversen, Glowinski and Axelrod, 1965b; Andén and Henning, 1966). Subcellular studies in mouse heart (Lundborg and Stitzel, 1968) show that this rather sudden recovery of uptake takes place in the granules. Similarly, in the rat brain recovery from the sedative effects of reserpine occurs at a time when monoamine levels are severely depressed; but the ability of the central

neurones to take up and retain exogenous noradrenaline (Glowinski, Iversen and Axelrod, 1966) or 5-HT (Brodie, Comer, Costa and Blabac, 1966) parallels the functional recovery.

It has been demonstrated that in the reserpinized animal newly formed noradrenaline storage granules, unaffected by reserpine, may reach nerve terminals at the time of functional recovery (Dahlström and Häggendal, 1966). Thus, both centrally and peripherally, all the drug-affected granules may have been exchanged for fresh granules at the time of complete restoration of noradrenaline levels.

6-Hydroxydopamine

Surgical sympathectomy and, more recently, immunosympathectomy have been extensively used for the structural, physiological and pharmacological investigation of the sympathetic nervous system. Latterly, much attention has been devoted to the study of 6-hydroxydopamine (2,4,5-trihydroxyphenylethylamine), a compound which produces a chemical sympathectomy when administered at sufficiently high dose - levels to animals (Thoenen and Tranzer, 1968).

6-Hydroxydopamine administration results in a significant and long-lasting depletion of the noradrenaline content of certain sympathetically innervated organs in a number of animal species (Porter, Totaro and Stone, 1963; Porter, Totaro and Burcin, 1965; Laverty, Sharman and Vogt, 1965). It has been variously postulated that damage to noradrenaline storage sites similar to that caused by reserpine, or replacement of the physiological transmitter by 6-hydroxydopamine and/or its metabolites as a "false transmitter" are possible mechanisms of action (Porter, Totaro and Stone, 1963, 1965; Stone, Porter, Stavorski, Ludden and Totaro, 1964; Laverty, Sharman and Vogt, 1965). Recently, electron microscopic investigation has revealed ultrastructural degenerative changes in the adrenergic nerves of the 6-hydroxydopamine-treated cat (Tranzer and Thoenen, 1968) and rat (Thoenen and Tranzer, 1968). The

effect of 6-hydroxydopamine is dose-dependent, higher doses leading to destruction of adrenergic nerve endings and consequently to dissipation of amine stores. Uptake of labelled noradrenaline is reduced in parallel with the observed decrease in endogenous amine concentration. The degree of sympathectomy varies with species and organ; in the rat, for example, the vas deferens is less susceptible to the noradrenaline - depleting effect of 6-hydroxydopamine than the heart (Thoenen and Tranzer, 1968; Laverty and Phelan, 1969).

The hypothesis of a 'chemical sympathectomy' (Thoenen and Tranzer, 1968) is supported by histochemical evidence (Malmfors and Sachs, 1968; Jonsson and Sachs, 1969; Devine, 1969; Knyihár, Ristovsky, Kálmán and Csillik, 1969). Whilst terminal adrenergic axons are affected by 6-hydroxydopamine, non-terminal axons and cell bodies appear to be relatively unaffected. In Vitro studies using mouse atria and iris indicate that 6-hydroxydopamine must be taken up by adrenergic nerve terminals to produce its effect (Jonsson and Sachs, 1969).

Systemic administration of 6-hydroxydopamine has no effect on mouse whole brain noradrenaline concentration (Laverty, Sharman and Vogt, 1965). Central administration of the drug, however, results in a long lasting depletion of endogenously formed noradrenaline and dopamine concentrations in rat brain (Ungerstedt, 1968, 1969; Bloom, Groppetti,

Revuelta and Costa, 1969; Uretsky and Iverson, 1969), as well as a reduction of the content of labelled catecholamines and their metabolites in all brain regions after intraventricular injection of ^{3}H-dopamine (Laverty and Taylor, 1969). Injection of 6-hydroxydopamine into the substantia nigra leads to complete degeneration of the nigro-neostriatrial dopamine neurone system, bringing in its train severe motor disturbances, and resulting in a caudate nucleus practically devoid of dopamine (Ungerstedt, 1968, 1969). In contrast, brain 5-HT levels are not decreased by 6-hydroxydopamine (Bloom, Groppetti, Revuelta and Costa, 1969).

Cardiac tyrosine hydroxylase activity disappeared in rats following administration of 6-hydroxydopamine (Mueller, Thoenen and Axelrod, 1969), but the enzyme showed a twofold increase in activity in the adrenal where a small increase in phenylethanolamine-N-methyl transferase was also observed. However, no change in adrenal catecholamine content occurred under these conditions, confirming an earlier finding (Thoenen and Tranzer, 1968).

Recently the synthesis of 2,4,5-trihydroxyphenylalanine (6-hydroxy-DOPA) has been accomplished (Langemann and Scheer, 1969; Ong, Creveling and Daly, 1969): this compound was found to undergo enzymatic decarboxylation in vitro and in vivo to form 6-hydroxydopamine and, in this way, caused depletion of noradrenaline both in the peripheral

and central nervous system.

The auto-oxidation of dopamine to 6-hydroxydopamine has been demonstrated in vitro and in vivo (Senoh, Creveling, Udenfriend and Witkop, 1959; Senoh, Witkop, Creveling and Udenfriend, 1959); 2,4-dihydroxy-5-methoxyphenylethylamine, the 3-O-methylated derivative of 6-hydroxydopamine, has been found in rat urine on administration of labelled dopamine to animals pretreated with an MAO inhibitor (Daly, Horner and Witkop, 1961).

CHAPTER 2

METHODS FOR ANALYSIS OF RAT URINE

Urine collection

Rats were placed separately in cylindrical (26.5 cm diameter) Perspex metabolic cages fitted with floors of stainless steel mesh resting on large glass funnels. The mesh was siliconized by treating with polymethylhydrogen siloxane (Hopkin and Williams Ltd.), thus preventing possible catalytic oxidation of catecholamines on contact with the metal. Faeces were separated from urine by interposing a conical glass bulb, apex downwards, between the tip of the large glass funnel and the urine collection vessel. Urine was collected for 24 hours into 6 N HCl (1ml) during which time the animal was fasted from solid food, but allowed water ad libitum. At the end of the collection period any urine on the funnel was washed into the collection vessel with distilled water. Specimens were stored at -15°C until required.

Purity of chemical reagents

Unless otherwise stated, all common chemical reagents referred to in this thesis are of A.R. or G.P.R. grade.

Estimation of 4-hydroxy-3-methoxyphenylglycol (HMPG) and 4-hydroxy-3-methoxymandelic acid (VMA) in rat urine

The quantification of HMPG and VMA in rat urine presents special problems which are not encountered with human urine. VMA is the major urinary metabolite of both noradrenaline and adrenaline in man (Armstrong, McMillan and Shaw, 1957; La Brosse, Axelrod and Kety, 1958), whilst HMPG is of lesser importance. In rats the position is reversed, as HMPG predominates in the urine (Kopin, Axelrod and Gordon, 1961). Common laboratory methods for the assay of VMA (Pisano, Crout and Abraham, 1962) and HMPG (Ruthven and Sandler, 1965) in human urine depend on the oxidation of these metabolites to vanillin, which is then measured spectrophotometrically. The presence of both compounds in the free state in a urine sample would thus lead to mutual interference in their chemical determination. The problem does not arise with human urine since VMA occurs largely in the free state, whereas HMPG is mainly excreted as conjugates (Axelrod, Kopin and Mann, 1959; Shimizu and La Brosse, 1969). In the rat, however, both metabolites are conjugated in the urine, VMA mainly as glucuronide (Armstrong and McMillan, 1959; see also page 173) and HMPG as a sulphate ester (Axelrod, Kopin and Mann, 1959). Since, in the present study, determination of total urinary HMPG and VMA depended on conversion of both compounds to vanillin,

initial separation of the metabolites on an anion exchange resin was necessary.

Chemical reagents and materials

Bis (4-hydroxy-3-methoxyphenylglycol) piperazine salt (Regis Chemical Co., Chicago, 111).

(±) -4-Hydroxy-3-methoxymandelic acid (British Drug Houses Ltd.).

Standard solution 4-hydroxy-3-methoxyphenylglycol (HMPG) containing 1 mg HMPG per 1 ml. Dissolve 31 mg HMPG piperazine salt in 25 ml water. Store at -15°C.

Standard solution 4-hydroxy-3-methoxymandelic acid (VMA) containing 1 mg VMA per 1 ml water. Store at -15°C.

Anion-exchange resin, AG 1-X2 (200 - 400 mesh, acetate form) (Bio-Rad Laboratories, Richmond, California).

1M Ammonium acetate buffer, pH 4.8, was prepared by adjusting a solution containing 77.1 g ammonium acetate in approximately 800 ml water to pH 4.8 with about 50 ml glacial acetic acid and then diluting with water to 1 litre.

1M Phosphate buffer, pH 7.4, was prepared by adding 1M potassium hydroxide (approximately 4.5 ml) to 50 ml 1M potassium dihydrogen phosphate until the pH was 7.4.

Procedure

Two 24 hour urine collections from the same rat were pooled and the resulting mixture divided equally. One half was estimated directly and the other after the

addition of internal standards (20 µg HMPG and 20 µg VMA). Samples were brought to pH 11 and a slight excess (about 0.4 ml) of saturated $BaCl_2$ was added to precipitate inorganic phosphate and sulphate which might inhibit the enzymatic hydrolysis. After centrifugation, the decanted supernatant solution was brought to pH 6.4 - 6.5 and incubated for 18 hours at 37°C with 0.2 ml of a β-glucuronidase-sulphatase preparation (Suc d'Helix pomatia, Industrie Biologique Francaise, Gennevilliers, Seine, France).

The hydrolysed urine was passed through a column (1 cm bore and 6 cm bed) of 4g anion-exchange resin, AG 1-X2 and the effluent was discarded. HMPG, weakly bound to the resin, was eluted with de-ionized water (25 ml), thus separating it from VMA which is held more firmly. No HMPG was present in the column effluent. VMA was then eluted from the resin with 30 ml of 1M ammonium acetate buffer (pH 4.8) (Kirshner, Goodall and Rosen, 1958).

The water eluate was acidified (1N and 0.1N HCl) to pH 4 and extracted twice with 30 ml of ethyl acetate. These and subsequent extractions in this procedure, were made by shaking for 5 minutes on a mechanical shaker. After centrifugation, portions (25 ml) of organic phase from each extract were combined (50 ml) in a round-bottomed flask and evaporated under vacuum at 35°C using a "Rinco" rotating evaporator. The dry residue was dissolved in 4N NH_4OH (11 ml) and portions (5ml) were pipetted into a small

beaker (25 ml) and treated for test and blank as described by Ruthven and Sandler (1965). The test was treated with 2% ($^{W}/v$) sodium metaperiodate, $NaIO_4$ (0.2 ml), to convert HMPG to vanillin. After 20 minutes, excess periodate was removed by adding 10% ($^{W}/v$) sodium metabisulphite, $Na_2S_2O_5$ (0.2 ml). Water (0.2 ml) and 10% ($^{W}/v$) $Na_2S_2O_5$ (0.2 ml) were added to the blank. The pH of test and blank samples was adjusted to 7.4 - 7.5 by the addition of 50% ($^{V}/v$) H_3PO_4.

The adjusted solutions were transferred to test tubes (length 150 mm) each fitted with a ground glass socket (B 24), and the vanillin was extracted by shaking for 5 minutes with toluene (20 ml). After centrifugation, 15 ml of the toluene layer was pipetted into a similar test tube and the vanillin was returned to aqueous solution by shaking for 5 minutes with 1.5 ml 1M K_2CO_3. The carbonate layer, after centrifugation, was transferred with a Pasteur pipette to a 10 mm cuvette (capacity 3.5 ml). Any haze present was dispersed by partially immersing the cuvette in water at 50°C for a few seconds. The cuvette was raised 5 mm in its carrier and, if necessary, incident light was partly masked to ensure an unobstructed light-path through the fluid in the cell. The optical density of the solution was read against water at 330,350, 360 and 380 mu using a Unicam SP 500 spectrophotometer.

The ammonium acetate buffer eluate from the anion exchange resin was brought to pH 1 with 50% ($^{V}/v$) H_2SO_4 (2 ml) and 15 ml aliquots were taken for test and blank determination. The solutions were saturated with solid NaCl, extracted twice (2 x 20 ml) with ethyl acetate and a portion (15 ml) of each extract combined and evaporated under vacuum at 35°C using a rotary evaporator (Rotary Evapo-Mix, Buchler Instruments, N.Y.). The residue was dissolved in 1M K_2CO_3 (1 ml) and VMA was assayed by the method of Pisano, Crout and Abraham (1962), starting after the first carbonate extraction. The test was treated with 2% ($^{W}/v$) metaperiodate solution (0.1 ml) and the tubes were placed in a water bath at 50°C for 30 minutes to effect the conversion of VMA to vanillin. After cooling, 0.1 ml of 10% ($^{W}/v$) metabisulphite solution was added to reduce residual metaperiodate. Water (0.1 ml) and 10% ($^{W}/v$) metabisulphite were added to the blank. The solutions were neutralized with 0.3 ml 5N acetic acid, followed by 0.6 ml 1M phosphate buffer pH 7.4 and the resulting mixture was extracted with 20 ml of toluene for 5 minutes on the mechanical shaker. After centrifugation, 15 ml of the toluene layer was transferred to a stoppered test tube containing 1.5 ml 1M K_2CO_3. After shaking for 5 minutes, followed by centrifugation to separate the organic and aqueous phases, the optical density of the carbonate layer was measured at 330, 350, 360 and 380 mμ in a cuvette as

described for HMPG.

The optical density readings were measured at the four different wavelengths to check the specificity of the estimations. The spectral curves obtained in the assay of HMPG were characteristic of those given by pure vanillin (Sandler and Ruthven, 1961), so that 24 hour excretion values could be calculated using the 360 and 380 mμ extinctions. Some distortion between 330 and 350 mμ was observed, however, in the curves obtained during the assay of VMA. p-Hydroxybenzaldehyde, produced in the estimation by the oxidation of urinary p-hydroxymandelic acid, was the probable cause of this interference and was corrected for by solving simultaneous equations as described by Pisano, Oates, Sjoerdsma and Udenfriend (1961), incorporating the extinctions at 330 and 350 mμ.

$$A_{330} = B + V = C_B B^1 + V$$
$$B_{350} = B^1 + V^1 = B^1 + \frac{V}{C_V}$$

where:

A_{330} and A_{350} = observed optical densities at 330 and 350 mμ of the experimental mixture.

B and B^1 = optical density at 330 and 350 mμ due to the p-hydroxybenzaldehyde in the mixture.

V and V^1 = optical densities at 330 and 350 mμ due to the vanillin in the mixture.

C_B = constant = $\frac{\text{optical density at 330 mμ}}{\text{optical density at 350 mμ}}$ of authentic p-hydroxybenzaldehyde.

C_v = constant = $\frac{\text{optical density at 330m}\mu}{\text{optical density at 350 m}\mu}$ of authentic vanillin.

The average recoveries of HMPG and VMA added to rat urine and carried through the procedure were 14% (21 determinations) and 22% (23 determinations) respectively.

Separation of HMPG from VMA on anion-exchange resin. AG 1-X2, acetate form.

(a) Two 24 hour rat urine samples were combined and hydrolysed with a β-glucuronidase-sulphatase preparation as described above. The urine was then centrifuged and divided into 2 equal portions, to one of which was added HMPG (10µg) and VMA (10µg) respectively. After passage of the urine samples through AG 1-X2 acetate (4g) as above, the effluents were discarded and the columns were eluted in three separate fractions with 20 ml water, 10 ml water and finally 30 ml 1M ammonium acetate buffer (pH 4.8).

The solutions were adjusted to pH 1 and extracted with ethyl acetate for 5 minutes on a mechanical shaker as follows; 20 ml water eluent - extraction with 25 and 20 ml ethyl acetate respectively; 10 ml water eluent - 20 and 15 ml ethyl acetate; 30 ml 1M ammonium acetate buffer (pH 4.8) eluent - 35 and 30 ml ethyl acetate. The combined ethyl acetate extract was evaporated in vacuo at 35°C on a rotary

evaporator and the dry residues were dissolved in 150 µl. methanol. Ascending chromatograms were run overnight in isopropanol: ammonia (0.880): water (160 ml : 8 ml : 32 ml) and then after drying, were run again at right angles to the original direction in anisole: glacial acetic acid: water (140 ml : 54 ml : 6 ml). After running for about 5 hours the chromatograms were dried for 1 to 2 hours in a current of warm air (about 35-40°C). HMPG and VMA were located by spraying the paper with 10% Na_2CO_3, drying, and then overspraying with a mixture prepared from ice cold solutions of 5 ml p-nitraniline (0.2%) 50 ml 0.1 N HCl and 1 ml sodium nitrite (5%), to which 10 ml 10% Na_2CO_3 solution was added after 2 to 3 minutes. The mixture was used immediately.

HMPG, but not VMA, was present in the 20 ml water eluate after passage of both urine and urine plus added standards through the column. Neither compound was detected in the 10 ml water eluates. VMA, added to the urine as an internal standard, was found in the 1M ammonium acetate eluate, but was barely detectable in the chromatogram of urine alone.

These results indicated an effective separation of VMA and HMPG in the column fraction procedure outlined above. They also confirmed that HMPG is present in much greater quantity in rat urine than VMA and stressed the

importance of removal of HMPG from samples of rat urine in which VMA was being estimated, since both compounds are converted to vanillin by $NaIO_4$.

(b) To confirm the findings obtained with paper chromatography, samples of rat urine to which had been added VMA (20µg) and normetadrenaline (20µg), which can also be converted to vanillin by treatment with $NaIO_4$, were carried through the entire procedure for estimation of HMPG. No increase in optical density was observed compared with untreated control urine, whereas addition of HMPG (20µg) gave a marked increase in extinction. Similarly, HMPG (20µg) did not interfere in the VMA estimation.

Differential estimation of normetadrenaline and metadrenaline in rat urine.

This method was a modification of that described by Anton and Sayre (1966).

Chemical reagents

(±) - Metanephrine ((±)-metadrenaline) hydrochloride (Calbiochem, Los Angeles, Calif.).

(±)- Normetanephrine ((±) - normetadrenaline) hydrochloride (Calbiochem).

Adrenaline Hopkin and Williams, Ltd.).

Standard solution metadrenaline containing 1 mg base per 1ml 0.01N HCl. Storage at -15°C.

Standard solution normetadrenaline containing 1 mg base per 1ml 0.1N HCl. Storage at -15°C.

Standard solution adrenaline containing 1mg base per 1ml 0.01N HCl. Storage at -15°C.

(±) - Normetanephrine - 7H^3 ((±) - normetadrenaline - 7 - ^3H) hydrochloride (3,700 mc/m-mole) in 0.01N acetic acid. (New England Nuclear Corp., Boston, Mass.).

S - adenosyl - (-) - methionine (S-methyl-^{14}C) (approx. 40 mc/m-mole in dilute H_2SO_4 (New England Nuclear Corp.).

Liquid scintillator: PPO (2,5 -diphenyloxazole), 10g, POPOP (1,4-di [2-(5-phenyloxazolyl)] benzene), 600 mg in 2 litres toluene.

Citric acid-sodium acetate buffer, 0.80 M, pH 5.0 was prepared by adjusting a 2.0M citric acid solution to pH 5.0 with a 2.0M solution of sodium acetate and then diluting with distilled water to 0.80M.

Borate buffer, 1.0M, pH10, was prepared by dissolving 30.92g of boric acid in 450 ml of distilled water and adjusting to pH 10 with solid and 5.0N NaOH. The solution was saturated with NaCl and n-butanol, readjusted to pH 10, made up to 500 ml with distilled water and filtered through two sheets of Whatman No. 1 paper.

Alkaline ascorbate. Dissolve 10 mg of ascorbic acid (British Drug Houses Ltd.) in 0.1 ml of distilled water, add 10 ml of 10N NaOH and mix thoroughly. This was prepared freshly.

Distilled water. The water was redistilled in all-glass stills a total of four times. The second distillation was carried out in the presence of EDTA (1g per litre).

Preparation of ^{14}C-methoxy-labelled metadrenaline

^{14}C-Methoxy-labelled metadrenaline was prepared by a procedure based on the work of Axelrod, Albers and Clemente (1959) and Inscoe, Daly and Axelrod (1965). With ^{14}C-S-adenosyl-L-methionine as methyl donor, adrenaline was O-methylated in the presence of rat liver catechol-O-methyl-

transferase. The resulting methoxy-amine was isolated and purified by extraction followed by chromatography.

Rat liver (5g) was homogenized in 5 vol. of cold (0-4°C) isotonic KCl (1.12%^{W}/v) centrifuged (14,000 g for 20 minutes at 0-4°C) and a portion (0.1ml) of the supernatant added to the following mixture: water (0.5ml), 0.5M phosphate buffer (pH 7.8) (0.2ml), 0.1M $MgCl_2$ (0.1ml), 0.1% (^{W}/v) adrenaline in 0.01N HCl (0.1ml) and ^{14}C-S-adenosyl-(-)-methionine (approx. 1μC).

After incubation at 37°C for 90 minutes, the labelled metadrenaline was extracted into isoamyl alcohol (2 X 5 ml) from the mixture, which had been previously made alkaline with 0.5 M borate buffer (0.5 ml, pH10). The combined extracts, acidified with glacial acetic acid (0.05 ml) were evaporated under vacuum at 40 - 45°C. ^{14}C-Metadrenaline was isolated from the residue by paper chromatography (n-butanol:glacial acetic acid:water, 12:3:5). It appeared to be chromatographically pure in this solvent and in tert.-amyl alcohol:0.880 ammonia, 4:1. The product was stored in solution (0.1N HCl) at -15°C.

Procedure for estimation of metadrenalines

(a) Hydrolysis and extraction

Rat urine (3.0 ml) was adjusted to pH 0.90 with 5.0N HCl in a 10 ml beaker. The sample was transferred

to a stoppered test tube (B24 socket, length 150 mm) and placed in a boiling water bath for 20 minutes to hydrolyse the metadrenaline conjugates.

To overcome inaccuracies due to erratic recoveries which were found using the original extraction procedure of Anton and Sayre (1966), labelled ^{14}C-metadrenaline (0.3 ml of solution giving 25,000 d.p.m.) and ^{3}H-normetadrenaline (0.3 ml of solution giving 115,000 d.p.m.) were added as internal standards to the hydrolysed urine before extraction. The amount of non-radioactive compound present in these standards was insufficient to affect the accuracy of the assay. The sample was poured back into the same 10 ml beaker, 1.0 ml of tris buffer (1.0M) added, and the pH adjusted to 6.8 to 7.2 with HCl or NaOH. The pH-adjusted solution was returned to the same B24 tube, and shaken with 5 ml of isoamyl alcohol for 5 minutes on a mechanical shaker to remove a fluorescent contaminant. After centrifugation at 1,200g for 5 minutes, the organic phase and the pigmented precipitate between the organic and aqueous phases were aspirated and discarded.

The treated urine was transferred to fresh B 24 tubes and 1.0ml of 1.0 M borate buffer, pH 10, was added with thorough mixing. The solution was saturated with 7g of dipotassium hydrogen phosphate (K_2HPO_4) powder, mixed for 20 seconds to saturate the sample and vigorously shaken with ether (15 ml) (2 minutes vortex mixing and 10 minutes

mechanical shaking) to extract the metadrenalines. After centrifugation, the ether layer was transferred to a tube containing 4 ml of 0.1 N HCl into which the metadrenalines were back-extracted (2 minutes vortex mixing and 10 minutes mechanical shaking). The ether was discarded and the acid phase transferred with a 5 ml pipette to a fresh B24 tube. Borate buffer, 0.1 M, pH 10 (1 ml) and K_2HPO_4 powder (9g) were added and mixed for 20 seconds, and the solution extracted with 15 ml ether as described above. After centrifugation, the ether layer was transferred to a fresh B24 tube containing 1.5 ml of 0.01N and shaken for 2 minutes on the vortex mixer and 10 minutes on the mechanical shaker. The ether phase was discarded after centrifugation, and the acid phase was stored in a small tube at -15°C until required.

The overall recovery of the procedure was calculated by comparing the initial counts added with those from each isotope measured in the final 0.01N HCl extract. An aliquot (0.1 ml) of the latter solution was dissolved in 4 ml of methanol and 15 ml of toluene liquid scintillator in a counting vial and its radioactivity measured with a "Tri-Carb" liquid scintillation spectrometer (Packard Co. Inc., La Grange, Illinois, U.S.A.) set for double-labelled counting. Quenching was measured by addition of ^{14}C-toluene (4.31×10^5 d.p.m./ml, Packard) and ^{3}H-toluene

(2.28×10^6 d.p.m./ml, Packard) respectively (usually 0.1 ml) as internal standards to the solutions after the first count. From these counts, after correction for background (0.1 ml of 0.01N HCl added to scintillator plus methanol), the radioactivity in the vials was calculated.

The recoveries of the labelled metadrenalines after the extraction procedure were thus determined accurately and were found to vary over a wide range, with an average recovery of about 40%. Vigorous mixing at the various stages of the extraction procedure was imperative since the overall recovery dropped from values as high as 60 -70% to as low as 5% when a horizontal mechanical shaker was used for equilibration.

(b) Fluorophor formation

Fluorophor formation in aliquots of the final extract in 0.01N HCl was carried out essentially as described by Anton and Sayre (1966). Aliquots (0.1 ml) diluted to 0.4 ml with 0.01 N HCl were assigned to test, test plus external standard (non-radioactive metadrenaline (10ng) or normetadrenaline (30ng) as appropriate) and blank tubes respectively. Two sets were prepared, one for periodate oxidation after adjusting to about pH 5.0 to determine metadrenaline plus normetadrenaline, and the other for oxidation at about pH 1.5 for the estimation of metadrenaline alone.

EDTA (-disodium, dihydrate, 50 mg/ml) (0.1 ml) was added to the test, test plus standard and blank samples respectively, followed by 0.05 ml of citric acid-acetate buffer, 0.80 M,pH 5 0 (high pH) or 0.05 ml 0.85 M citric acid solution (low pH). Periodate solution ($NaIO_4$, 20 mg/ml), 0.5 ml, was added with thorough mixing to the tests and test plus standard and 0.50 ml, water added to the blanks, and the samples were allowed to stand for exactly 4 minutes. Sulphite solution (Na_2SO_3, anhydrous powder, 100mg/ml), 0.1 ml, was then quickly added to stop the reaction, followed by alkaline ascorbate (0.3 ml) with thorough mixing after both additions. The solutions were transferred to a quartz cuvette and the relative fluorescence intensity (RFI) was determined at 409 and 519 mμ (uncorrected activation and fluorescence wavelengths) for metadrenaline plus normetadrenaline at about pH 5.0, and at 422 and 531 mμ for metadrenaline at about pH 1.5, using an Aminco-Bowman spectrophotofluorometer (American Instrument Co., Inc., Silver Spring, Maryland, U.S.A.) with slit arrangement no. 4.

Neglecting any fluorescence due to normetadrenaline in the sample oxidised at pH 1.5, the amount of metadrenaline or normetadrenaline in the test aliquot was calculated from the RFI of the appropriate external standard and test after correction for blank. Urinary levels of metadrenaline and normetadrenaline were calculated from these values and the overall recovery determined radioisotopically.

The estimation of total metadrenalines in rat urine (Ruthven and Sandler, 1965)

Chemical reagents and materials

'Amberlite' CG - 50 (H) Type I (100 - 200 mesh) ion exchange resin (British Drug Houses, Ltd.).

Preparation of cation exchange resin (pH 6.0 - 6.5)

'Amberlite' CG-50 (250 ml) in the acidic form and 750 ml water were stirred for 10 minutes. After settling (30 minutes) and removing the supernatant solution, water was added to make up the original volume. The process of suspension and sedimentation was repeated (about 8 times), until the supernatant liquid was clear or almost clear after allowing the resin to settle (15 minutes). The supernatant solution was removed and 750 ml water added to the resin, together with 500 ml 10N NaOH, run in with stirring over a period of 15 minutes. The stirring was continued for two hours, the resin was allowed to settle (15 minutes), the supernatant removed and the resin washed by decantation five times with 750 ml water. The resin, now in the sodium form, was then reconverted to the acidic form by adding 1,250 ml 6N HCl with stirring (30 minutes). After allowing to settle, the excess acid was sucked off and the resin washed five times, again by decantation, with 750 ml water, after which it was converted back to the

sodium form as described above. The washed (four times with 750 ml water) sodium resin was suspended in 750 ml water and glacial acetic (25 ml approx.) was added with continuous stirring, until the pH of the solution remained steady for 30 minutes at approximately 6.2 - 6.3. The prepared resin suspension was stored at $4^{o}C$ until required.

Procedure

Two 24 hour rat urines were pooled, divided into equal portions and, if necessary, diluted to 10 ml with water. One half was estimated directly and the other after addition of normetadrenaline (20 µg). The samples were then hydrolysed with a β-glucuronidase-sulphatase preparation as described in the HMPG/VMA method.

The hydrolysed urines were centrifuged, and after adjusting the pH of the supernatants to 6.2 - 6.3 with 0.1 N HCl or 0.1 NaOH, were passed at less than 1 ml / 1 minute through a column (10 mm bore and 5 cm bed, prepared from approx. 6 - 7 ml or resin suspension) of cation exchange resin (pH 6.0-6.5) previously freed from buffer by running 15 ml water through the column. After the hydrolysate has passed through, the column was washed with 5 ml water. The effluent from the column and the water wash were discarded. The metadrenalines were eluted from the washed column with 4N NH_4OH (10 ml). The eluate was mixed and divided. 5 ml was pipetted into a small beaker (capacity 25 ml) to

serve as test and the remainder was transferred to a similar beaker to act as blank. The test was treated with 2% ($^{W}/v$) $NaIO_4$ (0.1 ml) to convert the metadrenalines to vanillin and after 3 minutes the excess reagent was removed by adding 10% ($^{W}/v$) $Na_2S_2O_5$ (0.1 ml). Water (0.1 ml) and 10% ($^{W}/v$) $Na_2S_2O_5$ (0.1 ml) were added to the blank. The pH of test and blank was adjusted to 7.4 - 7.5 by the addition of 50% ($^{V}/v$) H_3PO_4. The adjusted solutions were transferred to stoppered test tubes (B 24 socket, length 150 mm) and the vanillin extracted by shaking for 5 minutes with toluene (20 ml). After centrifugation at 1,200g, 15 ml of the toluene layer was pipetted into a similar stoppered test tube and the vanillin returned to the aqueous phase by shaking for 5 minutes with 1.5 ml 1M K_2CO_3. The carbonate layer, separated by centrifugation, was transferred with the aid of a Pasteur pipette to a 10 mm cuvette (capacity 3.5 ml). Any haze which occasionally appeared in the solution was dispersed by partially immersing the cuvette in water for a few seconds at 50°C. The partly filled cuvette was placed in its carrier, its base raised 5 mm and the incident light partly masked when necessary to ensure an unobstructed light-path through the fluid in the cell. The optical density (O.D.) of the solution was then read against water at two wavelengths, 360 mμ and 380 mμ, using a Unicam SP 500 spectrophotometer.

If the extinctions at wavelength λ of the final K_2CO_3 extract, derived from test and blank after the estimation of v ml urine (V ml mean 24 hour volume) are indicated by T_λ and TB_λ and the corresponding extinctions after the addition of internal standard by S_λ and SB_λ, then the amount of metadrenalines in the specimen (24 hour), expressed as µg normetadrenalines, was:

$$\frac{(T_{360}-T_{380}) - (TB_{360} - TB_{380}) \times 20}{[(S_{360} - S_{380}) - (SB_{360} - SB_{380})] - [(T_{360}-T_{380})-(TB_{360}-TB_{380})]} \times \frac{V}{v}$$

Estimation of homovanillic acid (HVA) in rat urine

A large proportion of the HVA excreted in rat urine occurs in the free state (Shaw, McMillan and Armstrong, 1957). Indeed, according to weight, the albino rat excretes about 3 times as much free HVA as the adult human. In the present studies the sensitive fluorimetric procedure of Sato (1965) was used to quantify free HVA output in rat urine.

Chemical reagents and materials

Homovanillic acid (4-hydroxy-3-methoxyphenylacetic acid, (HVA) (Calbiochem, Los Angeles, Calif.).

Standard solution of HVA, 1 mg per ml water.

Anion exchange resin AG 1-X4 (100 - 200 mesh), chloride form. (Bio-Rad Laboratories, Richmond, California).

Ammonium acetate buffer (0.1M), pH 4.5: prepared by dissolving 3.85 g ammonium acetate in about 400 ml of water, adding glacial acetic acid to pH 4.5 and making up to 500 ml with water.

Tris buffer (0.05 M), pH 8.5; prepared by dissolving 3.025g tris-(hydroxymethyl)-amino methane (Koch-Light Laboratories, Ltd.) in about 400 ml water, adding glacial acetic acid to pH 8.5 and making up to 500 ml with water.

Procedure

Two 1 ml aliquots of rat urine (24 hour), one untreated and the other to which had been added 5µg HVA, were used as test and test plus internal standard respectively. They were mixed with 0.1M acetate buffer, pH 4.5 in a 10 ml beaker and adjusted to pH 4.5 with NaOH (2.5N and 0.1N) using a glass electrode. The samples were then applied to an anion exchange AG1-X4 chloride column (0.55 cm bore, 3 cm bed) followed by a 3 ml water wash. Both effluents and washings were discarded and the column was eluted with 18 ml 1.5 N NaCl. The recovery of HVA added to urine and passed through the column was found to be about 84%.

The eluates were collected in B 24 tubes (200 mm) acidified with 2 ml 2N HCl, saturated with NaCl and extracted twice with 20 ml toluene-chloroform mixture (4 parts of toluene to one part of chloroform) for 1 minute on a vortex mixer and 5 minutes on a mechanical shaker. After centrifugation at 1,200 g and removal of 2 x 15ml. of the organic phase, the extracts were combined and the HVA back-extracted into 0.05M tris buffer, pH 8.5 (4 ml) by the shaking procedure outlined above. The mixtures were centrifuged and the upper organic phase removed by suction on a water pump. The recovery of HVA after extraction into the toluene-chloroform mixture was found to be approx. 42%.

One ml of the tris buffer extract was placed in a test tube for the formation of the fluorophor while a second ml portion served as the sample blank. To the test sample was added 1 ml of 5N ammonia and 0.2 ml of potassium ferricyanide (0.01%) with thorough mixing. After exactly 4 minutes, 0.2 ml L-cysteine (0.1%) was added to stop the reaction. The blank was treated in the same manner as the test except that the sequence of the addition of cysteine and potassium ferricyanide was reversed. The relative fluorescence intensity of the samples was measured in an Aminco-Bowman spectrophotofluorometer at 320 mμ (activation) and 420 mμ (fluorescence) respectively (uncorrected wavelengths).

Gas chromatographic measurement of 4-hydroxy-3-methoxyphenylglycol and homovanillic acid in rat urine

(Based on the methods of Karoum, Ruthven, and Sandler, 1968; Karoum, Anah, Ruthven and Sandler, 1969)

Chemical reagents and materials

Siliconized acid-washed celites (85/100 and 100/120 mesh) (W.G.Pye, Ltd., Cambridge, England).

Methyl phenyl silicone gum (SE 52) (W.G. Pye, Ltd.).

1,4-Dioxane (Specially dried) (British Drug Houses, Ltd.).

Hexamethyldisilazane (HMDS) (Koch-Light Laboratories, Ltd.).
Trimethylchlorosilane (TMCS) (Hopkin and Williams, Ltd.).

Ethereal diazomethane (approx. 100mg/ml) was prepared by the reaction of p-tolylsulphonylmethylnitrosamide (Koch-Light Laboratories, Ltd.) with an ethanolic solution of KOH (Vogel, 1961). The solution was stored at -10°C when not in use.

Equipment and preparation of columns

Pye Panchromatograph (W.G. Pye, Ltd.) fitted with an argon ionisation detector.

Glass U-tube columns, 7ft x 5mm (internal diameter), were siliconised before packing by allowing them to stand overnight with a mixture of petroleum ether (B.P. 40/60°) :

TMCS : HMDS (100 : 4 : 2) before emptying and drying in a stream of argon gas at 200°C.

The columns were packed evenly with siliconised celite coated with SE 52 (10%). Freshly packed columns were preheated for 48 hours at 250°C in a stream of argon. The detector temperature was kept at 250°C with an ionising potential of 1250V. The sensitivity was varied between 10^{-8} to 10^{-9}A. full scale deflection.

Procedure

For the determination of free HVA, a 24 hour rat urine sample was divided into two equal portions, to one of which had been added 20µg HVA as internal standard. Total HMPG was determined after the pooling of two 24 hour rat urine samples, to one of which was added 20µg HMPG as internal standard, and hydrolysis of the urine with a β-glucuronidase-sulphatase preparation as described in the previous HMPG/VMA method.

The urines used for estimation of HVA were adjusted to pH 3 with NaOH (5N and 0.5N), saturated with NaCl, and extracted twice with ethyl acetate (30 ml, 1 minute vortex mixing and 5 minutes mechanical shaking). For the determination of HMPG, the urines were similarly extracted with ethyl acetate (2 x 20 ml) at pH 6. Aliquots of each ethyl acetate extract (HVA, 2 x 25ml; HMPG, 2 x 15ml) were combined and evaporated to dryness under vacuum at 35°C.

The methyl ester /trimethylsilyl ether (ME/TE) of HVA was formed as follows; the dry residue from the extracted urine in a B 24 tube was dissolved in methanol (0.2ml) and methyl esterified with 1.5ml of ethereal diazomethane for 1 minute. After evaporation in a stream of nitrogen, the dry residue was dissolved in 0.3ml of a 1:2 mixture of HMDS and dioxane, followed by 0.05ml of TMCS, and the contents were mixed thoroughly. The preparation was transferred to a 2ml test tube which was tightly stoppered with a plastic cap and left for 30 minutes at room temperature for completion of the reaction. The trimethylsilyl ether (TE) derivative of HMPG was prepared by dissolving the residue of the urine extract in 0.5ml methanol to wash down the sides of the tube; evaporation of the organic solvent with nitrogen and silylation of the residue with HMDS/dioxane and TMCS were performed as described above.

After centrifugation, 0.5µl volumes of the clear supernatant fluids were injected into the column with a Hamilton syringe. The derivatives of the phenolic compounds including HVA and HMPG, were separated isothermally on the 7ft 10% SE 52 column at 190°C. The peaks were characterized from their methylene unit value (MU) as described by Dalgliesh, Horning, Horning, Knox and Yarger (1966). This involved calibration of the chromatogram by the introduction

of even numbered straight chain hydrocarbons (C_{16} to C_{22}), n-alkane reference standards, into the column. Any peak MU value = $n+2y/x$, where y is the distance (mm) from the peak of this standard to that of a neighbouring reference standard C_n and x is the distance (mm) between the peak and that of a second hydrocarbon of chain length C_n+2. Quantification of HVA in the rat urine was achieved by measurement of peak height (mm) of test and test plus internal standard, with calculation of endogenous HVA and HMPG based on the relative height of the added standard. The results were expressed as μg free HVA or total HMPG excreted per 24 hours.

Estimation of 5-hydroxytryptamine (5-HT) in rat urine

Free 5-HT in rat urine was estimated essentially by the method of Oates (1961).

Chemical reagents and materials

Serotonin creatinine sulphate (Koch-Light Laboratories Ltd.).

Standard solution of 5-hydroxytryptamine (serotonin) 1 mg base per 10 ml water.

'Amberlite' CG-50 (H) Type I (100 - 200 mesh) (British Drug Houses, Ltd.).

Preparation of 'Amberlite' CG-50, pH 7.0

'Amberlite' CG-50 in the acidic form was stirred with distilled water (3 vol.) for 10 minutes. After settling (10 minutes) and decanting the supernatant solution, water was added to make up the original volume. The process of suspension and sedimentation was repeated 3 times. The supernatant solution was removed and distilled water (3 vol.) was added, and 10N NH_4OH (2 vol.) was run in with stirring over a period of 15 minutes, after which stirring was continued for 2 hours. After the resin had settled the supernatant was removed and the resin washed by decantation 3 times with water (3 vol.) as described above. The resin, now in the NH_4^+ form was reconverted to the acidic form by stirring for 30 minutes with 6N HCl

(5 vol.) and, after settling, the excess acid was removed. The resin was then washed 3 times by decantation with water and converted back to the NH_4^+ form, as above, by stirring for 2 hours with 10 N NH_4OH (2 vol). The washed (3 times with 3 vol. water) NH_4^+ resin was suspended in water (3 vol. and glacial acetic acid added with continuous stirring until the pH of the solution remained steady for 30 minutes at approximately 7.0.

Procedure

Two 24 hour rat urine samples were pooled, centrifuged and divided into two equal portions, to one of which had been added 1µg 5-HT (10µg of standard solution) as an internal standard. The urines were adjusted to pH 7.0 (NaOH) and applied to 'Amberlite' CG-50 (NH_4^+ form), pH 7.0 columns (0.55 cm bore, 5 cm bed), previously washed with 25 ml glass-distilled water. After passage of the sample through the columns, the latter were washed with 25 ml glass-distilled water. The 5-HT was then eluted from the columns with 3 ml of 3N HCl . After mixing, 1 ml of the eluate was added to a small test tube containing 0.3 ml concentrated HCl and the fluorescence intensity of the solutions was read in an Aminco-Bowman spectrophotofluorometer (slit arrangement no.4) at 295 mµ (activation) and 540 mµ (fluorescence), both uncorrected. Water, equal in volume to the urine portion estimated, was carried through the procedure as a blank. The amount of 5-HT in the urine

sample was calculated with reference to the internal standard.

The recovery of 5-HT added to the urine and carried through the procedure is about 90%.

The estimation of 5-hydroxyindoleacetic acid (5-HIAA) in rat urine

Free 5-HIAA in rat urine was determined by the method of Contractor (1966).

Chemical reagents and materials

5-hydroxyindolyl-3-acetic acid (Koch-Light Laboratories,Ltd.

Standard solution of 5-HIAA, 1 mg per ml water. Storage at -15°C.

Sephadex G-10 (Pharmacia Fine Chemicals, Uppsala, Sweden).

Procedure

Sephadex G-10 (1g) was slurried in 0.1 N HCl and allowed to settle in a glass column (1 cm bore). Two 1 ml aliquots of a 24 hour sample of rat urine, to one of which had been added 5 µg 5-HIAA as internal standard, were mixed with 0.1 N HCl (10 ml) and applied to two separate Sephadex columns. The latter were then washed with 10 ml 0.1 N HCl, followed by 5 ml distilled water. The absorbed 5-HIAA was eluted with 5 ml 0.02 N NH_4OH; 1 ml aliquots of test and test plus standard were diluted with 1 ml 6N HCl and the relative fluorescence intensity (R.F.I.) of the solutions determined in an Aminco-Bowman spectrophotofluorometer (slit arrangement no. 4) set for activation at 295 mµ and fluorescence at 535 mµ (uncorrected wavelengths). One

drop of a saturated solution of potassium persulphate in water was added to the samples which were well mixed. After 15 minutes, the blank readings were steady and their RFI was measured 20 minutes after persulphate addition. This blank value was subtracted from the original RFI to give the fluorescence intensity of 5-HIAA in the sample.

The recovery of 5-HIAA added to urine and carried through the procedure was about 95%.

Estimation of 5-hydroxytryptophol (5-HTOH) in rat urine

Total 5-HTOH in rat urine was determined by a method developed by Ruthven (1968).

Chemical reagents and materials

5-Hydroxytryptophol (Regis Chemical Co., Chicago, Ill., U.S.A.).

For experimental use, 5-HTOH (5 mg) was dissolved in water (5 ml) plus ascorbic acid (2 - 3 mg). Portions of this solution (0.1 ml) were dispensed into vials and freeze-dried so that each contained 100 µg 5-HTOH.

Sephadex G-10 (Pharmacia).

Sephadex G-15 (Pharmacia).

Procedure

Two 24 hour rat urine collections from the same rat were pooled and, after mixing, the solution was divided

into 2 x 10ml portions. If necessary, water was added to make the portions up to this volume. One sample was estimated directly and the other after addition of 5-HTOH (100µg) as internal standard. For hydrolysis, the urines were adjusted to pH 6 with NaOH (2N, dropwise), thus avoiding alkaline conditions under which 5-HTOH is very unstable. Saturated $BaCl_2$ (0.8ml) was added to precipitate inorganic phosphate and sulphate and, after centrifugation, the supernatant was adjusted to pH 6.4 - 6.5 and incubated for 18 hours at 37°C with β-glucuronidase-sulphatase preparation (Suc d'Helix pomatia).

After incubation, the solutions were centrifuged and the pH adjusted to pH 7 with NaOH (0.1N). Sephadex G-15 (3g) was allowed to swell in 0.2M ammonium acetate, pH 7, (15 ml) for 30 minutes and was then poured into a glass column (1cm bore, height 35 cm) and was allowed to settle by gravity on a base of cotton-wool over a glass-bead, avoiding air bubbles in the process. The hydrolysed urine samples were applied to separate columns and the effluent was collected into a calibrated measuring cylinder. This effluent included inorganic salts and aliphatic compounds present in rat urine. The columns were then eluted with 0.2M ammonium acetate, pH 7, (75 ml) and the eluent measured until a total volume of 40 ml fluid (i.e. urine effluent plus ammonium acetate eluent) had passed through the columns. This fraction, which was discarded, included

5-hydroxytryptophan (5-HTP) 5-HT and 5-HIAA present in rat urine (Ruthven, personal communication), but not 5-HTOH which was retained on the column. The next fraction of the eluent (40 - 50 ml) was collected separately but again did not contain any 5-HTOH. This was checked by taking an aliquot (1 ml) of it, adding conc. HCl (0.5 ml) and measuring its fluorescence intensity in comparison with that of the blank as described later. Similar test and blank readings indicated that no 5-HTOH was present in this fraction.

The final 0.2 M ammonium acetate fraction (50 - 85 ml), containing the urinary 5-HTOH, was collected and applied to a Sephadex G-10 column (2g, 1 cm bore), the gel having been slurried in water before placing in the column. The effluent was discarded, the column washed with water (10 ml) and 50% aqueous methanol (2.5 ml), and 5-HTOH was eluted from the column with 75% aqueous methanol (25 ml in 5 x 5 ml fractions). An aliquot (1 ml) of each of these fractions was diluted with 0.5 ml of concentrated HCl and the relative fluorescence intensity of the samples determined within 15 minutes on an Aminco-Bowman spectrophotofluorometer (slit arrangement no. 4) set for activation at 295 mμ and fluorescence at 535 mμ (uncorrected wavelengths). One drop of a saturated solution of potassium persulphate in water was then added to the samples, which were well mixed to aid blank formation. After 5 minutes

the fluorescence intensity reading was taken to determine 'blank' values. The individual readings for the 5 x 1 ml samples were added together to obtain the total test and blank readings. The calculation for the estimation of total 5-HTOH in rat urine was based on the reading obtained with added standard.

The use of 75% aqueous methanol to elute 5-HTOH from Sephadex G-10 has been found to increase the final fluorescence intensity of 5-HTOH 2.5 fold compared with elution using the same volume of water. The average recovery of 5-HTOH added to urine and carried through the procedure was 53% (mean of 10 experiments).

Estimation of dopamine (3-hydroxytyramine) in rat urine

Free dopamine was estimated in unhydrolysed urine essentially by the method of Sourkes and Murphy (1961).

Chemical reagents and materials

3-Hydroxytyramine hydrochloride (Koch-Light Laboratories, Ltd.).

Standard solution of dopamine, 1 mg base per 1 ml 0.01N HCl. Storage at -15°C.

Acetate buffer, pH6; prepared by adjusting 1M Na acetate to pH 6 with 1M acetic acid.

Aluminium oxide (alumina), neutral (Hopkin and Williams, Ltd.).

Preparation of alumina, acid-washed, for chromatographic use (Crout, 1961b).

Alumina (200 - 300g) was refluxed with 2N HCl (1 litre) for 30 minutes. The cloudy supernatant was poured off, the alumina stirred briefly in 1 litre of distilled water and the supernatant again decanted after 5 minutes. This washing and decanting process with distilled water was repeated until the water wash cleared after 5 minutes of settling, and had a reaction of pH 4-5. The alumina was allowed to dry overnight in an open pan at room

temperature and was then heated in an oven at 100°C for 2 hours.

Procedure

A 24 hour rat urine was diluted to 25 ml with distilled water. Two 10 ml portions of the solution, to one of which had been added 2µg dopamine as internal standard, were transferred to 25 ml beakers; after 10% EDTA (2.5ml) and alumina (0.5g) had been added, they were quickly adjusted to pH 8.4 on a glass electrode with NaOH (5N and 0.5N). The contents of the beakers were poured into B 24 tubes (150 mm) and shaken for 20 minutes on a mechanical shaker. When the alumina had settled, the supernatant was removed by suction (water-pump) and the alumina washed with 2x 5 ml portions of glass-distilled water, (manual shaking). The supernatants were again discarded and the adsorbed dopamine eluted with 0.5 M acetic acid (5ml) for 15 minutes on the mechanical shaker. After centrifugation, the clear supernatant was used for estimations.

Aliquots (2 x 1 ml), designated test and blank respectively, of the acetic acid extracts were mixed with 2 ml acetate buffer, pH 6; the final pH was about 5.3. Dopamine was oxidized with 0.01 N iodine solution (0.5 ml). At exactly 3 minutes, the oxidation of the test samples was terminated by adding 0.5 ml 4.5 N NaOH which was 0.2M with respect to Na sulphite. The dopamine fluorophor is

obtained by rearrangement in alkali in the presence of sulphite. The blanks were identical, except that the alkaline sulphite solution contained in addition ascorbic acid (2 mg), prepared freshly; this substance interferes with the final fluorescence when added at this stage. Three minutes after addition of the alkali, 1.0 ml of 5N HCl containing 2 mg ascorbic acid was added to the test samples to stabilize and intensify the dopamine fluorophor. The blanks received 1 ml of 5N HCl alone. After transfer to 25 ml beakers, the samples were irradiated under an ultraviolet lamp for 10 minutes at 254 mμ to produce maximal fluorescence and the relative fluorescence intensities were measured on an Aminco-Bowman spectrophotofluorometer (slit arrangement no. 4) at 330 mμ (activation) and 375 mμ (fluorescence) (uncorrected wavelengths). Endogenous free dopamine in the urine was estimated in relation to the values obtained with the internal standard.

The dopamine fluorescence was linear between 0.1 and 0.5 μg per ml and the recovery of dopamine added to urine and carried through the procedure was approx. 48%.

CHAPTER 3

STUDIES ON ENDOGENOUS CATECHOLAMINE AND 5-HYDROXYINDOLE METABOLISM IN IMMUNOSYMPATHECTOMIZED RATS

Noradrenaline is released at mammalian postganglionic sympathetic nerve endings (von Euler, 1956), but is also present at other sites in the body, including the adrenal gland (Holtz, Credner and Kroneberg, 1947) and discrete areas of the brain (Vogt, 1954). The permanent destruction of a major proportion of sympathetic neurones following administration of NGF antiserum to newborn animals (Levi-Montalcini and Booker, 1960b) provided suitable experimental conditions for the study of the metabolism of amines associated with the sympathetic nervous system.

Previous observations of endogenous catecholamine metabolism in the immunosympathectomized animal have been mainly confined to the measurement of tissue catecholamine levels (Levi-Montalcini and Angeletti, 1962; Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965; Klingman, 1965; Visscher, Lee and Azuma, 1965; Zaimis, Berk and Callingham, 1965); some data have also been published on the output of free noradrenaline and adrenaline (Brody, 1964; Carpi and Oliverio, 1964; Schönbaum, Johnson and Sellers, 1965, 1966). Unchanged amines form only a minor proportion of total

catecholamine derivatives in the urine, however, so that little is known of the overall effect of immunosympathectomy on endogenous catecholamine production and metabolism. In the present work the quantities of some of the major metabolites of noradrenaline and adrenaline excreted in the urine of normal and immunosympathectomized rats have been compared. The urinary output of the noradrenaline precursor, dopamine, and its O-methylated, oxidatively deaminated metabolite, homovanillic acid, has also been estimated in these animals in an attempt to estimate the proportion of urinary dopamine which is derived from the sympathetic nervous system.

An increased concentration of 5-HT has been reported in the gastro-intestinal tract of immunosympathectomized animals (Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965; Iversen, Glowinski and Axelrod, 1966; Thompson and Campbell, 1966b). The 5-HT content of the heart and submaxillary glands of the immunosympathectomized rat is similarly raised (Klingman, 1969). As these sites, particularly the gastro-intestinal tract (Erspamer, 1954b), contain a large proportion of total body 5-HT, the urinary output of 5-HT and its metabolites, 5-HIAA and 5-HTOH, has been measured in immunosympathectomized and control rats to determine whether or not the increase in tissue 5-HT levels is associated with changes in 5-HT metabolism.

Procedure

(a) Catecholamine and 5-hydroxyindole metabolites in the urine of immunosympathectomized rats

Twenty four hour urine samples were collected from male (455-775 g body weight) and female (210-585 g body weight), histologically proven immunosympathectomized (E. Zaimis, personal communication) Wistar rats, and their litter-mate controls, all aged 18-20 months. The treated rats had been injected subcutaneously with nerve growth factor antiserum (Abbott Laboratories, North Chicago, Illinois) for five days after birth, and the controls with an equivalent volume of 0.9% w/v NaCl. As supplies of an effective NGF antiserum in rats were scarce, these animals were kindly put at the author's disposal for urine collection by Professor E. Zaimis at the Royal Free Hospital School of Medicine, London. The degree of immunosympathectomy attained in such rats is illustrated in Fig. 5, showing the relative size of the superior cervical ganglion in a treated rat as compared to that in a control animal.

Male rats only were used for the determination of normetadrenaline, metadrenaline, HMPG and VMA in the urine of immunosympathectomized rats and their litter-mate controls, whereas urinary dopamine, HVA, 5-HT, 5-HIAA and 5-HTOH was estimated in rats of both sexes.

(b) Urinary HMPG determination in rats with a partial immunosympathectomy



Fig.5. Superior cervical ganglia of control rat (right) and rat treated with nerve growth factor antiserum (left), after Levi-Montalcini and Angeletti (1966).

The urinary excretion of HMPG was also determined in groups of rats which had undergone a partial immunosympathectomy. To achieve this condition, the rats were treated with a nerve growth factor antiserum which deprives mice of the bulk of their sympathetic nervous system, but which is much less effective in rats (D.C. Edwards, personal communication). The antiserum used was a sterile freeze-dried preparation (Burroughs Wellcome and Co., London), which had been prepared by injecting a horse with purified nerve growth factor from the submaxillary gland of the adult male mouse. Groups of rats were either injected with an isotonic solution of antiserum (B.W. 'normal strength'), prepared by reconstituting the serum in 10 ml of sterile distilled water, or with a hypertonic solution (B.W. 'double strength') prepared by reconstitution in 5 ml water. Control rats, of the same age, received an equivalent volume of 0.9% w/v NaCl.

Newborn rats, male and female, were injected subcutaneously for the first 5 days of life with either 'B.W. normal strength' antiserum, 'B.W. double strength' antiserum or 0.9% w/v NaCl respectively, in doses which increased as follows: 0.2 ml, 0.25 ml, 0.30 ml, 0.35 ml and 0.40 ml. Urine was collected for HMPG determination when the 'normal strength' rats and their respective controls were 8 months old, whereas, for the determination of HMPG output in the 'double strength' animals and their

controls, 5 of the animals in both the experimental and control groups were aged 2.5 months, whilst the remaining rats (3) in each group were 6 months old.

To ascertain the effectiveness of the Burroughs Wellcome antiserum in rats, the size of superior cervical ganglia dissected from both 'normal' and 'double strength' antiserum-treated animals was compared with that of ganglia taken from control rats of the same age. Typical results are illustrated in Fig. 6 and Fig. 7. Although there is a reduction in the size of the treated ganglia, this is not as pronounced as that found in fully immunosympathectomized rats (Fig. 5). Comparative photographs of superior cervical ganglia of mice treated with the Burroughs Wellcome antiserum are shown in chapter 5 (Fig.8).

(c) Conjugation of VMA in rat urine

The conjugation of VMA as a glucuronide in rat urine was reported by Armstrong and McMillan (1959). In order to confirm and, if necessary, extend this finding the following investigation was undertaken.

In two separate experiments, urine from fifteen (220 ml) and fourteen (231 ml) rats, respectively, was pooled. Three duplicate 25 ml portions of each specimen were taken, one sample of each pair containing 20 µg VMA added as internal standard. Free VMA was estimated in a pair of unhydrolysed urine samples. Free VMA plus VMA glucuronide was determined in a second pair of duplicate

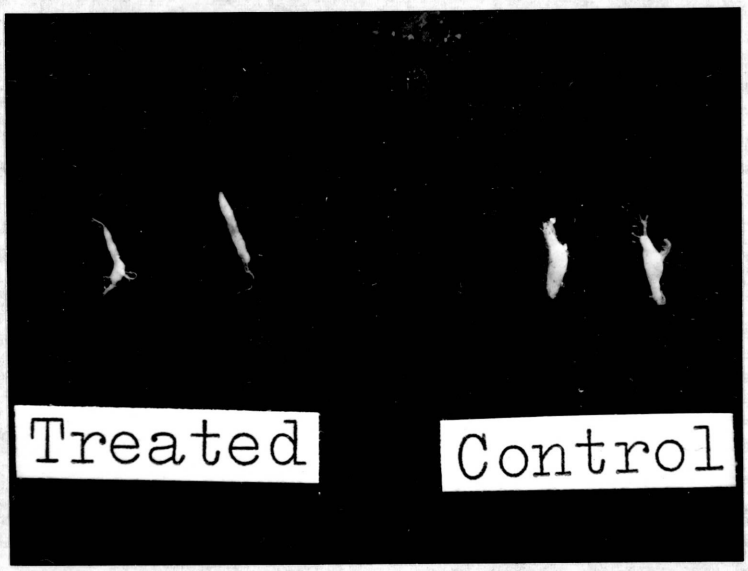


Fig.6. Superior cervical ganglia of control rat and rat treated with Burroughs Wellcome nerve growth factor antiserum ('double strength').

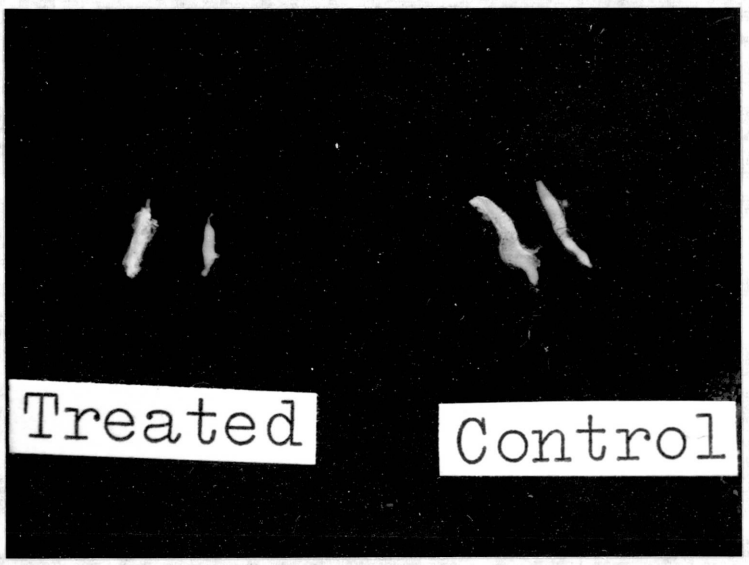


Fig.7. Superior cervical ganglia of control rat and rat treated with Burroughs Wellcome nerve growth factor antiserum ('single strength').

samples, following preliminary hydrolysis of the urine with a β-glucuronidase preparation (Type, II, Sigma Chemical Company, St. Louis, U.S.A.) at pH 6.8. Finally, 'total' VMA was measured in the third pair of urine samples after hydrolysis with a β-glucuronidase-sulphatase enzyme preparation. VMA was estimated by the spectrophotometric method described in chapter 2.

Results

Excretion of metabolites of noradrenaline and adrenaline in the urine of immunosympathectomized and control rats.

The levels of normetadrenaline, metadrenaline, HMPG and VMA excreted in the urine of immunosympathectomized (Abbott antiserum) and control rats are shown in Table 1. The results are expressed in terms of μg per 24 hr and μg kg per 24 hr.

HMPG output was significantly decreased ($P < 0.001$) in the urine of immunosympathectomized rats compared with controls. In contrast, VMA excretion, when expressed as μg per kg per 24 hr, was slightly increased in the treated rats, although the difference was significant only at the 5% level.

The levels of normetadrenaline and metadrenaline detected in the two groups of rats were not significantly different. To check the reliability of the fluorimetric

TABLE 1.

Excretion of metabolites of noradrenaline and adrenaline in the urine of immunosympathectomized (Abbott antiserum) and control rats.

	Immunosympathectomized rats			Control rats		
Metabolite	Mean excretion ± S.E.M.			Mean excretion ± S.E.M.		
Free plus conjugated	No.of expts.	µg per 24 hr	µg per kg per 24 hr	No.of expts.	µg per 24 hr	µg per kg per 24 hr
Normetadrenaline	6	2.18 ± 0.13	3.89 ± 0.35	7	2.54 ± 0.54	4.02 ± 0.87
Metadrenaline	6	0.77 ± 0.08	1.36 ± 0.16	7	0.87 ± 0.15	1.36 ± 0.21
HMPG	7	30.32 ± 1.11*	58.97 ± 2.71*	6	67.73 ± 3.73	108.00 ± 4.19
VMA	6	10.53 ± 0.84	19.99 ± 1.54**	6	9.20 ± 0.77	14.88 ± 1.40

Male rats only used in the experiment. Age 18-20 months

* $P < 0.001$ compared with controls

** $P < 0.05$ compared with controls

procedure described in chapter 2, and used above to assay the differential metadrenalines, total metadrenaline output (i.e. normetadrenaline plus metadrenaline) was also measured in the urine of five normal rats by the method of Ruthven and Sandler (1965) (see chapter 2). A mean excretion value of 3.70 ± 0.65 µg per 24 hours was obtained, which is in good agreement with the combined normal output of normetadrenaline plus metadrenaline shown in Table 1.

The concentrations of HMPG excreted in the urine of rats treated with either 'normal strength' or 'double strength' Burroughs Wellcome NGF antiserum at birth did not differ significantly from their controls (Table 2), although there was some tendency towards a decreased output of this metabolite in the urine of animals treated with the more concentrated antiserum. The mean 24 hour excretion of HMPG in the combined 2.5 and 6 month old control rats was lower than of the 8 month and 18-20 month control (Table 3) but, expressed on a weight basis, the mean level of HMPG found in the urine of the first group of rats tended to exceed that of the 18-20 month controls, although this finding was not statistically significant.

The results of the VMA conjugation studies in rat urine (Table 4) indicated that over sixty per cent was excreted in the form of a glucuronide, whilst about twenty per cent was found as a sulphate conjugate. Approximately twenty per cent of total VMA in the urine was present in the free state.

TABLE 2.

Excretion of total HMPG in the urine of rats treated with Burroughs Wellcome (B.W.) nerve growth factor antiserum.

	Treated rats			Control rats		
	Mean excretion ± S.E.M.			Mean excretion ± S.E.M.		
Experiment	No.of expts.	µg per 24 hr	µg per kg per 24 hr	No.of expts.	µg per 24 hr	µg per kg per 24 hr
B.W.'Normal strength'	6 (6♂, -♀)	68.57 ± 13.58	—	6 (6♂, -♀)	65.70 ± 9.48	—
B.W.'Double strength'	8 (4♂, 4♀)	31.43 ± 7.44	111.20 ± 18.65	8 (5♂, 3♀)	36.43 ± 5.74	132.37 ±28.1

B.W. 'normal strength' rats and controls - age, 8 months

B.W. 'double strength' rats and controls - age, either 2.5 months (5 rats) or 6 months (3 rats).

TABLE 3.

Variation of HMPG excretion with age in the urine of normal rats.

No. of rats	Age in months	Mean excretion ± S.E.M. µg per 24 hr	µg per kg per 24 hr
3♂, 2♀	2.5+	30.39 ± 8.16	137.40 ± 42.77
2♂, 1♀	6.0+	46.50 ± 2.37	123.96 ± 27.09
6♂	8.0*	65.70 ± 9.48	
6♂	18-20+	67.73 ± 3.73	108.00 ± 4.19

+ measured by spectrophotometry

* measured by gas liquid chromatography

TABLE 4.

Conjugation of VMA in rat urine.

	Urine No. 1		Urine No. 2	
	µg per 25 ml	% total VMA	µg per 25 ml	% total VMA
Total	28.4	100.0	18.3	100.0
Free VMA	4.4	15.6	3.4	18.5
Free VMA + VMA glucuronide	22.1	78.0	14.9	81.4
VMA glucuronide (by difference)	17.7	62.4	11.5	62.9
VMA sulphate (by difference)	6.3	22.0	3.4	18.6

'Total' VMA was estimated after enzymatic hydrolysis with a β-glucuronidase-sulphatase preparation. Free VMA was measured in unhydrolysed urine, and free VMA+glucuronide assayed after hydrolysis with a β-glucuronidase preparation.

Excretion of free dopamine and HVA in the urine of immunosympathectomized and control rats

Although there was a tendency for the immunosympathectomized rats to excrete lower amounts of HVA in the urine compared with the controls, this difference was not statistically significant (Table 5). The urinary output of dopamine was similar in both groups of rats.

Excretion of 5-hydroxyindole metabolites in the urine of immunosympathectomized and control rats

No significant differences were observed in the urinary excretion of free 5-HT and 5-HIAA between immunosympathectomized and control rats (Table 6). In contrast, although the amounts of 5-HTOH detected in the 24 hour urine samples used in the present experiment were similar for the two groups, when the results were expressed in terms of body weight, immunosympathectomized animals tended to excrete less than controls ($P = 0.05$).

General comment on the results.

There was a tendency for the animal to animal variation in both catecholamine and 5-hydroxyindole metabolite output values to be less in immunosympathectomized animals than in controls: 5-HIAA (µg per kg per 24 hr), normetadrenaline (µg per 24 hr) and HMPG (µg per 24 hr) showed a significant reduction in variation ($P < 0.05$), and all the other compounds measured exhibited this trend.

TABLE 5.

Excretion of free dopamine and HVA in the urine of immunosympathectomized and control rats.

	Immunosympathectomized rats			Control rats		
	Mean excretion ± S.E.M.			Mean excretion ± S.E.M.		
Compound	No.of expts.	µg per 24 hr	µg per kg per 24 hr	No.of expts.	µg per 24 hr	µg per kg per 24 hr
Dopamine	10 (8♂, 2♀)	2.90 ± 0.29	5.47 ± 0.44	8 (6♂, 2♀)	3.62 - 0.42	6.62 ± 0.79
HVA	9 (7♂, 2♀)	25.35 ± 3.15	52.77 ± 9.32	9 (5♂, 4♀)	31.97 ± 4.07	71.62 ± 11.69

TABLE 6.

Excretion of 5-hydroxyindole metabolites in the urine of immunosympathectomized and control rats.

	Immunosympathectomized rats			Control rats		
		Mean excretion ± S.E.M.			Mean excretion ± S.E.M.	
Compound	No.of expts.	µg per 24 hr	µg per kg per 24 hr	No.of expts.	µg per 24 hr	µg per kg per 24 hr
Free 5-HT	5 (4♂, 1♀)	1.39 ± 0.39	2.30 ± 0.15	(3♂, 2♀)	1.60 ± 0.22	3.04 ± 0.39
Free 5-HIAA	7 (6♂, 1♀)	44.48 ± 5.09	87.77 ± 6.69	7 (6♂, 1♀)	48.40 ± 8.93	86.31 ± 13.73
Free + conjugated 5-HTOH	5 (3♂, 2♀)	4.63 ± 0.96	8.88 ± 1.31*	5 (2♂, 3♀)	5.15 ± 0.43	13.99 ± 1.85

*P = 0.05

Discussion

HMPG was the only catecholamine metabolite, of those studied, to show a significant reduction in urinary output after immunosympathectomy. The observed decrease tended to run in parallel with the fall in free noradrenaline levels, previously reported in immunosympathectomized animals (Brody, 1964; Carpi and Oliverio, 1964; Schönbaum, Johnson and Sellers, 1965, 1966), suggesting that, since HMPG is the predominant metabolite of noradrenaline in rat urine (Kopin, Axelrod and Gordon, 1961), measurement of the concentrations of this metabolite in urine may provide a convenient index, during life, of the extent to which sympathetic nerve growth has been retarded in immunosympathectomized rats.

On appraisal of the results obtained in the partially immunosympathectomized rats, however, where no significant reduction in HMPG excretion was observed compared with controls, it would appear that the extent of the degeneration of the sympathetic nervous system resulting from the administration of NGF antiserum must be extensive (Fig. 5) before a fall in urinary HMPG output can be detected. Although the size of the affected sympathetic ganglia in rats treated with this less effective Burroughs Wellcome antiserum may be reduced by 50% or more, as shown in Fig.6 and Fig.7, the urinary output of HMPG, and presumably of

noradrenaline, in these animals can be effectively maintained at approximately normal levels. Whether or not an increased synthesis of noradrenaline occurs in the residual adrenergic nerves arising from the partially affected paravertebral ganglia, which would have shown almost complete atrophy after treatment with the more effective NGF-antiserum (Abbott), is unknown.

The present studies also establish a set of normal values for the urinary excretion of HMPG and other catecholamine metabolites in normal adult rats where firm data of this nature had previously been scanty. The daily output of HMPG in normal rat urine was seen to increase with age. When the urinary levels of the metabolite were expressed in terms of body weight, however, the differences between age groups disappeared. The youngest rats examined (2.5 months) exhibited a much wider scatter of individual HMPG values, particularly when expressed in terms of µg per kg per 24 hr, than the oldest rats (18-20 months) investigated. Although different experimental techniques were used to measure metabolite levels in the urine of 8 month and 18-20 month old normal rats, the mean daily excretion of HMPG was similar in the two groups of animals.

The daily excretion of noradrenaline has previously been shown to be similar in 8 and 20 month old rats (Herbeuval, Masse and Chollot, 1962), but in terms of body

weight, the 8 month old rats had a greater urinary output of the catecholamine.

The enzymes in the rat responsible for oxidative deamination and O-methylation of catecholamines, MAO and COMT respectively, undergo age changes depending on the tissue. For example, the MAO activity of rat heart increases with age (Novick, 1961; Studer, Baumgartner and Reber, 1964; Gey, Burkard and Pletscher, 1965; Horita, 1967; Prange, White, Lipton and Kinkead, 1967; de Champlain, Axelrod, Krakoff and Mueller, 1968) whereas the levels of COMT in this organ are independent of age (Gey, Burkard and Pletscher, 1965). In mid-aged rats (9 - 35 weeks), there is an increase in MAO and a relatively greater one in COMT activity of the liver. COMT activity in the kidney decreases with age (Prange, White, Lipton and Kinkead, 1967). These authors have also shown that the activity of both enzymes in brain is constant for all age groups. The net result of the various changes in enzyme activity with age on overall HMPG production can be seen in the results of the present investigation. (Changes in urinary excretion of normetadrenaline with age are discussed in the next chapter). The groups of 2.5 and 6 months old rats, used for investigation of normal uninary HMPG levels, included animals of both sexes whilst the older rats studied, 8 and 18 - 20 months old respectively, consisted entirely

of males. Since the MAO activity in both heart (Skillen, Thienes and Strain, 1962) and liver (Wurtman, Kopin and Axelrod, 1963) is greater in male than in female adult rats, this factor, as well as that of age, may contribute to the results obtained inthe present studies.

The comparatively low output of endogenous VMA in normal rat urine is further confirmation that reduction of the aldehyde formed by MAO, rather than oxidation as in the human, is the favoured metabolic route in this species (Kopin, Axelrod and Gordon, 1961). Whatever the species, several lines of evidence indicate that HMPG or DHPG, rather than VMA or DHMA, may be preferentially formed from noradrenaline in many peripheral tissues. This is true for such diverse tissues as rabbit atrium (Rutledge and Weiner, 1967), guinea-pig vas deferens (Weiner and Rabadjija, 1968), and rat submaxillary and sublingual glands (Jonason, 1969). The recent studies of Breese, Chase and Kopin (1969a) in rat brain, indicate that the presence of a β-hydroxyl group on the side chain favours intermediate aldehyde reduction, a finding which may apply equally well to the brains of other species in which a preferential formation of glycol metabolites from either exogenous or endogenous noradrenaline (Mannarino, Kirshner and Nashold, 1963; Rutledge and Jonason, 1967; Maas and Landis, 1968; Schanberg, Breese, Schildkraut, Gordon and Kopin, 1968; Jonason, 1969; Sharman, 1969a,b) is

well documented. The predominant output of VMA in human (Armstrong, McMillan and Shaw, 1957; La Brosse, Axelrod and Kety, 1958) and guinea-pig urine (Smith, 1961) may thus involve a secondary conversion of HMPG to VMA, a reaction which has been demonstrated in vivo in patients with neuroblastoma (La Brosse, 1966; La Brosse, Schweisguth and Bohuon, 1968).

The presence of a sulphate conjugate of HMPG and glucuronides of metadrenaline and normetadrenaline in rat urine is well established (Axelrod, Senoh and Witkop, 1958; Axelrod, Kopin and Mann, 1959), but the author is unaware of similar observations on VMA conjugation in the rat, apart from a passing reference of Armstrong and McMillan (1959) which states that the metabolite is conjugated as a glucuronide in the white rat. In the more detailed investigation reported herein, a small but significant proportion of the total quantity of VMA present in rat urine was found to be excreted in the free state; the major proportion of the acid was conjugated as a glucuronide, but a lesser degree of sulphate conjugation was also detected.

Despite an overall reduction in noradrenaline production in immunosympathectomized animals, as shown by the reduction in the endogenous tissue levels of the catecholamine (Levi-Montalcini and Angeletti, 1962; Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965; Klingman, 1965;

Visscher, Lee and Azuma, 1965; Zaimis, Berk and Callingham, 1965), the urinary output of VMA and normetadrenaline was not reduced. Indeed, VMA excretion, expressed in terms of body weight at least, may even have been increased in the treated rat. Additional VMA could arise from an increased adrenaline secretion from the adrenal medulla, but this possibility is unlikely since adrenaline output is normal in the urine of immunosympathectomized rats (Carpi and Oliverio, 1964), and no increase was detected in the urinary levels of metadrenaline in the present investigation. Adrenal noradrenaline concentration is raised, however, in immunosympathectomized animals (Berk, Filipe and Zaimis, 1965; Carpi and Oliverio, 1964; Visscher, Lee and Azuma, 1965) and, since the turnover of adrenal medullary catecholamines is more than doubled in immunosympathectomized mice (Iversen, Glowinski and Axelrod, 1966), it is likely that an increased secretion of noradrenaline into the circulation accounts for the anomalously high normetadrenaline and VMA which has been observed. Such an explanation, however, would demand a relatively greater production of acid at the expense of glycol from amine originating from the adrenal medulla compared with that derived from other tissues; this is unlikely since there is no change in the output of VMA in the urine of adrenalectomized rats (see chapter 5). The tendency for a greater proportion of amine secreted directly into the blood stream, compared with that metabolised within the cell to undergo primary O-methylation,

however, (Kopin and Gordon, 1962, 1963) could well make a proportionally large contribution to the normetadrenaline output. There is also evidence, arising from studies on the retention and metabolism of radioactive noradrenaline in immunosympathectomized animals (Iversen, Glowinski and Axelrod, 1966 ; Sjöqvist, Taylor and Titus, 1967), which indicates that an increased metabolism of the amine occurs in affected tissues, mainly accounted for by an increase in labelled normetadrenaline and acid metabolites. Thus any circulating noradrenaline secreted by the adrenal medulla, and taken up by immunosympathectomized tissues, would tend to undergo O-methylation rather than binding within the tissue. COMT activity in mouse tissues is not affected by immunosympathectomy (Iversen, Glowinski and Axelrod, 1966).

The pattern of catecholamine metabolism in the treated animal could be altered in favour of an increase in normetadrenaline output if MAO activity was decreased. Although such reductions in the activity of the enzyme have been reported, there is conflicting evidence regarding the sites affected and the extent of the decrease (Levi-Montalcini and Angeletti, 1962; Iversen, Glowinski and Axelrod 1966 ; Klingman, 1966a). The results reported in chapter 5 concerning the effects of NGF antiserum on MAO activity of the tissues of immunosympathectomized mice do not clarify the situation, possibly because a different preparation of anti-

serum was used from that employed in the other investigations.

The output of the noradrenaline precursor and endogenous catecholamine, dopamine, and its main O-methylated deaminated metabolite, HVA, was not significantly altered in the urine of immunosympathectomized rats compared with controls, data which are compatible with the view that the amount of dopamine associated with the sympathetic nervous system is at most a small percentage of the total body depot. Apart from its widespread distribution in various tissues (see chapter 1), a proportion of the amine excreted in the urine may arise from the direct renal decarboxylation of L-DOPA (Murphy and Sourkes, 1961). Conjugated HVA was not estimated in the present investigation, because in the rat the endogenous acid is mainly excreted in the free state (Shaw, McMillan and Armstrong, 1956), the rat excreting about three times as much free HVA as the adult human according to weight. Since intraneuronal catecholamines may be preferentially exposed to metabolic degradation by MAO, it is possible that the estimation of the oxidatively deaminated acid metabolite of dopamine, DOPAC, which is excreted in relatively large quantities in rat urine (Sourkes, Wiseman-Distler, Moran, Murphy and Saint Cyr, 1964), may provide a better assessment of the effect of immunosympathectomy on the metabolism of intraneuronal dopamine than does measurement of HVA.

The increased concentration of 5-HT in the gastro-intestinal tract (Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965; Iversen, Glowinski and Axelrod, 1966; Thompson and Campbell, 1966b) and submaxillary glands, heart and caecum of immunosympathectomized animals (Klingman, 1969) was not reflected in the present studies by a raised urinary output of 5-HT or 5-HIAA. The daily excretion of 5-HTOH, the alcohol formed by oxidative deamination of 5-HT and reduction of the intermediate aldehyde product, was also unchanged in the treated animal; when expressed in terms of body weight, however, the levels of this metabolite in the urine of the immunosympathectomized rat were reduced. This finding could be linked with decreases in MAO activity which have been described in various tissues of the immunosympathectomized animal (Levi-Montalcini and Angeletti, 1962; Iversen, Glowinski and Axelrod, 1966; Klingman, 1966a; see also chapter 5). Since the excretion of the quantitatively more important metabolite, 5-HIAA, is unchanged, however, it is unlikely that a reduction in MAO activity is responsible for the increased tissue concentrations of 5-HT found in these animals. In support of this conclusion, Klingman (1969) reported that administration of an MAO inhibitor to immunosympathectomized rats did not accentuate the rise in tissue 5-HT levels.

Recent observations (Klingman and Klingman, 1969) indicate that absence of sympathetic innervation may, in

fact, be responsible for the increased concentration of tissue 5-HT after immunosympathectomy; surgical sympathectomy of rat submaxillary glands resulted in significant increases of their 5-HT concentration and total 5-HT content. The raised concentration of the amine could thus result from a reduced ability of the tissue stores to discharge their 5-HT content, an effect which might be directly related to the impairment of the sympathetic nerve supply in the immunosympathectomized animal. In the gastro-intestinal tract, at least, there is evidence that such a relationship may exist between 5-HT storage and release, and sympathetic innervation. On the basis of histochemical studies Håkanson, Lilja and Owman (1969) have provided evidence indicating that gastric enterochromaffin cells receive an adrenergic innervation. Furthermore, stimulation of sympathetic nerves to the vasculature of isolated canine intestinal segments caused a significant release of 5-HT (Burks and Long, 1966).

In the normal rat the endogenous excretion of free 5-HT is seen to be very low in comparison with that of its deaminated metabolites. Similar values for the urinary output of the amine in the rat have been reported by Airaksinen, Paasonen and Pentola (1960). Unlike adrenaline or noradrenaline metabolism in the rat, where the glycol rather than the acid is the predominant oxidatively deaminated metabolite (Kopin, Axelrod and Gordon, 1961) the levels of

endogenous 5-HIAA in the urine are much higher than those of 5-HTOH. However, the relative proportion of 5-HTOH excreted in rat urine is greater than that reported to be present in human urine after administration of radioactive 5-HT (Davis, Cashaw, Huff and Brown, 1966).

An explanation of the tendency for the animal to animal variation of excreted compounds to be less in immunosympathectomized than in control rats is not readily apparent: its interpretation is made more difficult by the generality of its effect. The effect was noted not only in studies of catecholamine metabolites, but also of 5-HIAA.

CHAPTER 4

CATECHOLAMINE METABOLISM IN ADRENALECTOMIZED RATS

In chapter 3 the urinary output of catecholamine metabolites was determined in rats deprived of a major portion of their sympathetic nervous sytem. The present account deals with a similar investigation in adrenalectomized rats.

Changes in the urinary output of adrenaline and noradrenaline have been reported after bilateral adrenalectomy in man (von Euler, Franksson and Hellström, 1954) and rat (Imms and Jones, 1967), or following medullo-adrenalectomy in the dog (Schaepdryver, Preziosi and van der Stricht, 1959), rat (Biscardi, Carpi and Orsingher, 1964; Carpi and Oliverio, 1964), monkey (Harrison and Seaton, 1966) and cat (Sakai, 1967). Adrenaline excretion is abolished (Schaepdryver, Preziosi and van der Stricht, 1959; Carpi and Oliverio, 1964) or significantly reduced (von Euler, Franksson and Hellström, 1954; Harrison and Seaton, 1966; Sakai, 1967), whereas urinary noradrenaline levels are significantly unchanged (Schaepdryver, Preziosi and van der Stricht, 1959; Sakai, 1967) or increased (von Euler, Franksson and Hellström, 1954; Harrison and Seaton, 1966). In the present study a number of quantitatively important catecholamine metabolites have been measured in the urine of bilaterally

adrenalectomized rats in order to study, in more detail, the effect of this procedure on overall catecholamine production.

Procedure

(a) Total MHPG (spectrophotometric method), VMA, metadrenaline and normetadrenaline were estimated in 24 hour urine specimens from adrenalectomized and control rats by methods described in chapter 2. Free urinary HVA was measured by gas-liquid chromatography. Bilaterally adrenalectomized male Wistar rats (Scientific Products Farm, Canterbury, England) of the same age and weight range (280-350 g) were used in the study. The rats were housed under warm conditions and the treated animals allowed free access to solid food and 0.9% (w/v) NaCl solution. Control animals were given tap water instead of NaCl solution. During the urine collection period, animals were fasted from solid food but allowed 0.9% NaCl solution or water ad libitum.

(b) Later, the adrenalectomized rats, now weighing 372-522 g, were each treated daily for seven days with a subcutaneous injection of 10 mg per kg body weight hydrocortisone ("Efcortelan Soluble" - hydrocortisone sodium succinate for injection, B.P.) as replacement therapy. The concentrations of total MHPG and free HVA were re-estimated in adrenalectomized and control rat urine during treatment.

Results

There was a highly significant increase in urinary output of HMPG and, to a lesser extent, in that of HVA, in adrenalectomized rats compared with controls (Table 7). VMA and normetadrenaline excretion values were not significantly altered by adrenalectomy.

Administration of hydrocortisone for seven days to adrenalectomized rats abolished the rise in urinary HMPG and HVA output (Table 8); indeed, when expressed in terms of weight, the output of HMPG and HVA dropped significantly below normal levels. In accordance with the observations noted in chapter 3, the mean 24 hour urinary excretion of HMPG in the control rats increased with age, although this increase was not significant when expressed in terms of weight (Tables 7 and 8). Urinary HVA, however, was significantly reduced ($p < 0.001$) in the older controls when expressed on a weight basis.

Metadrenaline excretion was drastically decreased in adrenalectomized rats (Table 9), although measurable quantities of the metabolite could be detected in the urine of four out of six rats investigated.

TABLE 7

Excretion of total HMPG, VMA, normetadrenaline and free HVA in the urine of adrenalectomized and control rats.

	Adrenalectomized		Controls	
		Mean excretion ± S.E.M.		Mean excretion ± S.E.M.
Metabolite	Expts.	µg. per 24 hours	Expts.	µg per 24 hours
HMPG*	6	60.63 ± 2.63 (303.7 ± 20.0)	6	34.54 ± 2.55 (187.6 ± 13.8)
VMA	6	11.72 ± 1.43	6	13.50 ± 2.04
NMA	4	3.52 ± 1.45	5	5.10 ± 1.80
HVA+	6	55.92 ± 4.68 (207.0 ± 18.0)	6	44.45 ± 4.05 (160.2 ± 14.0)

* $p < 0.001$

+ $p < 0.05$ (Mann-Whitney test)

Values in parenthesis represent µg per kg per 24 hr

HMPG estimated by spectrophotometry.

HVA estimated by gas-liquid chromatography.

TABLE 8

Excretion of total HMPG and free HVA in the urine of adrenalectomized rats after treatment with 10 mg per kg hydrocortisone daily for 7 days.

	Adrenalectomized rats			Control rats		
		Mean excretion ± S.E.M.			Mean excretion ± S.E.M.	
Metabolite	No. of expts.	µg per 24 hr	µg per kg per 24 hr	No. of expts.	µg per 24 hr	µg per kg per 24 hr
HMPG	5	48.50 ± 6.09	90.4 ± 16.4*	6	60.06 ± 5.03	147.9 ± 13.0
HVA	5	29.58 ± 2.21	65.6 ± 5.3*	6	34.77 ± 2.07	86.5 ± 6.6

*$P < 0.05$

HMPG estimated by spectrophotometry

HVA estimated by gas-liquid chromatography

TABLE 9

Excretion of metadrenaline in the urine of adrenalectomized and control rats.

Adrenalectomized		Controls*	
Rat	µg per 24 hr	Rat	µg per 24 hr
1	0.35	1	0.70
2	—**	2	1.06
3	0.18	3	0.79
4	—**	4	0.33
5	0.24	5	1.77
6	0.11	6	0.89

* Mean ± S.E.M. = 0.92 ± 0.20 µg per 24 hr

** Below limits of sensitivity of the method

Discussion

The paradoxical increase in noradrenaline output after adrenalectomy in human (von Euler, Franksson and Hellström, 1954) and rat urine (Imms and Jones, 1967) respectively, is accompanied by a significantly elevated HMPG excretion in the rat at least. The cause of this effect is unknown, although Imms and Jones (1968) have suggested that bilateral adrenalectomy in rats results in an increase in sympathetic nerve activity, secondary to a decrease in blood pressure. In agreement with this hypothesis, it appears that a significant increase in rate of synthesis of cardiac noradrenaline occurs in adrenalectomized rats compared with normal or sham-operated controls, and that this rise can be prevented by ganglionic blockade (Westfall and Osada, 1969). An increased turnover of noradrenaline in rat heart has also been observed by Landsberg and Axelrod (1968) after hypophysectomy and adrenalectomy and, recently, Landsberg, de Champlain and Axelrod (1969) have demonstrated that increased de novo biosynthesis of catecholamines in the heart occurs concomitantly with the increased turnover. The raised urinary HMPG output after bilateral adrenalectomy reported in the present studies might also arise, at least in part, from an increased production of noradrenaline in such sites as the

brain (Javoy, Glowinski and Kordon, 1968).

HMPG, the major metabolite of adrenaline and noradrenaline in rat urine (Kopin, Axelrod and Gordon, 1961), appears to provide a better index of noradrenaline turnover than VMA or normetadrenaline, the urinary excretion of which was unchanged by adrenalectomy. A similar correlation was noted in rats after immunosympathectomy (see chapter 3), where a significant fall in urinary noradrenaline output is matched by a parallel change in HMPG excretion without any corresponding alteration in VMA or normetadrenaline output.

Treatment of the adrenalectomized rats with hydrocortisone resulted in a reduction of the elevated HMPG output to control values. There was even a tendency for the excretion values of this metabolite to drop below those of the control group, reaching significance ($P < 0.05$) when expressed in terms of body weight. These findings are compatible with the observations of Imms and Jones (1967) who showed that the chronic administration of corticosterone to adrenalectomized rats, in doses as low as 50 µg per 100 g twice daily, reduced the previously increased catecholamine excretion to almost normal levels. Furthermore, Westfall and Osada (1969) demonstrated that if adrenalectomized rats are treated with replacement doses of either hydrocortisone or desoxycorticosterone acetate no increase in the rate of synthesis of cardiac noradrenaline is observed.

These findings strongly suggest that the absence of adrenal cortical hormones in adrenalectomized rats is responsible for the changes observed in urinary noradrenaline and HMPG excretion. Neff, Ngai, Wang and Costa (1969), however, have recently reported a four fold increase in cardiac noradrenaline synthesis in the adrenal demedullated rat, whilst Harrison and Seaton (1966) observed a three-fold increase in the urinary levels of noradrenaline in demedullated monkeys. These results cast doubt on the physiological role, if any, of cortical steroids in the regulation of noradrenaline synthesis and excretion in the adrenalectomized animal. There is some evidence, however, to suggest that in demedullated rats the function of the adrenal cortex may be impaired for a period of time following surgical removal of the medullae; Imms and Jones (1967) reported an initial high rate of catecholamine excretion in rat urine after demedullectomy, which fell to normal levels as the cortex regenerated. In a similar experiment, Biscardi, Carpi and Orsingher (1964) observed an increased output of noradrenaline during two weeks following surgery, but normal levels of the amine were detected six weeks after removal of the medullae. If the effects of adrenalectomy on cardiac synthesis and urinary excretion of noradrenaline were to follow a similar time course, it is possible that cortical function was still impaired in the demedullated rats used by Neff, Ngai, Wang

and Costa (1969), even though their experiments were not conducted until at least fourteen days after surgery.

Such an explanation would not appear to be tenable, however, to explain the increased noradrenaline excretion in demedullated monkeys described by Harrison and Seaton (1966), since urine collections were made four months after surgery. In other species the effect of medullectomy on noradrenaline output in the urine appears to follow a different pattern. Thus, after bilateral adrenal medullectomy in cats, Sakai (1967) observed an increased noradrenaline excretion in the first twenty-four hours, but thereafter the output returned to normal levels. In the dog, no considerable changes were observed in the quantity of urinary noradrenaline excreted following removal of the medullae, although a tendency towards postoperatively increased values of the amine was detected (Schaepdryver, Preziosi and van der Stricht, 1959).

Regarding the tendency for HMPG levels in the urine of adrenalectomized rats to fall below those of the controls after treatment with hydrocortisone, it is of interest to note that Luft and von Euler (1952) reported a significant depression of noradrenaline excretion after chronic cortisone treatment in arthritic patients, though not after desoxycorticosterone administration; there were no effects on adrenaline excretion. The effect of hydrocortisone on the levels of urinary HMPG in the normal rat was not examined

in the present investigation, but future studies along these lines, examining as many different catecholamine metabolites as possible, would appear to be indicated.

In contrast with MHPG, the daily excretion of normetadrenaline in the urine of the adrenalectomized rats was not significantly altered from control values. The reason for this apparent discrepancy is not known. Cardiac COMT activity appears to be unchanged after bilateral adrenalectomy in rats (Avakian and Callingham, 1968). A slight reduction in hepatic COMT activity, however, has been reported in the hypophysectomized rat (Landsberg, de Champlain and Axelrod, 1969). Whether or not COMT activity in the liver is similarly decreased after adrenalectomy is unknown but, in any case, the observed increases in MAO activity which occur in certain tissues of these animals (see chapter 5), may favour the formation of deaminated rather than O-methylated metabolites. Moreover, additional normetadrenaline, which may be formed in the brain as a result of the increased noradrenaline turnover occurring in that site after adrenalectomy, (Javoy, Glowinski and Kordon, 1968), would be unlikely to pass out of the brain as unchanged amine to any great extent (Schanberg, Schildkraut, Breese and Kopin, 1968). Coward, Smith, Wilson and Robinson (1962), using a paper chromatographic procedure, noted that urinary normetadrenaline excretion seemed to be noticeably higher in patients with

bilateral adrenalectomy than in normal man. These differences between the human and the rat may be species dependent.

It is noteworthy that the mean daily urinary excretion of normetadrenaline in normal control Wistar rats weighing 280-350 g (Table 7) was greater than that observed for animals weighing 455-775 g (Table 1). Thus total normetadrenaline excretion appears to decrease with age. This finding may depend to some extent on the fact that MAO activity in certain tissues increases with the age of the rat (Novick, 1961; Studer, Baumgartner and Reber, 1964; Gey, Burkard and Pletscher, 1965; Horita, 1967; Prange, White, Lipton and Kinkead, 1967; de Champlain, Axelrod, Krakoff and Mueller, 1968), whereas renal COMT activity falls off with age and hepatic COMT increases only up to middle age (9-35 weeks), after which it is reduced (Prange, White, Lipton and Kinkead, 1967).

Four of the six adrenalectomized rats used in this investigation put out measurable amounts of metadrenaline in the 24 hour urine sample, a further indication of the existence of extra-adrenal sources of adrenaline. Other studies (von Euler, Franksson and Hellström, 1954; Schaepdryver and Smets, 1961; Harrison and Seaton, 1966; Sakai, 1967) have shown that extirpation of the adrenals or adrenal medullae is not followed by a complete disappearance of adrenaline from the urine. Coward, Smith, Wilson and Robinson (1962) detected urinary metadrenaline on the paper

chromatograms of the urines in two out of fourteen patients with bilateral adrenalectomy. The detection of phenylethanolamine N-methyltransferase, the enzyme which converts noradrenaline to adrenaline, in rabbit heart (Axelrod, 1962a) and the demonstration that labelled noradrenaline can be converted to adrenaline in rat heart (Potter, Bogaert and Schaepdryver, 1966), show that adrenaline may be formed in peripheral sites other than the adrenal medulla. Whilst there is substantial evidence indicating that brain tissue can form adrenaline (McGeer and McGeer, 1964; Ciaranello, Barchas, Byers, Stemmle and Barchas, 1969; Pohorecky, Zigmond, Karten and Wurtman, 1969), and low endogenous concentrations of the amine have been identified in the brain (von Euler, 1946c; Vogt, 1954; Holtz, 1959; Gunne, 1962), metadrenaline formed within the central nervous system may not easily cross the blood brain barrier so that only small amounts of the metabolite derived from this source may be present in the urine.

Dopamine forms a small proportion, only, of the catecholamine content of the adrenal medulla (Holtz, 1959), but has been detected in the adrenal venous effluent of the cat under in vivo conditions (Hempel and Männl, 1967). Paradoxically, adrenalectomy gave rise to an increased daily urinary output of the major metabolite of dopamine, HVA, which, however, was not significant when expressed in terms of body weight. This rise, proportionally not as

great as that of HMPG, may reflect accelerated production of precursor dopamine involved in the increased turnover and synthesis of noradrenaline in adrenalectomised animals (Landsberg and Axelrod, 1968; Neff, Ngai, Wang and Costa, 1969; Westfall and Osada, 1969). The accelerated turnover of noradrenaline in the brains of adrenalectomised rats (Javoy, Glowinski and Kordon, 1968) could also be associated with an increased turnover and synthesis of dopamine, the metabolism of which could, in turn, lead to increased levels of brain HVA; since this metabolite can pass out of the brain by an active transport process (Sharman, 1966; Werdinius, 1966) it could contribute to urinary HVA levels. Adrenalectomy has been reported to cause an increase, and hydrcortisone treatment a decrease, in the amine content of the tubero-infundibular dopamine neurones (Almayer and Donath, 1965). In a similar histochemical study, however, Fuxe and Hökfelt (1967) were unable to confirm these findings. Moreover, an increase of fluorescence intensity observed in dopamine cell bodies in such studies does not necessarily indicate an increased formation of amine. The administration of hydrocortisone to adrenalectomized rats in the present studies did, however, abolish the rise in urinary HVA output and, indeed, in terms of body weight, significantly reduced the urinary excretion of this metabolite below that of the controls.

The results shown in Tables 7 and 8 show that, in

contrast to HMPG, but like normetadrenaline, the excretion of HVA in rat urine is decreased with age. It would be of interest to compare the urinary excretion of DOPAC in rats of different age with that of HVA to see if the change in HVA excretion can be correlated with MAO and COMT activity.

CHAPTER 5

Studies on monamine oxidase

Current opinion favours the view that monoamine oxidase (MAO) activity within the sympathetic nerve is responsible for the deamination of intraneuronal noradrenaline, thereby modulating the level of the amine in the tissues (Kopin, 1966). Drugs such as reserpine which deplete catcholamine stores without arousing much sympathomimetic response greatly increase the excretion of deaminated metabolites, whereas drugs such as tyramine which evoke a sympathetic response cause some release of unchanged noradrenaline into the circulation; tyramine-released catecholamine is predominantly O-methylated (Kopin and Gordon, 1962, 1963).

Apart from intraneuronal enzyme, sympathetically innervated tissues also contain variable amounts of extra-neuronal MAO activity. The question arises as to how much intraneuronal enzyme activity there is in a particular tissue. After surgical sympathectomy, falls in MAO activity ranging from 10% to 60% have been described for various animal tissues (Burn and Robinson, 1952; Waltman and Sears, 1964; Snyder, Fischer and Axelrod, 1965; Iversen, Jarrott and Langer, 1968), although some authors detected no significant change (Armin, Grant, Thompson and Tickner,

1952; Burn, Philpot and Trendelenburg, 1954). The greatest reductions in MAO activity were observed in tissues with a rich sympathetic innervation such as nictitating membrane, iris, pineal gland and vas deferens. Results obtained in studies on immunosympathectomized animals have tended to be conflicting. Levi-Montalcini and Angeletti (1962) described large decreases (up to 70% of control values) in MAO activity of the spleen, heart and submaxillary glands after administration of NGF antiserum to animals. Significant, though smaller, decreases in MAO activity of the spleen, submaxillary glands, kidneys and liver of immunosympathectomized rat were also noted by Klingman (1966a). Iversen, Glowinski and Axelrod (1966), however, found decreased activity only in the salivary glands of the immunosympathectomized mouse, but no changes in other tissues examined. Thus it appears that only in tissues where intraneuronal enzyme constitutes an appreciable portion of total MAO activity is it possible to demonstrate a decrease after sympathetic nerve destruction.

Some recent investigations, using differential centrifugation and continuous sucrose gradient techniques, have shown that a considerable fraction of MAO activity in such tissues as heart, salivary glands, vas deferens and splenic nerve is present in the microsomal fraction; whether

or not this activity actually lies within the noradrenaline storage particles has tended to give rise to conflicting data, and may differ from tissue to tissue (Roth and Stjärne, 1966; Stjärne, Roth and Giarman, 1968; Jarrott and Iversen, 1968; de Champlain, Mueller and Axelrod, 1969). Because of these considerations, and the difficulty of separating intraneuronal from extraneuronal MAO activity in a crude tissue homogenate, it was decided to investigate the effect of a variety of procedures known to decrease tissue noradrenaline levels on MAO activity in subcellular compartments.

Investigations were therefore carried out in reserpine-treated and immunosympathectomized mice, as well as in mice pre-treated with 6-hydroxydopamine which causes a "chemical" sympathectomy (Thoenen and Tranzer, 1968) in animal tissues.

Parallel with the studies on catecholamine metabolism in the adrenalectomized rat described earlier, MAO activity in subcellular fractions of several tissues of these animals has been measured. In contrast with the experimental conditions listed above, which are associated with a lack of tissue noradrenaline, adrenalectomy brings about increased urinary concentrations of this catecholamine (von Euler, Franksson and Hellström, 1954; Imms and Jones, 1967) as well as enhanced turnover (Landsberg and Axelrod, 1968) and biosynthesis (Westfall and Osade, 1969) of cardiac

noradrenaline, and an increase in the MAO activity of rat heart (Avakian and Callingham, 1968; Westfall and Osada, 1969).

Methods

Chemicals

Kynuramine dihydrobromide (Sigma Chemical Company, St. Louis); 4-hydroxyquinoline (Koch-Light Laboratories Ltd.).

Drugs

"Serpasil" (reserpine) ampoules, 2.5 mg per ml. in reserpine diluent (Ciba). Reserpine diluent (citric acid (1 H_2O), 2.75 mg; benzyl alcohol, 20.6 mg; polyethylene glycol 300, 112.2 mg; water for injection to 1 ml). The author is grateful to Dr. R. Chambers (Hoechst Pharmaceuticals, Ltd.) for the supply of reserpine ampoules.

6-Hydroxydopamine hydrobromide was kindly supplied by Dr. A. Pletscher, Hoffman-La Roche and Co. Ltd., Basle, Switzerland. Prior to injection, the drug was dissolved in 0.001 N HCl saturated with nitrogen. 6-Hydroxydopamine is highly susceptible to oxidation at neutral and alkaline pH.

Antiserum to nerve growth factor (Burroughs Wellcome and

Co., London); this is a sterile freeze-dried serum, prepared by injecting a horse with purified nerve growth factor from the submaxillary gland of the adult male mouse. The contents of the bottle were dissolved by adding 10 ml of sterile distilled water and allowing the mixture to stand until solution was complete.

Drug treatment of animals.

(i) Reserpine

Male albino mice, Ash/Porton strain, weight 30-40 g (Scientific Products Farm, Canterbury, England), were used in the study. Each group of mice contained 5 animals. Separate groups of mice were injected with reserpine (5 mg per kg i.p) and killed at the following times after injection - 0, 1, 2, 4, 8, 12, and 24 hours. Control groups of mice were injected with reserpine diluent (0.25 ml i.p.) and killed at 0, 2, 8 and 24 hours. Liver, heart and brain were removed immediately and treated, as described below, for the estimation of MAO activity in subcellular fractions.

(ii) 6-Hydroxydopamine

Male and female albino mice (ratio 2:1 respectively) TO strain (A. Tuck and Son, Rayleigh, Essex), weight 30 - 40 g, were used as test and control animals. The test mice were injected intravenously with 2 x 50 mg per kg of

6-hydroxydopamine hydrobromide within a 24 hour period, and one week later with 2 x 100 mg per kg. This was the dose schedule found to be optimal for the most extensive destruction of adrenergic nerve endings in rats (Thoenen and Tranzer, 1968). The animals were sacrificed 9 days later, the required tissues were removed immediately, and treated as described for estimation of MAO activity in subcellular fractions.

(iii) Immunosympathectomy

Newborn male and female albino mice (ratio 1:1, respectively) TO strain, were injected subcutaneously for the first 5 days of life with increasing doses as follows: 0.07 ml, 0.1 ml, 0.1 ml, 0.12 ml, 0.15 ml. Thus, each received just over 0.5 ml. Dosage is not critical, and can be varied within wide limits and still give satisfactory results (D.C. Edwards, personal communication). The animals were allowed to mature for a 3 month period, after which they weighed 30 - 40 g. They were then killed, and tissues were immediately extracted and treated as described for estimation of MAO activity in subcellular fractions.

To prove that immunosympathectomy had in fact occurred, superior cervical ganglia were dissected from two treated and two control animals. A drastic reduction is size was shown to occur in the ganglia of the animals treated with NGF antiserum (Fig. 8). This feature is

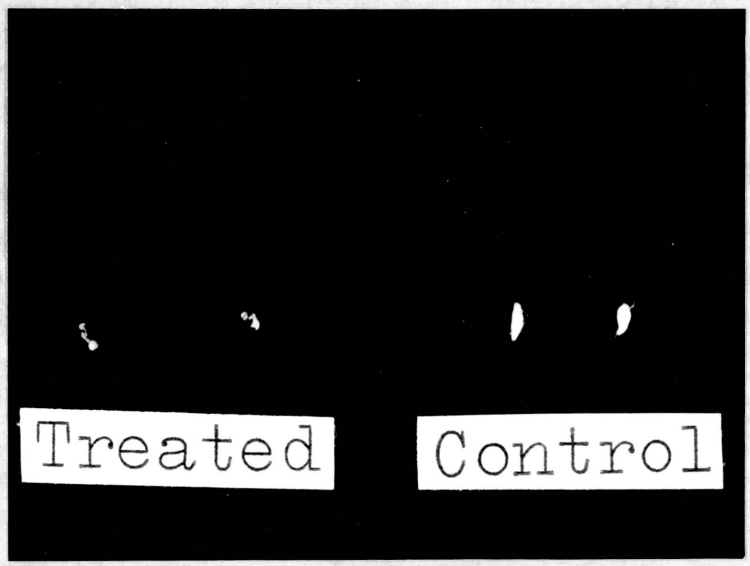


Fig. 8. Superior cervical ganglia of control mouse and mouse treated with Burroughs Wellcome nerve growth factor antiserum.

characteristic of immunosympathectomized animals.

(iv) Adrenalectomized rats

The adrenalectomized and control rats used in the studies described in chapter 4 were kept for 8 weeks after the administration of the final dose of hydrocortisone. They were then sacrificed, the required tissues obtained immediately, and MAO activity estimated in subcellular fractions.

Subcellular fractionation of tissues

After removal from test animals, tissues were washed with ice-cold normal NaCl solution, quickly weighed, and stored at $4^{o}C$ until required. The tissues were homogenized in ice-cold 0.3 M sucrose solution in a glass homogenizer with Teflon pestle. They were then submitted to subcellular fractionation by a method based on that described by Hawkins (1952). Cell debris was removed by centrifugation at 1000 x g for 30 minutes (M.S.E. "High Speed 17" Refrigerator Centrifuge) and discarded. This treatment sediments unbroken tissue cells, large cell fragments, nuclei and red blood cells. The supernatant, containing soluble cell components and particulate elements, was centrifuged at 17,500 x g for 1 hour to sediment the "mitochondrial" fraction which was retained. The second supernatant was centrifuged at 108,000 x g for 1 hour (M.S.E. Automatic "Superspeed 50" Refrigerator Centrifuge) to sediment the "microsomal" fraction.

Both the mitochondrial and microsomal fractions were suspended in phosphate buffer (0.05 M, pH7.4) and stored at -15°C. The final "supernatant" fraction after centrifugation at 108,000 g was also retained. All operations were carried out between 0°C and 4°C.

Comment on the procedure

The subcellular fractionation procedure employed in this study is arbitrary. Any MAO activity present in the gross cell debris was not measured. Activity ascribed to the so-called final "supernatant" fraction was shown to be mainly associated with particulate matter. Samples of normal mouse heart, brain and liver final "supernatant" were subjected to centrifugation at 200,000 x g for 1 hour. 73, 64 and 71% respectively of the MAO activity was found to be associated with the sediment, the remainder in the supernatant. There is no reason to suppose that centrifugation at a sufficiently high force would not remove all the enzyme activity from solution. With these considerations in mind, it is obviously important to standardize conditions as far as possible. Test and control tissues were therefore homogenized in the same manner, at the same intensity, and for the same length of time.

Estimation of MAO activity

The MAO activity of the various tissue fractions was estimated spectrophotofluorimetrically, using kynuramine

as substrate. The experimental procedure was the same as that employed by Kraml (1965), except that half-quantities of reagents were used. In this method the fluorescence intensity of 4-hydroxyquinoline (4HQ), which arises from the spontaneous cyclization of the intermediate aldehyde formed by the oxidative deamination of kynuramine (Weissbach, Smith, Daly, Witkop and Udenfriend, 1960), is measured. Thus, the assay does not depend on the tissue sample containing aldehyde dehydrogenase.

Procedure

Aliquots of tissue fractions were used with MAO activity yielding a fluorescence intensity lying between 0.1 and 1 µg 4-hydroxyquinoline on a standard curve. A linear response could, however, be obtained for values up to 10 µg 4-hydroxyquinoline. Phosphate buffer (0.05M, pH 7.4) was added to make the volume up to 1.25 ml. The solutions were placed in a metabolic incubator at 37°C and allowed to equilibrate. Kynuramine (0.25 ml) was added at timed intervals, and the mixture incubated for a period of 20 minutes with air as the gas phase. The reaction was stopped by adding 1.0 ml 10% trichloroacetic acid (TCA), and precipitated proteins were spun down at 1,200 x g. To 1.0 ml of 1N NaOH in a quartz cuvette was added 0.5 ml supernatant. The solution was activated at 315 mµ and the

fluorescence measured at 380 mμ on an Aminco-Bowman Spectrophotofluorometer, using slit arrangement No.4. Blanks, consisting of enzyme preparation mixture to which 10% TCA had been added before incubation, were carried through the entire procedure. Standard 4-hydroxyquinoline (0.1 μg, 0.25 μg, 0.5 μg and 1.0 μg, all in duplicate) plus phosphate buffer to 1.5 ml were similarly incubated, with the addition of TCA after the incubation. Standard blanks consisted of buffer alone plus 10% TCA. MAO activity in the tissue fractions was read from a standard 4-hydroxyquinoline curve, and expressed in terms of μg of 4-hydroxyquinoline formed per minute incubation per g wet weight of tissue.

Results

In the following section, the term 'total MAO activity' refers to the sum of mitochondrial, microsomal and supernatant enzyme activity, but does not include any activity which might be associated with the gross cell debris.

(i) Mouse MAO activity after reserpine treatment

Total MAO activity increased significantly in the heart and the liver, the maximum effect in both occurring 2 hours after reserpine administration (Figs.9 and 10). The increase in cardiac activity was followed by a fall which continued until at least 24 hours after

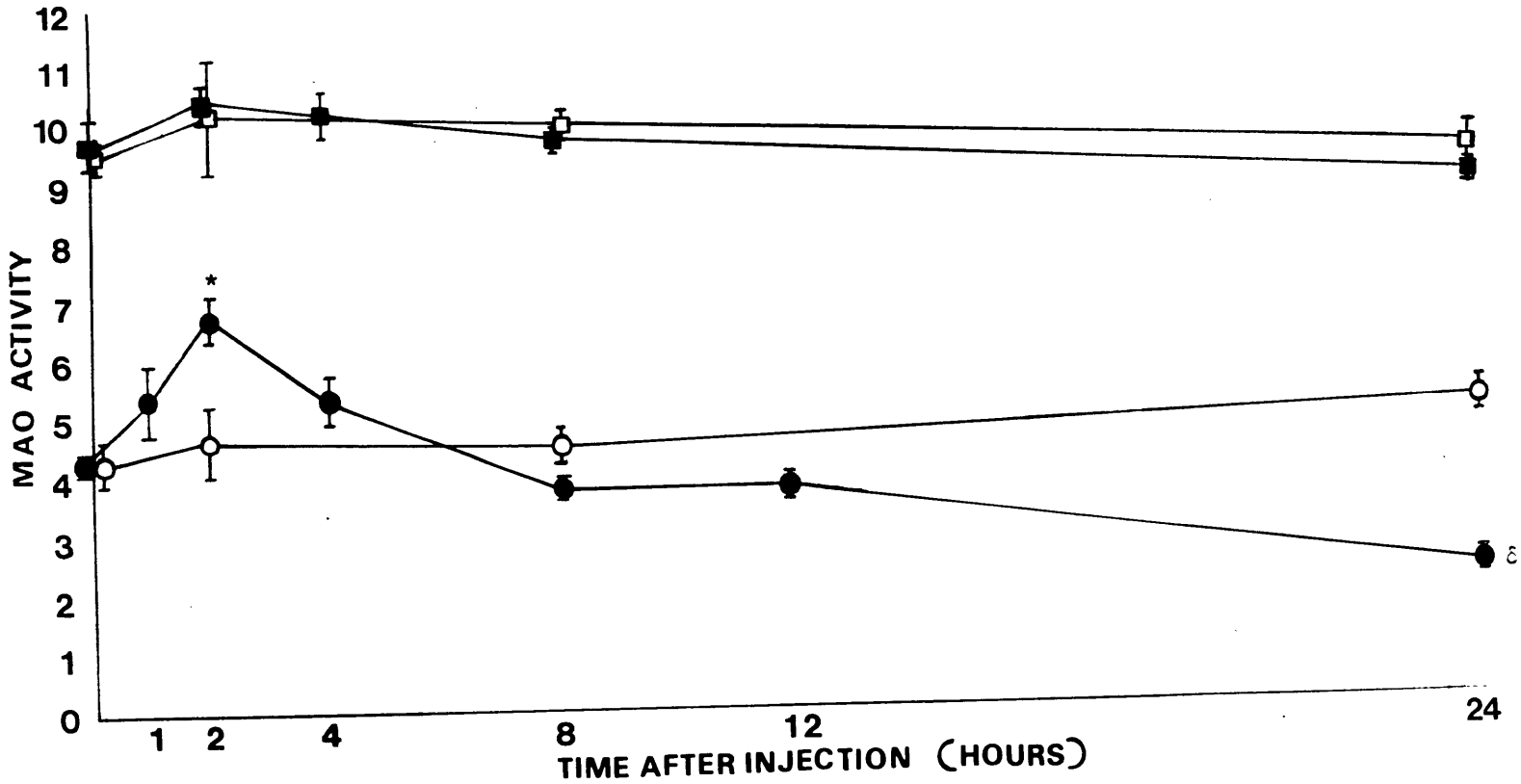


Fig. 9 Sum of MAO activity (µg 4-hydroxyquinoline formed per min per g wet weight tissue) in subcellular fractions of mouse brain after reserpine, 5 mg per kg i.p. (■—■—■) and reserpine diluent (□—□—□) and in mouse heart after reserpine (●—●—●) and reserpine diluent (○—○—○) * $p < 0.025$, ε $p < 0.001$

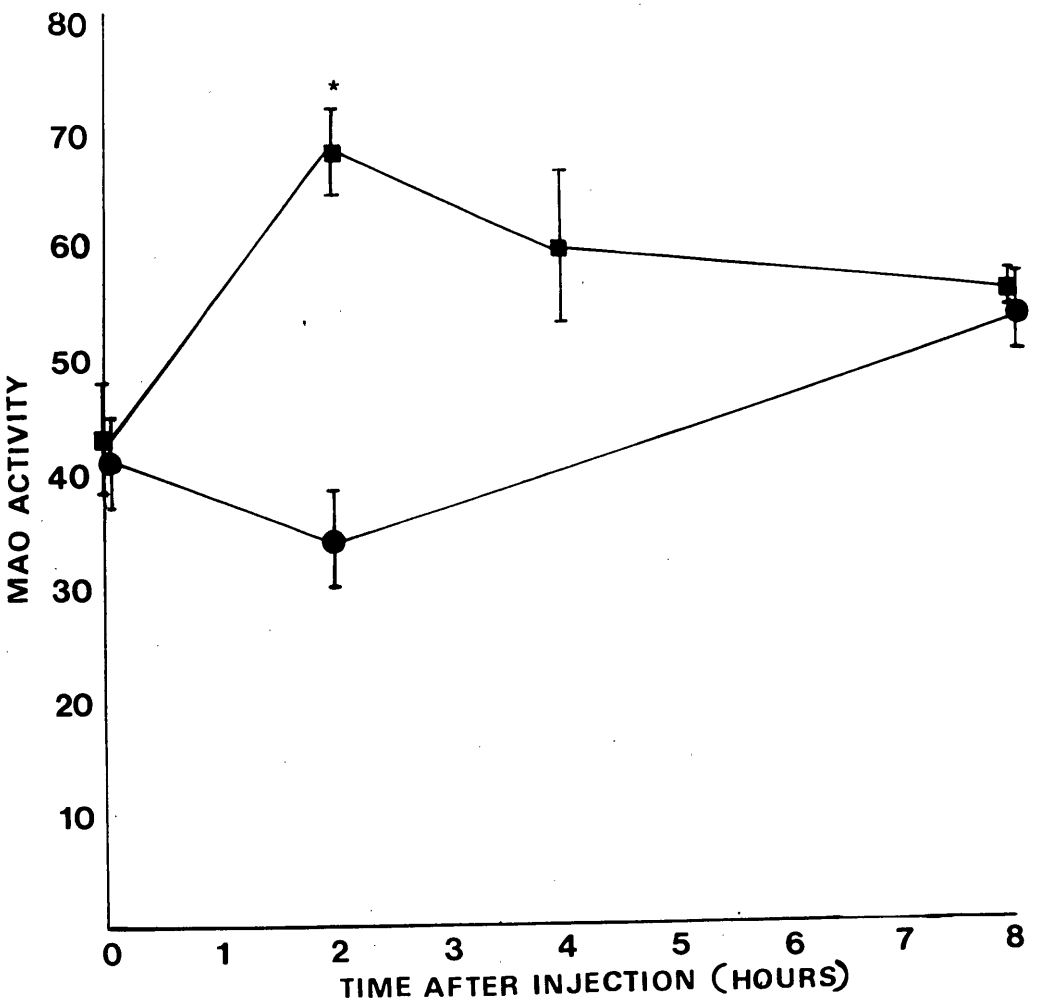


Fig. 10 Sum of MAO activity (μg 4-hydroxyquinoline formed per min per g wet weight tissue) in subcellular fractions of mouse liver after reserpine, 5 mg per kg i.p. (■—■—■) and reserpine diluent (●—●—●) * $p < 0.001$

treatment with the drug. As the mitochondrial activity is large compared with the other fractions, changes in total activity in heart and liver homogenates mirror those of the mitochondrial fraction (Table 10). Thus, after an initial rise reaching its peak at 2 hours, activity of the heart mitochondrial fraction fell, and at 24 hours after injection was significantly less ($P < 0.001$) than that of control samples. On the other hand, despite there being no significant change in total MAO activity in the reserpinized mouse brain homogenates compared with control values, alterations in enzyme activity were detected in the subcellular fractions of this organ; mitochondrial activity showed an increase, (Table 10) which was still significant at 24 hours. In the liver, the increase was more rapid, and by 8 hours had fallen to control levels.

In contrast to the mitochondrial fraction, MAO activity of the microsomal fraction of both brain and heart showed a significant and prolonged fall after reserpine treatment (Table 11). After 24 hours, activity was significantly less ($P < 0.01$ in the heart and < 0.001 in the brain) than control values. Liver microsomal activity, however, showed a threefold increase 2 hours after injection. The increase observed in the control liver microsomal fraction was slower in onset, and had declined to initial values after 24 hours.

TABLE 10.

MAO activity (μg 4-hydroxyquinoline formed per min per g wet weight) of mouse brain, heart and liver mitochondrial fractions after reserpine (5 mg per kg i.p) and reserpine diluent.

TISSUE		TIME AFTER INJECTION					
		0 HR	1 HR	2 HR	4 HR	8 HR	12 HR
BRAIN	RESERPINE	6.33±0.31	7.04±0.18	7.00±0.11*	7.37±0.30	7.21±0.13 §	6.73±0.15
	CONTROL	5.98±0.13	—	6.00±0.27	—	6.34±0.10	—
HEART	RESERPINE	3.15±0.10	4.17±0.33	5.64±0.41*	4.34±0.40	2.77±0.12	2.72±0.20
	CONTROL	3.04±0.25	—	3.12±0.31	—	3.37±0.29	—
LIVER	RESERPINE	37.70±4.63	54.67±3.18	54.36±4.30*	48.69±6.99	44.08±2.70	34.21±3.99
	CONTROL	34.43±3.36	—	29.51±5.36	—	44.11±4.52	—

		24 HR
BRAIN	RESERPINE	6.52±0.09*
	CONTROL	5.87±0.17
HEART	RESERPINE	1.65±0.15 §
	CONTROL	3.85±0.18
LIVER	RESERPINE	32.47±4.70
	CONTROL	30.41±1.61

The values are the mean of 5 experiments ± S.E.M.

* $P < 0.01$

§ $< P$ 0.001

TABLE 11.

MAO activity of mouse brain, heart and liver microsomal fractions after reserpine (5 mg per kg i.p.) and reserpine diluent. For explanation of legend see Table 10.

TISSUE		TIME AFTER INJECTION					
		0 HR	1 HR	2 HR	4 HR	8 HR	12 HR
BRAIN	RESERPINE	2.40±0.03	2.32±0.08	1.77±0.15	1.54±0.06	1.31±0.09§	1.35±0.06
	CONTROL	2.45±0.12	—	2.31±0.22	—	2.54±0.11	—
HEART	RESERPINE	0.82±0.05	0.86±0.06	0.73±0.04	0.62±0.02	0.60±0.07	0.58±0.04
	CONTROL	0.85±0.11	—	0.83±0.08	—	0.77±0.07	—
LIVER	RESERPINE	3.72±0.32	7.58±1.11	11.96±0.82§	6.99±0.07	6.32±0.92	6.22±0.65
	CONTROL	4.89±0.72	—	3.25±0.36	—	7.77±0.91	—

		24 HR
BRAIN	RESERPINE	1.51±0.12§
	CONTROL	2.76±0.24
HEART	RESERPINE	0.36±0.04*
	CONTROL	0.90±0.11
LIVER	RESERPINE	—
	CONTROL	4.57±0.22

The values are the mean of 5 experiments ± S.E.M.

* $P < 0.01$

§ $P < 0.001$

Enzyme activity of the supernatant fraction (Table 12) increased significantly in the brain and the liver; the peak in the former occurred at about 2 hours after reserpine, and in the latter at 4 hours. Supernatant activity in the heart was not altered significantly in the reserpinized animal.

(ii) Mouse MAO activity after 6-hydroxydopamine treatment

Treatment with 6-hydroxydopamine significantly decreased the MAO activity in the microsomal fraction of both the heart and the liver (Table 13), but only in the latter tissue was there a significant decrease in total tissue activity. In the vas deferens, however, a significant increase in the microsomal activity was detected. The mitochondrial fraction of the brain similarly showed an increase, in contrast with significant decreases in microsomal and supernatant activity.

The only other tissue fraction showing any alteration in MAO activity was the duodenal supernatant, where a signfficant fall occurred after 6-hydroxydopamine. No alteration was detected in subcellular fractions of submaxillary gland, spleen and kidney.

(iii) MAO activity in immunosympathectomized mice

Total MAO activity was significantly decreased in heart and liver (Table 14), whereas significant increases were observed in the submaxillary gland and, to an even

TABLE 12.

MAO activity of mouse brain, heart and liver supernatant fractions after reserpine (5 mg per kg i.p) and reserpine diluent. For explanation of the legend see Table 10.

TISSUE		TIME AFTER INJECTION					
		0 HR	1 HR	2 HR	4 HR	8 HR	12 HR
BRAIN	RESERPINE	1.01±0.03	——	1.62±0.19*	1.34±0.08	1.30±0.07	——
	CONTROL	1.10±0.04	——	0.98±0.16	——	1.13-0.16	——
HEART	RESERPINE	0.30±0.02	0.32±0.01	0.37±0.07	0.36±0.02	0.41±0.04	0.53±0.06
	CONTROL	0.42±0.03	——	0.46±0.05	——	0.38±0.02	——
LIVER	RESERPINE	1.50±0.51	——	1.69±0.47*	3.88±0.74	2.11±0.62	——
	CONTROL	1.44±0.44	——	1.41±0.09	——	1.94±0.44	——

	24 HR
BRAIN RESERPINE	0.94±0.11
BRAIN CONTROL	1.01±0.05
HEART RESERPINE	0.31±0.04
HEART CONTROL	0.41±0.04
LIVER RESERPINE	1.80±0.75
LIVER CONTROL	2.01±0.25

The values are the mean of 5 experiments

± S.E.M.

* $P < .05$

TABLE 13.

MAO activity in the tissues of mice treated with 6-hydroxydopamine. Activity expressed in terms of μg 4-hydroxyquinoline formed per min. per g wet weight of tissue ± S.E.M.

TISSUE		MITOCHONDRIAL FRACTION	MICROSOMAL FRACTION	SUPERNATANT FRACTION	SUM OF FRACTIONS
HEART	CONTROL	10.40±1.73 N.S.	3.03±0.30 P<0.01	0.86±0.08 N.S.	14.29±1.94 N.S.
	6 - HD.	8.05±1.24	1.80±0.19	1.02±0.11	10.78±1.10
BRAIN	CONTROL	20.39±0.49 P<0.02	2.49±0.14 P<0.001	0.17±0.20 P 0.05	23.05±0.47 N.S.
	6 - HD.	24.00±1.11	0.83±0.11	0.11±0.01	24.95±1.20
LIVER	CONTROL	28.98±3.52 N.S.	12.37±1.71 P<0.05	4.66±0.49 N.S.	46.01±3.06 P<0.02
	6 - HD.	23.42±1.27	7.90±1.01	4.17±0.76	35.49±1.44
SUBMAXILLARY GLAND	CONTROL	1.98±0.36 N.S.	0.71±0.16 N.S.	0.23±0.05 N.S.	2.93±0.52 N.S.
	6 - HD.	1.76±0.24	0.65±0.12	0.22±0.02	2.65±0.34
SPLEEN	CONTROL	1.85±0.20 N.S.	0.39±0.09 N.S.	0.24±.10 N.S.	2.48±0.21 N.S.
	6 - HD.	2.02±0.20	0.25±0.02	0.24±.04	2.51±0.22
DUODENUM	CONTROL	8.66±3.11 N.S.	3.12±0.76 N.S.	0.42±0.13 P<0.05	12.21±3.92 N.S.
	6 - HD.	7.42±1.44	2.37±0.35	0.16±0.02	9.95±1.73
VAS DEFERENS	CONTROL	2.56±0.54 N.S.	0.36±0.09 P<0.05	0.27±0.07 N.S.	3.19±0.60 N.S.
	6 - HD.	2.10±0.33	0.80±0.20	0.15±0.04	3.05±0.37
KIDNEY	CONTROL	2.50±0.27 N.S.	0.24±0.02 N.S.	0.03±0.01 N.S.	2.77±0.28 N.S.
	6 - HD.	2.56±0.23	0.29±0.04	0.04±0.002	3.05±0.23

5 animals in each control and 6-hydroxydopamine group.
For details of 6-hydroxydopamine dosage schedule see text.
N.S. Not significant at a 5% level of probability.

TABLE 14.

MAO activity of immunosympathectomized and control mouse tissues. Activity expressed in terms of μg 4-hydroxyquinoline formed per min per g wet weight of tissue ± S.E.M.

TISSUE		MITOCHONDRIAL FRACTION	MICROSOMAL FRACTION	SUPERNATANT FRACTION	SUM OF FRACTIONS
HEART	CONTROL	6.51 ± 0.52 N.S.	3.77 ± 0.31 $P < 0.005$	0.71 ± 0.06 N.S.	10.99 ± 0.35 $P < 0.005$
	IMMUNO.	4.97 ± 0.83	2.09 ± 0.22	1.24 ± 0.30	8.31 ± 0.49
LIVER	CONTROL	22.43 ± 0.37 $P < 0.01$	11.31 ± 1.82 $P < 0.05$	4.04 ± 0.34 $P < 0.005$	37.78 ± 2.25 $P < 0.005$
	IMMUNO.	14.59 ± 2.03	6.01 ± 1.18	1.79 ± 0.30	22.47 ± 2.05
SUBMAXILLARY GLAND	CONTROL	0.69 ± 0.10 N.S.	0.21 ± 0.04 $P < 0.05$	—	0.91 ± 0.09 $P < 0.05$
	IMMUNO.	1.55 ± 0.42	0.36 ± 0.04	—	1.91 ± 0.40
SPLEEN	CONTROL	1.33 ± 0.13 N.S.	0.16 ± 0.02 N.S.	0.16 ± 0.01 N.S.	1.65 ± 0.15 N.S.
	IMMUNO.	1.10 ± 0.13	0.20 ± 0.02	0.19 ± 0.01	1.48 ± 0.14
SMALL INTESTINE	CONTROL	0.51 ± 0.18 $P < 0.001$	0.26 ± 0.08 $P < 0.005$	0.38 ± 0.08 N.S.	1.15 ± 0.29 $P < 0.005$
	IMMUNO.	4.51 ± 0.74	2.36 ± 0.44	0.30 ± 0.10	7.24 ± 0.99
VAS DEFERENS	CONTROL	1.61 ± 0.56 N.S.	0.66 ± 0.21 N.S.	—	2.27 ± 0.66 N.S.
	IMMUNO.	1.48 ± 0.55	0.58 ± 0.21	—	1.65 ± 0.67
UTERUS	CONTROL	1.39 ± 0.25 N.S.	0.49 ± 0.09 N.S.	0.28 ± 0.10 N.S.	1.73 ± 0.36 N.S.
	IMMUNO.	2.01 ± 0.93	0.23 ± 0.09	0.70 ± 0.43	2.94 ± 0.73

Control and immunosympathectomized mice - 5 animals per group.

N.S. Not significant at a 5% level of probability.

greater extent, in the small intestine. No change in enzyme activity was detected in the subcellular compartments of the spleen, vas deferens and uterus. Whilst the decrease in liver was a reflection of reduced activity in all three subcellular fractions, microsomal activity, only, was significantly depressed in the heart. Submaxillary mitochondrial activity was unaffected, but enzyme activity increased in the microsomal fraction. In the small intestine the activity of both mitochondrial and microsomal fractions was substantially increased. Enzyme levels were too low to be detected in aliquots of submaxillary gland and vas deferens supernatants taken for assay.

(iv) MAO activity in adrenalectomized rats

Total MAO activity of heart, brain and vas deferens homogenates was significantly increased in adrenalectomized rats (Table 15), but there was no significant overall change in other tissues examined.

There was a greater than two-fold increase in mitochondrial activity in the heart, and significant increases were detected in the mitochondrial fraction of the brain, vas deferens and duodenum. The rise in duodenal mitochondrial activity was counter-balanced, however, by a significant decrease of activity in the supernatant, similar to that noted in the submaxillary gland supernatant. Only in the heart was supernatant activity increased after adrenalectomy.

TABLE 15.

MAO activity of adrenalectomized and control male rat tissues. Activity expressed in terms of ug 4-hydroxyquinoline formed per min. per g wet weight of tissue ± S.E.M.

TISSUES		MITOCHONDRIAL FRACTION	MICROSOMAL FRACTION	SUPERNATANT FRACTION	SUM OF FRACTIONS
HEART	CONTROL	5.21 ± 0.40 $P < 0.001$	2.36 ± 0.32 N.S.	0.59 ± 0.12 $P < 0.05$	8.15 ± 0.46 $P < 0.001$
	ADRENEC	12.25 ± 2.09	3.16 ± 0.35	1.26 ± 0.29	16.67 ± 1.03
BRAIN	CONTROL	3.31 ± 0.43 $P < 0.05$	0.26 ± 0.03 N.S.	0.10 ± 0.01 N.S.	3.67 ± 0.27 $P < 0.001$
	ADRENEC	5.42 ± 0.63	0.20 ± 0.03	0.18 ± 0.03	5.79 ± 0.40
VAS DEFERENS	CONTROL	1.38 ± 0.09 $P < 0.01$	0.42 ± 0.05 $P < .05$	0.32 ± 0.04 N.S.	2.11 ± 0.18 $P < 0.01$
	ADRENEC	1.96 ± 0.10	0.64 ± 0.05	0.31 ± 0.05	2.92 ± 0.22
DUODENUM	CONTROL	3.75 ± 0.04 $P < 0.01$	0.80 ± 0.03 N.S.	1.35 ± 0.14 $P < 0.001$	5.90 ± 0.47 N.S.
	ADRENEC	4.61 ± 0.05	0.62 ± 0.06	0.51 ± 0.07	5.74 ± 0.25
SUBMAXILLARY GLAND.	CONTROL	2.54 ± 0.12 N.S.	0.71 ± 0.06 N.S.	0.35 ± 0.02 $P < 0.02$	3.59 ± 0.27 N.S.
	ADRENEC	2.67 ± 0.13	0.81 ± 0.08	0.25 ± 0.02	3.73 ± 0.39
LIVER	CONTROL	11.14 ± 1.13 N.S.	2.69 ± 0.36 N.S.	2.66 ± 0.22 N.S.	16.49 ± 1.13 N.S.
	ADRENEC	14.61 ± 2.13	2.16 ± 0.20	3.12 ± 0.33	19.89 ± 1.41
SPLEEN	CONTROL	1.80 ± 0.11 N.S.	0.22 ± 0.02 N.S.	0.15 ± 0.02 N.S.	2.17 ± 0.21 N.S.
	ADRENEC	1.99 ± 0.14	0.19 ± 0.02	0.20 ± 0.04	2.38 ± 0.08

Control - 6 animals per group.

Adrenalectomized - 5 animals per group.

N.S. Not significant at 5% level of probability.

Microsomal MAO activity was not significantly altered by adrenalectomy in any tissue apart from the vas deferens, where an increase was observed.

Discussion

Several factors may influence the MAO activity of a tissue, including differences in strain, sex and age within an animal species. In the present work, for instance, total heart and brain MAO activity was lower in one strain of mouse investigated, Ash/Porton (Fig. 9) than in the other, TO (Tables 13 and 14); within a strain, mean tissue enzyme activity in a group of mice with a higher proportion of males (Table 13) tended to be greater than corresponding values in a group (Table 14) where the male/female ratio was equal. Others have shown that MAO activity in the adult rat heart (Skillen, Thienes and Strain, 1962; Prange, White, Lipton and Kinkead, 1967) and liver (Wurtman, Kopin and Axelrod, 1963; Prange, White, Lipton and Kinkead, 1967) is related to sex, males having significantly more enzyme activity per unit weight of tissue or tissue protein, and similar considerations may apply to mice. The influence of age is particularly important in the rat, where heart MAO activity is known to increase markedly during growth (Novick, 1961; Studer, Baumgartner and Reber, 1964; Gey, Burkard and Pletscher, 1965; Horita, 1967; Prange, White,

Lipton and Kinkead, 1967; de Champlain, Axelrod, Krakoff and Mueller, 1968). This process appears to be specific for the heart, and appears to be species specific for the rat, since it has not been observed in other animals, including the mouse (Studer, 1966; Horita, 1967). In the present study, possible difficulties in interpretation of results stemming from these factors, were largely overcome by using in each experiment control and test animals of similar strain and age, and in the same sex ratio.

Studies of subcellular distribution of MAO in rat liver have shown that the enzyme is predominantly mitochondrial (Cotzias and Dole, 1951; Hawkins, 1952; Baudhuin, Beaufay, Rahman-Li, Sellinger, Wattiaux, Jacques and de Duve, 1964; Ostwald and Strittmatter, 1963; Zile and Lardy, 1959; de Champlain, Mueller and Axelrod, 1969), and during the present investigation, MAO activity was also found to be mainly localized the mitochondrial fraction of rat and mouse liver. The presence of about 16% of normal rat liver MAO activity in the microsomal fraction is a finding in broad agreement, also, with the results of other workers (Ostwald and Strittmatter, 1963; Baudhuin, Beaufay, Rahman-Li, Sellinger, Wattiaux, Jacques and de Duve, 1964; Jarrott and Iversen, 1968). As the rat liver mitochondrial enzyme is probably associated with the outer membrane of the mitochondrion (Schnaitman, Erwin and Greenawalt, 1967; Sottocasa, Kuylenstierna, Ernster and Bergstrand, 1967; Tipton, 1967;

Boadle and Bloom, 1969), it is possible that apparent microsomal activity arises from a shearing off of the mitochondrial membrane during homogenisation; it appears, however, that the subcellular distribution of rat liver MAO may not exactly parallel that of succinic dehydrogenase activity (Ostwald and Strittmatter, 1963; Baudhuin, Beaufay, Rahman-Li, Sellinger, Wattiaux, Jacques and de Duve, 1964) which is usually considered to be a marker enzyme for mitochondria (Slater and Planterose, 1960). Thus, a true extra-mitochondrial location for a proportion of the MAO activity cannot be ruled out.

The results presented in this chapter confirm previous findings (Bogdanski, Weissbach and Udenfriend, 1957; Weiner, 1960; Rodríguez de Lorez Arnaiz and de Robertis, 1962) that brain MAO is also predominantly associated with the mitochondrial fraction. The proportion of microsomal MAO activity in the brain of one strain of control mouse (Ash/Porton) was, however, markedly higher than in the other strain (TO). The possible significance of the brain microsomal fraction is discussed later, in the light of effects produced by reserpine administration.

Although considerable activity was observed in the microsomal fraction of the heart and submaxillary glands after differential centrifugation, a greater proportion was again located in the mitochondrial compartment. This

finding does not confirm the observations of de Champlain, Mueller and Axelrod (1969), who reported that MAO activity in the rat heart is mainly localized in the microsomal fraction. This disagreement may be accounted for, in part, by differences in rat strain and in the experimental procedure adopted. The major portion of enzyme activity was localized in the mitochondrial fraction of all other normal mouse or rat tissues investigated including vas deferens, in agreement with previous findings (Jarrott and Iverson, 1968; de Champlain, Mueller and Axelrod, 1969).

Although depletion of tissue noradrenaline levels is an effect common to reserpinized, immunosympathectomized and 6-hydroxydopamine-treated animals, the ultrastructural alterations induced by these drugs is essentially different. The precise mode of action of reserpine is still largely unknown. It does not appear to damage the external membrane of the neurone; its effect appears to be related in some way to impairment of catecholamine uptake and release mechanisms associated with the amine storage granules (For references see chapter 1). Reserpine-induced changes in the ultrastructure of heart mitochondria are discussed later. The anatomical lesions produced by antiserum to NGF are more extensive. Histological studies have shown that the cell population of paravertebral sympathetic ganglia

is markedly reduced (Levi-Montalcini and Booker, 1960b; Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965) with resulting atrophy of the postganglionic sympathetic axons. Administration of 6-hydroxydopamine, in sufficiently high dosage, has been shown to produce ultrastructural neuronal damage in cats (Tranzer and Thoenen, 1968) and rats (Thoenen and Tranzer, 1968), but the degenerative process appears to be confined to adrenergic nerve endings. Non-terminal axons and cell bodies seemed to be relatively unaffected (Malmfors and Sachs, 1968). Such differences in drug action must be borne in mind when considering the effect of these drugs on MAO activity. For example, if intraneuronal MAO is distributed along the length of the postganglionic sympathetic neurone, it is likely that the quantitative effect on MAO activity might differ in immunosympathectomized mice compared with animals treated with 6-hydroxydopamine.

During the present work reserpine appeared to cause both increases and decreases in MAO activity, and the time after injection at which the maximum effect was seen differed not only from tissue to tissue, but also between the subcellular fractions of a single organ. It is difficult to correlate changes in tissue MAO activity with reserpine-induced depletion of monoamine levels (see chapter 1) because of differences in species, dosage and route of administration.

Recent studies (Izumi, Oka, Yoshida and Imaizumi,

1967, 1969; Youdim and Sandler, 1968) have shown that reserpine can enhance the activity of MAO in tissues. The increased enzyme activity in the heart and brain mitochondrial fractions observed by these authors, is in general agreement with the present results, although the increase reported by Youdim and Sandler (1968) in the combined microsomal-supernatant activity of rat heart and brain, 5 hours after reserpine, could not be confirmed by extrapolation of the present data. On the otherhand, Cervoni (1969) has reported a fall in the MAO activity of cat nictitating membrane 24 hours after reserpine administration; total MAO activity in mouse heart is also significantly reduced at this time (Fig.9). The results presented here show that changes in MAO activity after reserpine are time dependent, whereas data of other authors were obtained at one specific time after reserpine administration.

Whereas MAO activity of the microsomal fraction of both the brain and heart showed a significant and prolonged fall after reserpine, that of the liver was markedly increased. The maximum increase of both mitochondrial and microsomal MAO activity in the liver was observed at 2 hours. This parallel behaviour of the liver subcellular fractions is compatible with the view that the liver microsomal fraction is part of the large mitochondrial fraction, which has become sheared off from the outer membrane

during homogenisation. Although an increase in mitochondrial MAO activity was observed in the heart and the brain, the decrease in microsomal MAO activity of mouse heart and brain after reserpine makes it more difficult to accept this explanation for the findings in these organs, despite the fact that, hitherto, brain MAO has been thought to be associated exclusively with mitochondria (Bogdanski, Weissbach and Udenfriend, 1957; Weiner 1960; Rodríguez de Lorez Arnaiz and de Robertis, 1962). The enzyme activity of the supernatant fraction increased significantly in all three tissues after reserpine administration. The drug thus appears to cause a decrease in the ratio of microsomal to supernatant MAO activity in both heart and brain, as opposed to liver, where its administration results in a sharp but transient increase in ratio. Reserpine thus seems, in heart and brain at least, to cause a leakage of MAO from the microsomal to the supernatant fractions. It is interesting to note that in the hearts of reserpine-pretreated animals, exogenous noradrenaline appears to accumulate in the supernatant as opposed to the microsomal fraction (Campos and Shideman, 1962; Iversen, Glowinski and Axelrod, 1965b).

It is difficult to weight the relative importance of the MAO activity associated with each subcellular fraction with respect to the inactivation of physiclogically

released endogenous monoamines and of amines released by reserpine. A close association has been noted between microsomal MAO activity and noradrenaline storage particles in the heart (Stjärne, Roth and Gierman, 1968; de Champlain, Mueller and Axelrod, 1969) and in bovine splenic nerve (Roth and Stjarne, 1966). The transient increase in MAO activity of the mitochondrial fractions of all three tissues after reserpine, parallels the monoamine release (for references, see review by Carlsson, 1966). These compounds might act as substrate activators of the enzyme. On the other hand, the apparent increase in activity might derive from increased access of substrate to enzyme, due to membrane permeability changes caused by reserpine. Histochemical evidence exists of a derangement in lipid and glycogen metabolism, and depression of mitochondrial oxidative enzymes, in the myocardium of reserpinized mice (Sun, Sohal, Colcolough and Burch, 1968); ultrastructural studies have shown signficant changes of mitochondrial architecture in the hearts of reserpine-treated animals (Wilcken, Brender, MacDonald, Shorey and Hinterberger, 1967; Sun, Sohal, Colcolough and Burch, 1968). In the former study, mitochondrial changes paralleled noradrenaline depletion. Biochemical evidence also points to the existence of abnormal mitochondrial function after large doses of reserpine, uncoupling of oxidative phosphorylation having

been observed in the mitochondria of the heart (Schwartz and Lee, 1960), brain (Abood and Romanchek, 1957) and liver (Kirpekar and Lewis, 1960). Whether or not the shift of MAO activity from microsomal to supernatant fractions in the brain and heart is similarly due to a direct physical effect of reserpine on the particle membranes, leading to rupture and release of soluble enzyme, is unknown.

The effects of immunosympathectomy on adrenergic degeneration are permanent, whereas those due to 6-hydroxydopamine appear to be reversible. In the present study, mice were not killed until 9 days after the last dose of 6-hydroxydopamine. This dosage schedule was, however, the same as that used in rats by Thoenen and Tranzer (1968) to obtain the most complete sympathectomy. Two weeks after the last injection of 6-hydroxydopamine they found, at most, only an insignificant recovery in noradrenaline content of all organs studied. Laverty, Sharman and Vogt (1965) found that the level of mouse heart noradrenaline was still low, after a single injection of 10 mg. per kg i.v., as long as 14 days after the injection. Thus, in the present study, it is not unreasonable to assume that there was still a substantial degeneration of adrenergic nerve terminals in treated mouse tissues 9 days after the final administration of 6-hydroxydopamine.

A significant reduction in total hepatic MAO activity was observed in both immunosympathectomized and

6-hydroxydopamine-treated mice. Whereas this decrease was spread through all subcellular fractions in immunosympathectomized mice, it was confined to the microsomal fraction in animals treated with 6-hydroxydopamine. Similarly, although MAO activity was significantly reduced in the cardiac microsomal fraction in both groups, only in immunosympathectomized mice was a significant reduction in total activity observed. It is noteworthy that a significant reduction in heart microsomal MAO activity occurred in animals treated with reserpine, antiserum to NGF, and 6-hydroxydopamine, pointing to an association between this fraction and intraneuronal MAO activity, at least in mouse heart. The reductions observed in liver MAO, which have also been reported by Klingman (1966a) using crude liver homogenates, are more difficult to explain. Intraneuronal MAO activity might be expected to form a small proportion of the total MAO activity of this organ, if the relatively low noradrenaline content of rat liver (Klingman, 1965) compared with other organs is used as a guide; thus the present findings are rather unexpected.

No alteration in MAO activity was observed in subcellular fractions of vas deferens and uterus from immunosympathectomized mice. In the vas deferens of 6-hydroxydopamine-treated mice, a rise in microsomal enzyme

activity was detected, which was not, however, mirrored by the total activity. The peripherally located adrenergic ganglia, which supply nerves to the vas deferens and the uterus (Sjöstrand, 1965; Sjöberg, 1967), are relatively resistant to the effects of NGF-antiserum (Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965; Levi-Montalcini and Angeletti, 1966) so that a decrease in MAO activity of these organs would not be expected. The decrease in noradrenaline content and ultrastructural alteration of the vas deferens induced by 6-hydroxydopamine in rats, were less extensive than similar changes in other organs (Thoenen and Tranzer, 1968; Laverty and Phelan, 1969), but it is difficult to explain the increase in microsomal MAO activity detected in this organ.

As noted previously, the lesion produced by 6-hydroxydopamine appears to be mainly confined to adrenergic nerve endings. MAO activity may, however, have a wider distribution along the postganglionic neuron. These considerations might help to explain why MAO activity in organs with a rich sympathetic innervation, such as the spleen or submaxillary glands, is not reduced after 6-hydroxydopamine treatment. Other workers have reported variable, but significant, reductions in MAO activity of spleen and salivary or submaxillary glands of immunosympathectomized animals (Levi-Montalcini and Angeletti, 1962; Klingman, 1966a;

Iversen, Glowinski and Axelrod, 1966), but their findings could not be confirmed in the present study; indeed, an increase in activity was observed in microsomal and total fractions of the submaxillary gland of the treated mouse. Equally puzzling is the marked increase in enzyme activity detected in the small intestine of the immunosympathectomized mouse. Although an increase in intestinal 5-HT has been reported in animals treated with NGF-antiserum (Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965; Iversen, Glowinski and Axelrod, 1966 ; Thompson and Campbell, 1966b), this finding is not mirrored by a rise in the urinary output of deaminated metabolites of this monoamine (chapter 3). The efficacy of the antiserum used in the present study is proved, however, by the drastic reduction observed in the size of the superior cervical ganglia of treated mice. No previous work, however, has been published in which this particular antiserum has been used. Unlike NGF antisera of rabbit (Levi-Montalcini and Angeletti, 1966) and bovine origin (Abbott Laboratories, Chicago, Ill., U.S.A.), used to prepare immunosympathectomized animals previously, the present antiserum derived from the horse. At present, however, no explanation for the increases in MAO activity observed in certain tissues of the immunosympathectomized mouse can be put forward.

These studies indicate that MAO activity in the duodenum of the mouse is greater than that found in more

distal regions of the small intestine. Histochemical studies in the rat and other animals have previously shown that a large proportion of duodenal MAO is located in extraneuronal sites, particularly in the mucosal epithelium (Glenner, Burtner and Brown, 1957); drug-induced changes in intraneuronal MAO activity may be therefore difficult to detect.

Kidney levels of MAO are low in the rat (Bhagvat, Blaschko and Richter, 1939) but, in confirmation of other work (Hope and Smith, 1960), vigorous enzyme activity was detected in mouse kidney. Subcellular MAO activity was, however, unaltered in this organ following administration of 6-hydroxydopamine. Systemic administration of 6-hydroxydopamine has previously been shown to reduce the noradrenaline content of the kitten hypothalamus, but the drug had no effect on whole brain concentrations of noradrenaline in the mouse (Laverty, Sharman and Vogt, 1965) when administered by this route. 6-Hydroxydopamine is thus unlikely to cross the blood-brain barrier. The cause of the shifts in enzyme activity observed in the subcellular fractions of mouse brain after 6-hydroxydopamine is unknown, but, since the drug is unlikely to enter the brain, and total MAO activity is unaffected, it may arise from homogenisation artifacts.

Total MAO activity in a number of different organs was significantly increased in adrenalectomized rats, confirming and extending the findings of other workers in

the heart (Avakian and Callingham, 1968; Westfall and Osada, 1969). Indeed, the heart was the organ most affected and the only one to show a rise in supernatant MAO activity. Whether the increased enzyme activity and compartment shifts are adaptive responses to facilitated production of its substrate is unknown; in rat heart (Landsberg and Axelrod, 1968) and brain (Javoy, Glowinski and Kordon, 1968) an enhanced noradrenaline turnover is known to accompany the potention of MAO activity observed after adrenalectomy. Alternatively, they might stem from an action of glucocorticoids on the permeability of intracellular membranes to amines. The reduction of MAO levels in the adrenalectomized heart to normal, following treatment with hydrocortisone (Avakian and Callingham, 1968), is a pointer in this direction.

CHAPTER 6

SOME INVESTIGATIONS ON THE METABOLIC AND BIOSYNTHETIC ACTIVITY OF NEUROBLASTOMA TUMOUR IN TISSUE CULTURE

The urinary excretion of catecholamine metabolites in rats treated with antiserum to NGF has been described in a previous chapter. Contrasting with the deficiency of noradrenaline and the consequent effects on catecholamine metabolism in the immunosympathectomized animal is the over-production of catecholamines and their metabolites found in functional neural crest tumours. Moreover, evidence has been reported by Burdman and Goldstein (1965) that NGF activity is higher in the sera of neuroblastoma patients than in normal sera, and increases with the size of the tumour. This establishes an involvement, though not necessarily a role, for NGF in the pathology of the tumour (Varon, 1968).

Early observations on the increased urinary excretion of catecholamines and their major metabolites in patients with tumours of neural crest origin (Mason, Hart-Mercer, Millar, Strang and Wynne, 1957) have been confirmed many times (Isaacs, Medalie and Politzer, 1959; Greenberg and Gardner 1959; Stickler, Hallenbeck and Flock, 1959; Sandler and Ruthven, 1959; von Studnitz, 1960; Voorhess and Gardner, 1961b; La Brosse and Karon, 1962.). Tumour tissue analysis has been scanty. La Brosse and co-workers (La Brosse, Belehradek, Barski, Bohuon and Schweisguth, 1964) incubated

tritium-labelled noradrenaline with isolated neural crest tumours in tissue culture. Following paper chromatography of the culture medium, the developed chromatograms were scanned for radioactivity. The authors claimed to detect the formation of 4-hydroxy-3-methoxyphenylglycol and normetadrenaline as well as the sulphate conjugate of normetadrenaline, but not of VMA. In this experiment, labelled noradrenaline was incubated with the tissue culture for 48 hours at 37°C. It is known that adrenaline and noradrenaline are destroyed within a few minutes in Krebs bicarbonate buffer, pH 7.4 at 37°C (Häggendal and Svedmyr, 1967), although the presence of tissue (rat diaphragm) has an inhibitory effect on the destruction of adrenaline. On the other hand, noradrenaline can be stored for at least four hours without destruction at room temperature in sterile sodium bicarbonate solution, pH 7.9 (Häggendal and Johnsson, 1967). Nevertheless, the lengthy incubation period of 48 hours may have led to some breakdown of noradrenaline in the experiment of La Brosse, Belehradek, Barski, Bohuon and Schweisguth (1964). In addition, no initial fractionation of possible metabolites of ^{3}H-noradrenaline, prior to chromatography, was undertaken in the experiment. Removal of residual ^{3}H-noradrenaline is desirable because of the large initial count (7.5 µc) added to the medium. The salts and nutrients, in addition to the protein present in the medium, are likely to interfere with the running of a paper chromatogram and lead to erroneous conclusions as a result of the high ^{3}H-noradrenaline count

present.

Because of these objections, it was decided to re-investigate the metabolism of labelled noradrenaline incubated with a continuous cell line of a neuroblastoma tumour, using a more detailed analytical approach.

As an extension of these studies, labelled DOPA was similarly added to a neuroblastoma tissue culture and the supernatant was examined after incubation to detect the possible presence of labelled dopamine and noradrenaline.

Investigations

A. Incubation of ^{14}C-noradrenaline with neuroblastoma tissue culture

Specification of ^{14}C-noradrenaline

DL-Noradrenaline (carbinol-^{14}C) DL-bitartrate (^{14}C-NA) crystals; Specific activity, 41.2 mc/m mole. (Radiochemical Centre, Amersham).

Preparation of solution of ^{14}C-noradrenaline for incubation with tissue culture.

The ^{14}C-noradrenaline, as supplied, was dissolved in 1.0 ml of 0.1 N acetic acid. To prepare sterile ^{14}C-noradrenaline for the 48 hour incubation experiment, a portion of the solution was passed through a millipore filter into a sterile bijou bottle and the sterile filtrate was stored at -15°C.

Breakdown of ^{14}C-noradrenaline during incubation at 37°C

This experiment was conducted in an attempt to find the longest period of incubation of ^{14}C-noradrenaline

in culture medium at 37°C compatible with minimal breakdown of the compound.

Procedure

(a) Incubation and paper chromatography

^{14}C-noradrenaline solution (20 µl , 2 µc) was added to each of four 15 ml samples of Eagle's tissue culture medium (Burroughs Wellcome and Co., Ltd., London) which included 10% calf-serum. The preparations were incubated at 37°C and pH 7.4-7.5 for periods of 30, 60 and 120 minutes and 48 hours respectively. After incubation, 50 µl of each sample was applied as a 2 cm streak to a 4 cm wide strip of Whatman No. 1 paper. Chromatograms were developed overnight by descending chromatography in butan-1-ol: acetic acid: water (4: 1: 1); 50 µl of ^{14}C-noradrenaline solution in 15 ml of Eagle's medium plus calf-serum, which had not been incubated at 37°C prior to chromatography, was used as control. Authentic noradrenaline (10 µg) was used as carrier and marker on the chromatograms.

(b) Location and measurement of radioactivity by automatic scanning.

Developed chromatograms were dried and labelled with 1 µl of a non-volatile highly active ^{14}C - compound, the 'hot spot' being placed at a point 2 cm from the origin on the side remote from the solvent front. Chromatograms were then joined end to end with adhesive tape, and the first and last strips attached to feeder spools of a windowless 4π Geiger gas-flow automatic scanner ("Scanogram II" Model RSC - 160 - Baird

Atomic, Cambridge, Mass., U.S.A.). This instrument was coupled to a research ratemeter (Model 432 A-Baird Atomic) and a rectilinear recording milliammeter (Texas Instruments Inc., Houston, Texas, U.S.A.). Chromatograms were scanned in an atmosphere of Geiger gas (helium 98.5%, butane 1.5%), after which the 'cold' noradrenaline was located by spraying with ferricyanide - phosphate buffer (pH 7.4) reagent (potassium ferricyanide (K_3 Fe $(CN)_6$), 0.44 g per 100 ml prepared in 0.1 M phosphate buffer (pH 7.4)). With the aid of the 'hot spot', the charts showing the location and intensity of radioactive peaks on the chromatograms were aligned with the sprayed strips, so that radioactivity due to ^{14}C-noradrenaline and/or its degradation products could be demonstrated.

Results

Incubation with culture medium for 48 hours led to virtually complete breakdown of ^{14}C-noradrenaline. Whilst there was some degradation of this compound after 60 minutes incubation, this was less marked than that observed after 120 minutes. No break down of ^{14}C-noradrenaline was detected after 30 minutes incubation.

Incubation of ^{14}C-noradrenaline with neuroblastoma tissue culture at 37°C

An incubation time of 30 minutes at 37°C was chosen for a detailed study of the metabolism of ^{14}C-noradrenaline by neuroblastoma cells in tissue culture since, under these con-

ditions, no breakdown of the compound was detected in the experiment described above. Some preliminary work was also carried out employing an incubation time of 48 hours as used in the original study of La Brosse, Belehradek, Barski, Bohuon and Schweisguth (1964), in addition to an intermediate period of 2 hours.

Incubation procedure

The author thanks Dr. A. E. Claireux (Dept. of Morbid Anatomy, The Hospital for Sick Children, London), who supplied the tissue culture material and provided laboratory facilities for the incubation experiments. The neuroblastoma tissue cultures were continuous cell lines derived from histologically-proven differentiating tumours. The incubations were carried out by Mrs. G. Adron and Miss D. Chambers, to whom the author is very grateful.

In each experiment, ^{14}C-noradrenaline solution was added to 15 ml of the following: Eagle's tissue culture medium alone (including 10% calf serum); control non-catecholamine secreting tumour cells in tissue culture, plus medium; and neuroblastoma cells in tissue culture, plus medium. Ependymoma tumour cells were used as control for the 30 minutes experiment, and He La cells for the 2 and 48 hour experiments. After incubation of 10 µc (0.1 ml) ^{14}C-noradrenaline test and control samples for 30 minutes at 37°C, or 2 µc (20 µl) in the 2 and 48 hour experiments respectively, the pH of the decanted supernatants was adjusted to between 1 and 2, after which they were stored at -15°C until required.

Column fractionation of ^{14}C-noradrenaline metabolites

Reagents and materials

0.2M Sodium acetate reagent: 27.2 g hydrated (A.R.) salt dissolved in 1 litre distilled water.

Sodium acetate solution of hydrated (A.R.) salt dissolved in distilled water, 50% (^{W}/v).

Ascorbic acid, 5% (^{W}/v) in distilled water, freshly prepared.

Ethylenediamine tetraacetic acid (EDTA) (disodium salt, A.R.) 10% ^{W}/v) in water.

0.2M Sodium acetate, pH 8.6, containing approximately 0.2% (^{W}/v) EDTA and 0.1% (^{W}/v) ascorbic acid. To prepare, add 10% (^{W}/v) EDTA (2 ml) and 5% (^{W}/v) ascorbic acid (2 ml) to 0.2M sodium acetate (100 ml), adjust the pH to 8.6 with 0.1N NaOH: prepared freshly.

Alumina (aluminium oxide, MFC, purified neutral) was prepared for chromatographic use (Crout, 1961b) as described in chapter 2.

Amberlite CG50 (H) Type 1 (100 - 200 mesh) ion exchange resin (pH 6.0 to 6.5) was prepared as described in chapter 2.

DL-Noradrenaline bitartrate (Koch-Light Laboratories Ltd., Colnbrook, England).

(DL) - Normetadrenaline hydrochloride, (Calbiochem, Los

Angeles, California, U.S.A.).

(±) -4-Hydroxy-3-methoxymandelic acid. (British Drug Houses Ltd., Poole, England). (VMA)

Bis (4-hydroxy-3-methoxyphenylglycol) piperazine salt (HMPG) (Regis Chemical Co., Chicago, Ill., U.S.A.). (HMPG)

DL-3,4-Dihydroxymandelic acid. (Calbiochem. Los Angeles, California, U.S.A.) (DHMA).

Dihydroxyphenylglycol was a gift from Dr. J. Axelrod, National Institutes of Health, Bethesda, Maryland, U.S.A. (DHPG).

Standard solution noradrenaline, 1 mg per ml in 0.01N HCl.

Standard solution 4-hydroxy-3-methoxymandelic acid, 1 mg per ml in water.

Standard solution 4-hydroxy-3-methoxyphenylglycol, 1 mg per ml in water.

Standard solution dihydroxymandelic acid, 1 mg per ml in 0.01N HCl.

All solutions stored at -15°C when not in use.

Standard solution dihydroxyphenylglycol, 1 mg per ml in 0.01 N HCl, was prepared freshly before use.

Liquid scintillator (Davis, Brown, Huff and Cashaw, 1967):

500 ml toluene, 500 ml methyl alcohol, 500 ml p-dioxane, 100 g naphthalene, 6g 2,5-diphenyloxazole (PPO), and 150 mg 1,4-bis-2 (5-phenyloxazolyl)-benzene (POPOP).

Standard scintillator: PPO (10g), POPOP (600 mg) in 2 litres toluene.

15 ml of standard scintillator was added to the radioactive sample in 4 ml of methanol before counting.

Procedure

Sodium acetate buffer (50%, 0.2 ml) and non-radioactive carriers (100 µg each of noradrenaline, normetadrenaline, DHMA, HMPG and VMA) were added respectively to 5 ml of neuroblastoma supernatant, control culture supernatant, and the medium control. After the solutions had been carefully adjusted with NaOH (0.1 N) to pH 8.4, they were poured on to separate columns (bore 0.55 cm) of alumina (0.5 g), previously stirred with sodium acetate reagent. Column effluents were collected, the columns washed with 4 ml of sodium acetate reagent and 4 ml of water, and the washings added to the effluents. Adsorbed ^{14}C-noradrenaline was eluted from the alumina columns with 3.5 ml of 0.2N acetic acid, followed by 3 ml water which was added to the acetic acid eluate. Any catechol acids formed from ^{14}C-noradrenaline were then eluted with 3.5 ml 0.2N HCl, followed by 3 ml of water which was added to the HCl eluate.

Small portions (0.5 ml) of each of the effluents

plus washings from the alumina columns were set aside for liquid scintillation counting. The remainder of the solutions, adjusted to pH 6.5 with 0.1N HCl, were passed through an Amberlite CG 50 column, pH 6.0 - 6.5 (1 cm bore, 6-7 cm bed). Effluents were collected, and 0.5 ml of each set aside for estimation of radioactive count. This fraction would contain any ^{14}C-VMA or ^{14}C-HMPG formed by the neuroblastoma tissue culture cells.

In order to remove any ^{14}C-noradrenaline which may have passed through the alumina column into the column effluent, and which thereafter had become adsorbed on the Amberlite resin, the latter columns werewashed with 3 separate 10 ml quantities of 4% (^{W}/v) boric acid (Mattok and Wilson, 1965), followed by 2 separate 10 ml water washes. Any ^{14}C-normetadrenaline adsorbed on the columns was then eluted with 4N NH_4OH (Ruthven and Sandler, 1965). From each fraction, 0.5 ml was used for estimation of radioactivity in a Tri-Carb liquid scintillation spectrometer set for ^{14}C-counting. The solution was dissolved in 16 ml of liquid scintillator (Davis, Brown,Huff and Cashaw, 1967), using 0.5 ml of water in 16 ml scintillator as a blank. Correction for quenching was made by re-counting each fraction after the addition of an internal standard of ^{14}C-toluene. From these counts, after correction for background, the corrected recovery of ^{14}C-radioactivity in each fraction was calculated and expressed as a percentage of the original radioactivity in the supernatant.

Results

The distribution of radioactivity in the various column fractions, following incubation of ^{14}C-noradrenaline for 30 minutes, 2 hours and 48 hours respectively, is shown in Table 16. The amount of radioactivity present in the 30 minute acetic acid fraction represents the major proportion of the counts in both neuroblastoma and control supernatants. This is due to residual ^{14}C-noradrenaline activity. The distribution of radioactivity observed in the two controls is similar to that seen in the neuroblastoma culture supernatant and, whilst ^{14}C-noradrenaline breakdown would appear to be insubstantial, there is no major fraction of radioactivity in the neuroblastoma supernatant which is not also present in the controls. This finding indicates that no significant conversion of ^{14}C-noradrenaline to its metabolites has occurred during the 30 minute incubation.

After 2 hours incubation, the distribution of radioactivity in the neuroblastoma fractions and in the medium is similar. The small percentage of the total count present in the acetic acid fraction indicates that substantial oxidative degradation of ^{14}C-noradrenaline has occurred in the medium. Over 50% of the recovered activity was present in the acetic acid fraction of the control culture supernatant, however, so that a lesser degree of breakdown occurred in this control.

Following incubation for 48 hours, there was a

TABLE 16.

Incubation of ^{14}C-DL-noradrenaline with neuroblastoma tissue culture for various incubation times at 37°C. Analysis of radioactivity after column fractionation of supernatant.

Fraction	Percentage radioactivity after incubation times of 30 min. 2 hr and 48 hr.								
	Eagle's Medium			Control Culture			Neuroblastoma Culture		
	30 min	2 Hr	48 Hr	30 min	2 Hr	48 Hr	30 min	2Hr	48 Hr
0.2N Acetic Acid	77.79	15.08	8.17	78.03	44.11	25.30	83.17	17.53	17.16
0.2N HCl	13.06	20.67	18.59	14.97	17.19	9.33	11.14	19.95	14.18
CG 50 Effluent	6.64	30.49	45.57	5.09	11.64	42.45	4.72	30.15	42.71
Boric Acid I	3.75	11.67	15.05	2.62	6.15	16.86	3.27	12.57	18.55
Boric Acid II	0.29	0.95	1.04	0.21	0.64	0.60	0.25	1.69	0.88
Boric Acid III	0.15	0.47	0.43	0.15	0.23	0.37	0.23	0.53	0.34
Water Wash I	0.16	0.27	0.38	0.10	0.05	0.28	0.10	0.26	0.28
Water Wash II	0.04	0.17	0.50	0.05	0.15	0.25	0.08	0.16	0.32
4N NH_3 Eluate	1.90	10.32	12.80	2.07	5.39	14.37	1.32	11.63	12.21
Total Recovery	103.78	90.09	102.53	103.29	85.55	109.81	104.28	94.47	106.63

further decrease in the proportion of residual ^{14}C-noradrenaline activity present in the acetic acid fractions of the controls and the neuroblastoma supernatant, compared with the results obtained in the 2 hour experiment. As in the 2 hour investigation, relatively less breakdown of added ^{14}C-noradrenaline was detected in the control culture supernatant.

Any metabolism of ^{14}C-noradrenaline occurring in the neuroblastoma tissue culture was therefore difficult to detect in the latter two experiments because of extensive breakdown in the controls. In each study, no major fraction of radioactivity was present in the neuroblastoma supernatant which was not also present in one or both of the controls.

Analysis of column fractions by paper chromatography.

This investigation was restricted to analysis of the column fractions obtained in the 30 minute and 2 hour experiments.

(a) Examination of the 0.2N HCl eluate for ^{14}C-dihydroxymandelic acid (^{14}C-DHMA)

Portions of the acid eluates of the 30 minute experiments, containing the same radioactive count, were taken to dryness *in vacuo* on a rotary evaporator (Rotary Evapo-Mix, Buchler Instruments, N.Y.) at 40°C and the residues were dissolved in 0.2 ml methanol. A slight modification of this procedure was adopted for the 2 hour acid eluates. They were initially extracted with 25 ml ethyl acetate by

90 seconds vortex mixing, followed by 5 minutes mechanical shaking. The organic phase was removed and taken to dryness in vacuo on a rotary evaporator. The residue was dissolved in 0.1 ml acidified methanol (1% (^{v}/v) glacial acetic acid in methanol). Portions of the methanol solutions, 100 µl (30 minute experiment) and 50 µl (2 hour experiment) respectively, were applied as a 2 cm streak to a 4 cm strip of Whatman No. 1 paper. Chromatograms were developed by descending chromatography overnight in butan-1-ol: acetic acid: water (4:1:1), with 10 µg authentic DHMA added as carrier and marker. The developed chromatograms were dried and scanned for radioactivity on the automatic scanner as described earlier.

When compared with controls, no additional peaks of radioactivity were visible on the neuroblastoma chromatogram charts, indicating that no large scale conversion of ^{14}C-noradrenaline to ^{14}C-DHMA had occurred in either experiment. The sensitivity of the automatic scanner was not high enough to detect ^{14}C-compounds present in small amount. The following procedure was therefore adopted for this purpose; chromatogram strips were cut longitudinally into 2 halves, each 2 cm wide. One half was sprayed with ferricyanide-phosphate buffer (pH 7.4) reagent to locate the marker DHMA. The equivalent test area on the unsprayed duplicate strip, plus areas of equal size immediately preceding and following the test area, were cut out and immersed in 4 ml methanol

in a counting vial. Standard liquid scintillator (15 ml) was added, and radioactivity was determined in a Tri-Carb liquid scintillation spectrometer. An average was taken of background readings from the two adjacent areas to each test area, to give a mean background count.

Results

In the 2 hour experiment, after correction for background, the counts in the DHMA area of the neuroblastoma chromatogram exceeded those obtained in the equivalent position of the control strips (Table 17). High background radioactivity present in the chromatograms of the 30 minute experiment was responsible for a negative count in the test area of the controls and prevented a meaningful evaluation of the results.

(b) Examination of Amberlite CG50 effluent for ^{14}C-HMPG and ^{14}C-VMA

Portions of the resin effluents giving the same radioactive count were adjusted to pH 1 (5N and 1N HCl), saturated with NaCl, and extracted with ethyl acetate (2 x 20 ml, 30 minute expt; 1 x 30 ml, 2 hour expt.). After separation and evaporation to dryness of the organic phases, the residues were taken up in methanol. (0.2ml, 30 minute expt; 0.1 ml, 2 hour expt.). In the 30 minute experiment, 2 x 0.1 ml portions of the methanol extract were applied to separate strips of Whatman No. 1 paper as described above. Chromatograms were developed by descend-

TABLE 17.

Incubation of ^{14}C-DL-noradrenaline with neuroblastoma tissue culture for 2 hours at 37°C. Radioactivity in area of chromatogram corresponding to authentic dihydroxymandelic acid. (DHMA).

Experiment	Count per 10 min * §
Neuroblastoma	1352
He La Control	283
Medium	380

* Corrected for background

§ Uncorrected for solvent quenching.

Solvent system:- butan-1-ol: acetic acid: water (4:1:1)

ing chromatography overnight in isopropanol: ammonia (880); water (8:1:1) and butan-1-ol: acetic acid: water (4:1:1) solvent systems respectively. Authentic HMPG (10 µg) and VMA (10 µg) were added as carrier and marker substances. In the 2 hour experiment 50 µl of methanol extract was chromatographed in the isopropanol-ammonia solvent system. On development of the chromatograms, scintillation counting of the test areas of the strips, corresponding to marker HMPG and VMA, was undertaken as described under (a). Nitraniline reagent was prepared as follows; p-nitraniline (0.25 g) was dissolved in 2 ml conc. HCl and 98 ml water (5 vol.). Sodium nitrite, 0.5% ($^{W}/v$) in water, was added until the p-nitraniline was decolorized, and the mixture was then diluted with water to 20 vol. The dried isopropanol/ ammonia chromatograms were sprayed with sodium carbonate (anhydrous, 10% ($^{W}/v$) in water) and, after drying in a stream of warm air, were over-sprayed with diazotized p-nitraniline to locate cold VMA and HMPG; ferricyanide-phosphate buffer (pH 7.4) reagent was employed for location of marker compounds on the butanol/ acetic strips.

Results

The findings of the 30 minute incubation experiment are presented in Table 18. There was no evidence of the presence of ^{14}C-VMA or ^{14}C-HMPG.

Similarly in the 2 hour experiment, no additional radioactivity was detected in areas of the neuroblastoma

TABLE 18.

Incubation of ^{14}C-DL-noradrenaline with neuroblastoma tissue culture for 30 minutes at 37°C. Radioactivity in areas of chromatograms corresponding to authentic VMA and HMPG.

Experiment	VMA counts per 10 min* § isoprop/NH_3	butanol/ acetic	HMPG counts per 10 min * § Isoprop/NH_3	Butanol/ Acetic
Neuroblastoma	429	222	-15	1586
Ependymoma	1025	217	2	1553
Medium	775	327	74	1316

* Corrected for background

§ Uncorrected for solvent quenching.

chromatogram corresponding to authentic VMA and HMPG greater than the counts found in equivalent areas of the control chromatrograms.

(c) Examination of 4N NH_4OH eluate for ^{14}C-normetadrenaline

Portions of the 30 minute eluates, with the same radioactive count, were evaporated to dryness in vacuo on a rotary evaporator at 40°C. To exclude an unwanted white deposit present on evaporation of the solution, and soluble in methanol, the residues were extracted twice with 2 x 10 ml volumes of ether (Anton and Sayre 1966). Ether was removed by evaporating in vacuo and the residues were dissolved in 0.2 ml methanol. Methanol extracts were chromatographed overnight as described above, using isopropanol: ammonia (880): water (8:1:1) as solvent system and 10 µg authentic NMA as carrier and marker. Nitraniline reagent was used to locate cold NMA. The procedure for scintillation counting of test areas of the strips, described previously, was used to estimate the radioactivity on the chromatograms.

Results.

The count in the test area of the neuroblastoma chromatogram exceeded that of both the controls (Table 19).

Further analysis of neuroblastoma supernatant for ^{14}C-dihydroxyphenyglycol (^{14}C-DHPG) and ^{14}C-normetadrenaline activity.

Sodium acetate reagent (3 ml) was added to

TABLE 19

Incubation of ^{14}C-DL-noradrenaline with neuroblastoma tissue culture for 30 min at 37°C. Radioactivity in area of chromatogram corresponding to authentic normetadrenaline.

Experiment	Counts per 10 min.* §
Neuroblastoma	667
Ependymoma control	52
Medium	8

* Corrected for background.

§ Uncorrected for solvent quenching

Solvent system:- isopropanol: ammonia (880): water (8:1:1).

portions, containing the same radioactive count, of the 30 minute neuroblastoma and control supernatants respectively The pH of each solution was adjusted to 8.4 and catechol compounds were adsorbed on alumina as described previously in this chapter. The effluents were set aside for ^{14}C-normetadrenaline analysis, together with sodium acetate reagent (1 ml) which was used to wash the columns. The latter were then eluted with 3.5 ml 0.2N HCl, followed by 3.5 ml distilled water. The eluate was adjusted to pH 7 with NaOH (2.5N and 0.1N), saturated with solid NaCl and shaken with 25 ml ethyl acetate (2 minutes vortex mixing and 5 minutes mechanical shaking) to extract any ^{14}C-DHPG present. The ethyl acetate was taken to dryness in vacuo on a rotary evaporator at 35°C. The residue was dissolved in 0.1 ml methanol which was applied to a chromatogram for overnight descending chromatography in the solvent system butan-1-ol: acetic acid: water (4:1:1) by the procedure described earlier in this chapter. Authentic DHPG (10 µg) was used as carrier and marker. Liquid scintillation counting of the radioactivity present in the test areas of the strips was estimated as before, using ferricyanide-phosphate buffer reagent (pH 7.4) to locate cold DHPG on the strips.

The effluents from the alumina column were treated as follows: 3 ml borate buffer, pH 10, was added, and the samples were adjusted to pH 10 with 0.5N and 0.1N

NaOH. Any ^{14}C-normetadrenaline present was then extracted with 25 ml isoamyl alcohol (2 minutes vortex mixing, 10 minutes mechanical shaking). After centrifugation, the isoamyl alcohol layers were removed and evaporated *in vacuo* on a "Rinco" evaporator in round-bottomed flasks. The residue was taken up in 5 ml of methanol, which was re-evaporated *in vacuo* on a rotary evaporator at 35°C. The residues were dissolved in 0.1 ml methanol and were chromatographed overnight, using 10 ug authentic normetadrenaline, in the solvent system isopropanol: ammonia (880): Water (8:1:1). Radioactivity in areas of the chromatograms corresponding to authentic NMA was estimated by the procedure described previously.

Results

The amount of radioactivity in the area of the neuroblastoma chromatogram corresponding to authentic DHPG was of a similar order to that found in equivalent areas of the controls (Table 20), indicating that no conversion of ^{14}C-noradrenaline to ^{14}C-DHPG had occurred.

High background radioactivity was present in all 3 chromatograms of the 30 minute normetadrenaline experiment, the counts in the neuroblastoma and control culture strips being lower than those of the adjacent areas of the strips used to estimate background. Thus the presence of ^{14}C-normetadrenaline in the neuroblastoma supernatant, suggested by the findings in Table 19, was not confirmed.

TABLE 20

Incubation of ^{14}C-DL-noradrenaline with neuroblastoma tissue culture for 30 minutes at 37°C. Radioactivity in areas of the chromatograms corresponding to authentic dihydroxyphenylglycol (DHPG).

Experiment	Counts per 10 min * §
Neuroblastoma	20
Ependymoma control	13
Medium	52

* Corrected for background

§ Uncorrected for solvent quenching

Solvent system:- butan-1-ol: acetic acid: water (4:1:1)

Further investigation of the neuroblastoma supernatant for the presence of ^{14}C-dihydroxymandelic acid.

Portions (5 ml) of the 30 minute neuroblastoma and control supernatants, including 100 µg authentic DHMA in each sample as carrier, were treated as described on page 249 of this chapter up to the collection of 0.2N HCl eluates of the alumina column plus water washings. The eluates were then investigated for ^{14}C-DHMA activity as described on page 253 except that 0.1 ml portions of the final methanol extract (0.2 ml) were chromatographed in the solvent systems butan-1-ol:HCl (upper phase of n-butanol saturated with 1N HCl) and methanol: butan-1-ol: benzene: water (4:3:2:1) respectively. Authentic DHMA was located in each case with ferricyanide-phosphate buffer (pH 7.4) reagent.

Results

The results obtained using the methanol:butanol: benzene: water solvent system are presented in Table 21. The count obtained for the neuroblastoma exceeds that of the control culture, but is less than that of the medium.

In the other solvent system used, butanol:HCl, high background radioactivity resulted in a negative count in the DHMA test areas of all 3 chromatograms, so that no conclusions could be drawn from the experiment.

General conclusions

The main conclusion of the investigation is that

TABLE 21

Incubation of ^{14}C-DL-noradrenaline with neuroblastoma tissue culture for 30 minutes at 37°C. Radioactivity in area of chromatogram corresponding to authentic dihydroxymandelic acid (DHMA)

Experiment	Counts per 10 min * §
Neuroblastoma	2748
Ependymoma	1334
Medium	5495

* Corrected for background

§ Uncorrected for solvent quenching.

Solvent system:- methanol: butan-1-ol: benzene: water, (4:3:2:1).

no large scale conversion of ^{14}C-noradrenaline to ^{14}C-normetadrenaline, ^{14}C-DHMA, ^{14}C-DHPG, ^{14}C-HMPG or ^{14}C-VMA by neuroblastoma cells in tissue culture could be detected in the present study. There was little evidence of even a minor amount of ^{14}C-noradrenaline metabolism. The presence of traces of ^{14}C-DHMA in the neuroblastoma supernatant after 30 minutes and 2 hours incubation was indicated after chromatography in a butanol: acetic acid: water solvent system, but was not confirmed in butanol: HCl and methanol: butanol: benzene: water systems (30 minutes expt). Similarly, a small amount of additional radioactivity was found in the normetadrenaline position of the neuroblastoma chromatogram in an isopropanol: ammonia: water solvent system after initial ether extraction, but this finding was not subsequently confirmed in the same solvent system, after initial extraction of the eluate with isoamyl alcohol at pH 10.

B. <u>Incubation of ^{14}C-DOPA with neuroblastoma tissue culture</u>

<u>Specification of ^{14}C-DOPA</u>

DL-3-(3,4-dihydroxyphenyl)-alanine-2-^{14}C (^{14}C-DOPA) crystals; specific activity 31.2 mc/mmole (Radiochemical Centre, Amersham).

<u>Preparation of ^{14}C-DOPA solution for incubation with tissue culture</u>

The ^{14}C-DOPA (50 µc) was dissolved in 0.5 ml of

0.1N acetic acid.

Incubation procedure

^{14}C-DOPA solution (0.1 ml, 10uc) was added to 15 ml of control ependymoma tissue culture cells in Eagle's medium plus 10% calf-sefum, and to neuroblastoma tissue culture cells in medium (15 ml) respectively. The samples were incubated at 37°C for 30 minutes, after which the pH was adjusted to 1-2 with 6N HCl. The solutions were stored at -15°C until required.

Separation of ^{14}C-DOPA from ^{14}C-noradrenaline and ^{14}C-dopamine

The large radioactive count of ^{14}C-DOPA used as precursor in the experiment means that a satisfactory separation of this compound from both of its metabolites, ^{14}C-dopamine and ^{14}C-noradrenaline is necessary, particularly when, at best, only small amounts of metabolites are likely to be formed. An efficient system to separate ^{14}C-dopamine from ^{14}C-noradrenaline was also required; that described by Rutledge and Weiner (1967), based on a procedure by Häggendal (1963), was satisfactory.

Reagents and materials

Cation exchange resin AG 50W-X8 (100-200 mesh, hydrogen form) (Bio-Rad Laboratories, Richmond, Calif.)

Ethylenediamine tetraacetic acid (EDTA) (disodium salt, A.R.) 0.1% (^{W}/v) in water.

Di-sodium hydrogen orthophosphate, Na_2HPO_4. 12 H_2O (A.R.).

Sodium dihydrogen phosphate, NaH_2PO_4. $2H_2O$ (A.R.).

0.1M sodium phosphate buffer (pH 6.5): prepared by mixing 0.1 M Na_2HPO_4. $12H_2O$ and 0.1 M NaH_2PO_4. $2H_2O$ solutions to obtain a pH of 6.5.

Preparation of analytical grade cation exchange resin AG 50W - X8 (100-200 mesh) Na^+ form from the hydrogen form. Resin (100 g) in the H^+ form was poured into a large column, 3 cm in diameter, plugged with absorbent cotton wool. For conversion to the Na^+ form, the resin was washed with 2 volumes of 1N NaOH / 1 volume of resin, at a flow rate of 1 ml/min/sq.cm of bed. Complete conversion to the Na^+ form was indicated when the column effluent was pH 9 or over. The resin was then washed with 2 volumes of distilled water to one volume resin.

Test of procedure

It was necessary to test the system used to separate ^{14}C-DOPA from ^{14}C-dopamine and ^{14}C-noradrenaline because of the large count of ^{14}C-DOPA used in the experiment. This was done by carrying a solution of pure ^{14}C-DOPA alone through the procedure, monitoring the radioactivity recovered at each stage. 1 µl ^{14}C-DOPA solution (128,000 c.p.m., uncorrected) was added to 5 ml of Eagle's medium, with 100 µg cold DOPA as carrier. The pH was adjusted to

6.5 on a pH meter using NaOH, and the adjusted solution was passed through a Dowex 50 - X8 column (Na^+ form, 5 by 35 mm, previously prepared by mixing the resin with sodium phosphate buffer, pH 6.5). For separation of ^{14}C-DOPA from ^{14}C-dopamine and ^{14}C-noradrenaline, the effluent was collected, after which the resin was washed with 15 ml 0.1% (W/v) Na_2 EDTA, 15 ml of 0.1 M Na phosphate buffer (pH 6.5) and 10 ml glass distilled water, all of which were collected separately. The column was then eluted with 18 ml 1N HCl and 20 ml 2N HCl, which were also collected separately. The initial column effluent (0.1ml) plus 0.9 ml water, and 1 ml of each of the other eluates were counted for 10 minutes in scintillation fluid (equal parts by volume of ethanol, dioxane, xylene; PPO, 5 g per litre; POPOP, 50 mg per litre, naphthalene 80g) for 10 minutes in the Tri-Carb liquid-scintillation spectrometer: 1 ml water in scintillation fluid was used as a blank. Correction for quenching was made by recounting each fraction after the addition of an internal standard of ^{14}C-toluene. The corrected recovery of ^{14}C-radioactivity in each fraction was calculated, and expressed as a percentage of the original radioactivity added to the column. (Table 22).

Table 22 shows that about 25% of ^{14}C-DOPA is adsorbed by the resin on passage through the column. Most of this, however, is removed by subsequent elutions with 0.1% EDTA and phosphate buffer. The large count of ^{14}C-DOPA added to the column will not, therefore, cause a major interference in the detection of any ^{14}C-dopamine or ^{14}C-

TABLE 22

Percentage radioactivity in column fractions after passage of ^{14}C-DOPA solution (0.1 µc) through a cation exchange resin AG 50W-X8 Na^+ form.

Fraction	% Radioactivity
Column effluent	75.59
0.1% EDTA	19.54
0.1M Phosphate buffer	2.88
Water wash	0.17
1N HCl elution	0.85
2N HCl elution	1.72
Total ^{14}C recovery	100.75

noradrenaline eluted from the column with 1 and 2N HCl, since paper chromatography will remove any residual amounts of DOPA still contaminating the dopamine and noradrenaline fraction.

Analysis of neuroblastoma tissue culture supernatant on cation exchange resin AG 50W - X 8 Na^+ form

Neuroblastoma tissue culture supernatant (5 ml) and ependymoma tissue culture supernatant (5 ml) were adjusted to pH 6.5 after the addition of 100 µg. each of authentic DOPA, dopamine and noradrenaline as carriers. The solutions were passed through the cation exchange resin and treated exactly as described for ^{14}C-DOPA in the test experiment. The 1N and 2N HCl fractions were retained for further analysis by paper chromatography.

The percentage radioactivity found in each fraction after the passage of the supernatants through the cation exchange resin is shown in Table 23.

There was no marked difference between distribution of radioactivity found in the different column fractions, after passage of the neuroblastoma and ependymoma supernatants through the cation exchange resin. This suggests that no significant conversion of ^{14}C-DOPA to ^{14}C-catecholamines has occurred in the neuroblastoma tissue culture.

Identification of sources of radioactivity in 1N and 2N elutes by paper chromatography.

Portions of the 1N HCl eluates containing equiva-

TABLE 23

Incubation of ^{14}C-DOPA (10 µc) with neuroblastoma tissue culture for 30 minutes at 37°C. Percentage radioactivity in column fractions after passage of supernatant through cation exchange resin AG 50W - X8 Na^+ form.

Fraction	% Radioactivity	
	Ependymoma Supernatant	Neuroblastoma Supernatant
Column effluent	64.04	69.13
0.1% EDTA	28.42	21.31
0.1M phosphate buffer	2.80	2.79
Water wash	0.26	0.09
1N HCl elution	0.35	0.39
2N HCl elution	0.36	0.41
Total ^{14}C recovery	96.23	94.12

lent amounts of radioactivity were evaporated to dryness in vacuo on a rotary-evaporator at 40°C. A white solid residue was present which proved to be soluble in methanol. Any catechol compounds present were therefore extracted into 2 ml of acid acetone (0.5 ml concentrated HCl made up to 100 ml with acetone), leaving the white residue behind. The acid acetone extract was re-evaporated to dryness as above, the residue dissolved in 0.1 ml methanol, and chromatographed overnight (descending) in the solvent system methanol: butan-1-ol: benzene: water (4:3:2:1), with 10 µg cold DOPA, dopamine and noradrenaline respectively as markers. The catechol compounds were located on one half of the chromatogram, as described previously, with ferricyanide-phosphate buffer (pH 7.4) reagent. The location of noradrenaline was additionally confirmed by the fluorescence of its oxidation product under ultra-violet light at 254 and 345 mµ. DOPA is well separated from noradrenaline and dopamine in this system. Radioactivity in areas of the chromatograms corresponding to authentic dopamine and noradrenaline was estimated by the procedure described on page 254. The 2N HCl eluate was treated in the same manner to determine radioactivity in the area to which authentic dopamine runs on the chromatogram.

Results

On the neuroblastoma 1N HCl eluate chromatogram, the radioactivity in positions corresponding to authentic

noradrenaline and dopamine exceeded that present in equivalent positions of the ependymoma chromatogram (Table 24). Radioactivity in the dopamine position of the neuroblastoma 2N HCl eluate chromatogram was, however, lower than that present in the ependymoma control.

Further analysis of the 1N HCl eluate by chromatography in butanol: acetic acid: water

The previous experiment on the 1N HCl eluate was repeated up to extraction of the residues into 0.1 ml methanol. Overnight descending paper chromatography of the extracts was undertaken using the solvent system butan-1-ol: acetic acid: water (4:1:1). The authentic catecholamines were located with ferricyanide-phosphate buffer (pH 7.4). Radioactivity in areas corresponding to dopamine and noradrenaline was estimated as before.

Results

No additional counts could be detected in the area of the neuroblastoma chromatogram corresponding to noradrenaline but, again, radioactivity found in the neuroblastoma dopamine position exceeded that present in the ependymoma control (Table 25).

General conclusions

No significant conversion of ^{14}C-DOPA to ^{14}C-dopamine and ^{14}C-noradrenaline could be detected in this

TABLE 24

Incubation of ^{14}C-DOPA (10 µc) with neuroblastoma tissue culture for 30 min at 37°C. Radioactivity in areas of the chromatograms corresponding to authentic dopamine and noradrenaline.

Experiment	Area examined	Counts per 10 min. * §
Neuroblastoma 1N HCl eluate	Noradrenaline position	253
Ependymoma 1N HCl eluate	Noradrenaline position	35
Neuroblastoma 1N HCl eluate	Dopamine position	623
Ependymoma 1N HCl eluate	Dopamine position	298
Neuroblastoma 2N HCl eluate	Dopamine position	3884
Ependymoma 2N HCl eluate	Dopamine position	4094

* Corrected for background

§ Uncorrected for solvent quenching

Solvent system:- methanol: butan-1-ol: benzene: water (4:3:2:1).

TABLE 25.

Incubation of ^{14}C-DOPA with neuroblastoma tissue culture for 30 minutes at 37°C. Radioactivity in areas of chromatogram corresponding to authentic dopamine and noradrenaline.

Experiment	Area examined	Counts per 10 min.*§
Neuroblastoma 1N HCl eluate	Noradrenaline position	1954
Ependymoma 1N HCl eluate	Noradrenaline position	2486
Neuroblastoma 1N HCl eluate	Dopamine position	9709
Ependymoma 1N HCl eluate	Dopamine position	7596

* Corrected for background

§ Uncorrected for solvent quenching

Solvent system:- butan-1-ol: acetic acid: water (4:1:1).

study. The presence of traces of ^{14}C-dopamine in the 1N HCl eluate was suggested by experiments using 2 different solvent systems but, since the counts in the dopamine position were also relatively high in the control, this additional radioactivity could fall within the range of experimental error. There was no evidence of any ^{14}C-dopamine in the 2N HCl eluate. Chromatography in a butanol/acetic acid/water solvent system did not confirm the possible presence of traces of ^{14}C-noradrenaline, indicated previously in a methanol/butanol/benzene/water system.

DISCUSSION

In this chapter, the ability of a continuous cell line of neuroblastoma cells to metabolize ^{14}C-noradrenaline and to synthesize ^{14}C-dopamine and ^{14}C-noradrenaline from precursor ^{14}C-DOPA, has been examined by a variety of experimental techniques. As only a small proportion of patients with secreting neuroblastoma show a marked elevation in blood pressure (Voorhess and Gardner 1962; Sourkes, Denton, Murphy, Chavez and St. Cyr 1963; Von Studnitz, Käser and Sjoerdsma, 1963), and because this situation points to metabolic breakdown of pressor noradrenaline before release into the circulation, studies on the metabolism of ^{14}C-noradrenaline within tumour cells in tissue culture assume added significance.

No significant conversion of ^{14}C-noradrenaline to any of the ^{14}C-metabolites investigated could be conclusively demonstrated in the present experiments. The results fail to confirm the findings of La Brosse, Belehradek, Barski, Bohuon and Schweisguth (1964), who reported the presence of ^{3}H-HMPG, ^{3}H-normetadrenaline and the sulphate conjugate of ^{3}H-normetadrenaline in the culture medium, after incubation of ^{3}H-noradrenaline with neural crest tumour in tissue culture for 48 hours. A further study (La Brosse, Schweisguth and Bohuon, 1968) again pointed to ^{3}H-HMPG formation under similar experimental conditions. It is possible that the neuroblastoma cells used in the present work may be derived from a tumour which was a relatively inactive secretor of noradrenaline. Cases, although extremely rare, have been reported of neuroblastoma patients with an elevated urinary excretion of dopamine or HVA, but with no increase in VMA excretion (Bell and Steward, 1961; von Studnitz, 1961b). A more plausible explanation would be loss of metabolic activity during the course of continuous sub-culturing of the original tumour cells. In their studies, La Brosse, Belehradek, Barski, Bohuon and Schweisguth (1964) had access to fresh post-operative tumour tissue.

There are, however, other noteworthy differences between the latter experiments and those reported in this chapter. Chief among these is the lengthy incubation period, 48 hours, employed by La Brosse, Belehradek, Barski,

Bohuon and Schweisguth (1964), compared with the shorter times of 30 minutes or 2 hours in the present investigation. Complete degradation of added ^{14}C-noradrenaline was observed after 48 hours in the initial breakdown studies reported herein and, even after periods as short as 1 or 2 hours, considerable breakdown was found to occur. This might suggest that radioactivity attributed by La Brosse, Belehradek, Barski, Bohuon and Schweisguth (1964) to noradrenaline metabolites, might in fact be due to breakdown products. This view is reinforced by the fact that unlike the present work, they did not exclude the presence of residual ^{3}H-noradrenaline counts by column chromatography prior to paper chromatography. Close examination of their chromatograms does, indeed, reveal some ^{3}H-noradrenaline breakdown after 48 hours, but if metabolic products were formed relatively early during the incubation time, particularly methoxy compounds, these would tend to be far more stable than the labile ^{3}H-noradrenaline precursor. In addition, the protein content of the culture fluid used by La Brosse, Belehradek, Barski, Bohuon and Schweisguth (1964) (30% pooled human plasma or a mixture of 15% pooled human plasma and 15% calf-plasma) was about 3 times as high as that in the fluid in the present studies (10% calf-serum), where noradrenaline broke down completely in 48 hours; on the other hand, since the stability of noradrenaline in plasma is comparatively high (Bain, Gaunt and Suffolk, 1937; Cohen, Holland and Goldenberg, 1958),

It is possible that breakdown may have been less in the medium of La Brosse, Belehradek, Barski, Bohuon and Schweisguth (1964).

Lack of essential co-factors in the medium is another possible explanation for the inability to detect metabolites in the present study, although a similar criticism can be levelled against the experiments of La Brosse, Belehradek, Barski, Bohuon and Schweisguth, (1964). Thus, Goldstein, Anagnoste and Goldstein (1968) found that p-hydroxyphenylacetaldehyde was the major deaminated metabolite of ^{3}H-tyramine incubated with neuroblastoma cells in tissue culture at 37°C. Addition of $NADPH_2$ was, however, necessary for further conversion of the aldehyde to p-hydroxyphenylethanol. Furthermore, aldehyde dehydrogenase activity was found to be very low in cultured neuroblastoma cells. Investigation of neuroblastoma cells incubated with ^{14}C-noradrenaline for aldehyde products of MAO activity, rather than acid or alcohol metabolites, might thus be more fruitful in future studies. La Brosse, Belehradek, Barski, Bohuon and Schweisguth (1964) tentatively identified ^{3}H-4-hydroxy-3-methoxymandelaldehyde in one of their experiments.

It may be inferred from the observations of Goldstein, Anagnoste and Goldstein (1968) that MAO is present in cultured neuroblastoma cells, but studies showing the presence of enzymes involved in catecholamine metabolism in the tumour tissue have been limited to those of Amar-Costesec, Bohuon and Schweisguth(1965) for MAO, and La Brosse

and Karon (1962) for COMT. Evidence is not yet available concerning the intracellular localisation of these enzymes in neuroblastoma tissue.

Incubation of the neuroblastoma tissue culture cells with ^{14}C-DOPA was restricted to a period of 30 minutes in the present work. This served a dual purpose; it minimized degradation of the added compound, and decreased the tendency for any ^{14}C-dopamine or ^{14}C-noradrenaline formed to undergo further metabolism on longer incubation. However, no significant conversion of ^{14}C-DOPA to either compound was detected although there was a suggestion that traces of ^{14}C-dopamine might have been formed. Any small amount, which might have been present, was eluted from the cation exchange resin with 1N HCl, and not with 2N HCl as previously described (Rutledge and Weiner, 1967). Comparison was made with one control, ependymoma tissue in culture, and not with the medium also, as in the noradrenaline experiments. The presence of ^{14}C-dopamine, without its further conversion to ^{14}C-noradrenaline, is quite possible; patients with neuroblastoma have been described in whom urinary dopamine and HVA concentrations were elevated, but noradrenaline metabolites were present in normal amount (Bell and Steward, 1961; von Studnitz, 1961b).

The finding presented here with ^{14}C-DOPA, coupled with those obtained during the ^{14}C-noradrenaline experiments, are compatible with the interpretation that we are dealing with non-secreting neuroblastoma tissue in culture; the same

continuous cell line was used in both studies. In future investigations with more active material, it will probably be worth bearing in mind the observation of Goldstein (1966) that dopamine-β- hydroxylase activity was not high in tissue from his case of neuroblastoma. Thus there may be little tendency for ^{14}C-DOPA to be converted to ^{14}C-noradrenaline in this type of experiment.

In conclusion, the results described in this chapter show that previous findings in this area of research (La Brosse, Belehradek, Barski, Bohuon and Schweisguth, 1964; La Brosse, Schweisguth and Bohuon, 1968) must be treated with caution, and that further investigation and refinement of techniques is necessary before any definite conclusions may be drawn.

CHAPTER 7

General discussion

Despite permanent and specific destruction of a major portion of the sympathetic nervous system, the immunosympathectomized animal develops normally in most respects (Levi-Montalcini and Angeletti, 1966). Thus, there did not appear to be any secondary changes which might have exerted any influence on the present studies. It would not appear unreasonable to regard the observed changes as stemming from the degree of sympathetic denervation effected by immunosympathectomy. The adrenal medulla is the other main peripheral source of catecholamines in the body (Malmejac, 1964), so that a similar study of overall catecholamine metabolism in the adrenalectomized rat was undertaken for comparative purposes.

The separate investigations undertaken in the immunosympathectomized (chapter 3) and adrenalectomized (chapter 4) rat appear to be complementary to each other in many respects. Despite the fact that a significantly reduced output of noradrenaline occurs in the urine of immunosympathectomized rats (Brody, 1964; Carpi and Oliverio, 1964; Schönbaum, Johnson and Sellers, 1965, 1966), no change was observed in the urinary excretion of normetadrenaline (chapter 3). Iversen, Glowinski and Axelrod (1966), however, have reported an increased turnover of catecholamines

in the adrenal medulla of immunosympathectomized mice, so that normetadrenaline arising from the O-methylation of noradrenaline released from the adrenal medulla, may contribute to the unchanged urinary concentration of this metabolite recorded in the immunosympathectomized rat. It is possible that an increased release of catecholamines from the adrenal medulla could be involved in the maintenance of homeostasis in the immunosympathectomized animal. Conversely, the increased concentration of catecholamines in the urine of adrenalectomized rats (Imms and Jones, 1967) has been attributed to an increase in sympathetic nerve activity (Imms and Jones, 1968), secondary to a decrease in blood pressure. The reported increase in noradrenaline turnover (Landsberg and Axelrod, 1968) and synthesis (Westfall and Osada, 1969) in the hearts of adrenalectomized rats is probably reflected in the raised urinary output of HMPG, as may be the increased turnover of noradrenaline in the brains of such animals (Javoy, Glowinski and Kordon, 1968); since, however, the brain is unaffected by NGF antiserum (Levi-Montalcini and Angeletti, 1966), the contribution of HMPG arising from this source to the levels of the metabolite excreted in the urine is unlikely to be changed in the immunosympathectomized animal.

The present investigations also reveal that under conditions such as immunosympathectomy, where the activity of the sympathetic nervous system is impaired, or adrenalectomy, where its activity may be increased, the urinary

excretion of HMPG, rather than noradrenaline itself, may be utilized as a convenient index of noradrenaline production by the nerves. Relatively large quantities are present in normal rat urine. The increased amounts found in the urine of adrenalectomized rats appear to be more closely related to removal of the adrenal cortex, rather than the medulla however, since, on treatment with hydrocortisone, the urinary output of the metabolite returned to normal levels. This aspect has been discussed in chapter 4.

A logical extension of the studies reported herein would be an investigation of overall catecholamine metabolism in rats following combined immunosympathectomy and adrenal demedullation. Whether or not such an investigation would shed light upon the precise source of the VMA and normetadrenaline detected in the immunosympathectomized rat is doubtful, however, since adrenal demedullation was shown by Carpi and Oliverio (1964) to bring about a constant eliminiation of noradrenaline in the urine of the immunosympathectomized animal; these authors have suggested that in such animals there exists a noradrenergic system, the activity of which, latent in basal conditions, becomes evident in emergency conditions. An assessment of endogenous catecholamine metabolism in rats which have undergone chemical sympathectomy with 6-hydroxydopamine

(Thoenen and Tranzer, 1968), would also extend the present studies in the immunosympathectomized animal. The finding of measurable quantities of metadrenaline in the urine of adrenalectomized rats (chapter 4) provides further evidence for extra-adrenal sources of adrenaline. In contrast with the PNMT of the adrenal medulla (Wurtman and Axelrod, 1965, 1966), however, the enzyme left in the rat after adrenalectomy does not appear to be dependent on the presence of corticosteroids for its activity.

Of the two main enzymes involved in catecholamine metabolism, COMT is thought to have an exclusively extraneuronal location in peripheral tissues (Kopin and Gordon, 1962; Iversen, Glowinski and Axelrod, 1966; Iversen, Jarrott and Langer, 1968), whilst some MAO activity, at least, resides within the sympathetic neurone (Kopin, 1966). Pharmacological investigation of enzymic activity was therefore restricted to the latter enzyme in the present studies.

Treatment of mice with some drugs which are known to interfere with the function of the sympathetic nervous system, namely NGF antiserum, 6-hydroxydopamine and reserpine (see chapter 1), resulted in changes of MAO activity in various subcellular compartments of certain tissues (chapter 5). The pattern of results obtained was complex, but allthree drug-treatments caused a reduction in cardiac microsomal MAO activity, a finding which may be of significance, in view of a postulated association of intraneuronal MAO activity with the heart microsomal fraction (Stjärne,

Roth and Giarman, 1968; de Champlain, Axelrod and Mueller, 1969). Increased MAO activity, particularly in the mitochondrial fraction, was observed in heart, brain and vas deferens of adrenalectomized rats. Since an increased turnover of noradrenaline is known to occur in the heart (Landsberg and Axelrod, 1968) and brain (Javoy, Glowinski and Kordon, 1968) of such animals, an increased turnover of the amine might also be expected to occur in the vas deferens. Furthermore, administration of hydrocortisone abolishes the increased cardiac MAO activity in adrenalectomized rats (Avakian and Callingham, 1968; Westfall and Osada, 1969); it remains to be seen whether or not the corticosteroid would have a similar effect on the MAO activity of the brain and vas deferens.

There was no significant change in the output of deaminated metabolites of 5-HT in the urine of immunosympathectomized rats (chapter 3), apart from a small reduction in 5-HTOH excretion when expressed in terms of rat weight. These findings suggest that the increased concentration of 5-HT found in the gastrointestinal tract (Hamberger, Levi-Montalcini, Norberg and Sjöqvist, 1965; Iversen, Glowinski and Axelrod, 1966; Thompson and Campbell, 1966b) and other tissues (Klingman, 1969) of immunosympathectomized animals, is not related to an alteration in MAO activity. A possible relationship between increased 5-HT content and impairment of sympathetic innervation in these animals was discussed in

chapter 3. Such a postulate might also be tested in animals which have undergone a chemical sympathectomy with 6-hydroxydopamine. Very recently, Klingman and Klingman (1969) reported that after sympathetic denervation of rat submaxillary glands by means of superior cervical ganglionectomy, the 5-HT concentration per unit weight and total 5-HT content of the organ was increased.

In addition to the information on the urinary output of important catecholamine and 5-hydroxyindole metabolites in immunosympathectomized or adrenalectomized rats (chapters 3 and 4), these studies provide a comprehensive set of data on the endogenous output of these compounds in normal rat urine. Relatively few studies of this nature have been undertaken in rats, in contrast with the large body of similar information available in man. A detailed study of the extent of VMA conjugation, however, such as that described herein for rat urine, might profitably be made on human urine; it is generally assumed that the endogenous acid is excreted exclusively in the free state in man, but Goodall and Alton (1968a) have recently identified the 4-O sulphate of VMA in human urine as a metabolite of noradrenaline formed from infused dopamine. The need for such a study is emphasized by the fact that although HMPG was shown to be a catecholamine metabolite by Axelrod, Kopin and Mann in 1959, only recently has its glucuronide been detected as a major conjugate in man (Shimizu and La Brosse, 1969).

In contrast to the adrenaline and noradrenaline degradation pathway, the intermediate aldehyde formed from endogenous 5-HT by MAO was preferentially oxidised to 5-HIAA, rather than reduced to 5-HTOH (chapter 3). The urinary output of catecholamine metabolites in the rat appears to be related to the age of the animal, but experiments conducted throughout the life-sycle of the rat are required for a more complete knowledge of this relationship.

Unlike the excretion pattern of catecholamine metabolites in the immunosympathectomized rat, a raised output of these compounds is a constant finding in the urine of neuroblastoma patients (see chapter 1). In addition, an increased NGF titre is found in sera of children with neuroblastoma (Burdman and Goldstein, 1964). The failure to demonstrate further metabolism of noradrenaline on incubation of labelled amine, or its precursor, with a continuous cell line of neuroblastoma in tissue culture (chapter 6), may indicate that it is necessary to use fresh tumour tissue for such studies. A future re-investigation of the problem might also include an evaluation of co-factor requirements for noradrenaline metabolism in tissue culture.

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