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CURLE, P.F.

1986

A STUDY OF THE EFFECTS OF OXYPERTINE ON MONOAMINERGIC PATHWAYS IN RAT BRAIN USING A NEW HPLC/ECD METHOD

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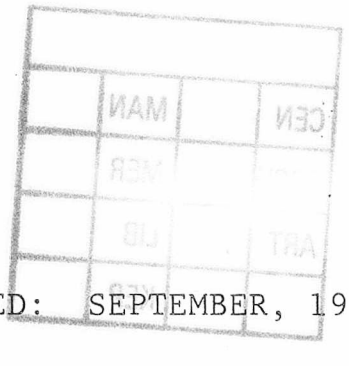
SUBMITTED TO THE C.N.A.A. AS PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

THE SPONSORING ESTABLISHMENT: SCHOOL OF PHARMACY, ROBERT GORDON'S INSTITUTE OF TECHNOLOGY, ABERDEEN

THE COLLABORATING ESTABLISHMENT: DEPARTMENT OF MENTAL HEALTH, CLINICAL RESEARCH CENTRE, ROYAL CORNHILL HOSPITAL, ABERDEEN.



SUBMITTED: SEPTEMBER, 1986

Abstract

A Study of the Effects of Oxypertine on Monoaminergic Pathways in Rat Brain using a New HPLC/ECD Method

Peter F. Curle

A new HPLC/ECD system for the measurement of the biogenic amines and their major metabolites has been developed. It comprises of a two column system, one for the analysis of the biogenic amines and one for the metabolites. The preparation of brain samples for analysis has been made as simple as possible (homogenisation in perchloric acid, plus centrifugation) so that all the compounds of interest are present in the sample.

The biochemical action of oxypertine has been assessed using the above system. Comparing the action of oxypertine against the action of reserpine has shown a difference between the two compounds. Reserpine causes at least 90% reduction in neuronal amine content while oxypertine caused no reduction in neuronal amine content. These results combined with the results of behavoural studies, suggest that oxypertine disrupts the reserpine resistant (non-vesicular) pool of amines.

The Kinetics of amine turnover were also investigated. A computer programme for the analysis of catecholamine turnover rates and times was developed. The programme includes the calculations required for the calculation of the SEM's for turnover rate and time. These differ from previous calculations, as the mathematical assumption on which the calculations are based differ. The effect of oxypertine on catecholamine turnover rates and times was investigated using the above mathematical model and also in the analysis of oxypertine effect on the non-vesicular pool of amines.

ACKNOWLEDGEMENTS

I should particularly like to express my thanks to Dr. K. E. Kendle for his advice and help throughout the research and Miss C. Geddes for her unlimited enthusiasm.

My thanks also to Dr. R. B. Taylor, Dr. T. Palomo and Prof. G. Ashcroft for their advice and help when asked for. Thanks also to the technical secretarial staff of the School of Pharmacy R.G.I.T. and the inmates of PC6 who were always good for a drink.

TO SUSAN

LOVE PETER

CONTENTS

AIM OF THE INVESTIGATION

The proposal study is designed to examine the biochemical action of oxypertine on the biogenic amines and their metabolites in selected rat brain areas. Originally synthesised in the late 1950's, oxypertine was classified as a reserpine like compound, ie. an amine depleter, and remained a compound of little interest until the late 1970's, when Sterling (1979) suggested its use in the treatment of withdrawn psychotics. Palomo and Russell (1983) examined the behavioural effects of oxypertine on amphetamine-induced behaviour in rats and noted that doses of 1mg/kg and 4mg/kg increased the rats exploratory behaviour. Further behaviour studies by Palomo and Reid (1983), 1984) resulted in the suggestion that oxypertine may deplete the readily synthesised (non-vesicular) pool of neuronal catecholamines as opposed to the reserpine releasable (vesicular) pool of catecholamines and they concluded that biochemical data was essential to confirm their behavioural evidence. Amphetamine-induced behaviour in rats is used as an animal model for schizophrenia. With oxypertine increasing exploratory behaviour in amphetamine-induced behaviour in rats, the idea emerged that oxypertine may be useful in the treatment of some forms of schizophrenia. This though, still requires much work, as the complex nature and causes of schizophrenia are little understood.

At present there are several methods available for the estimation of the biogenic amines and their metabolities (see later introduction), but the recent developments in high-performance

liquid chromatography combined with those in electrochemical detection allows for the recording of levels in the low picogramme range. It is suggested that a new HPLC/EDC system for the measurement of the biogenic amines and metabolites be developed so that all the compound of interest (amines and metabolites) can be recorded from a single sample. An attempt will also be made to simplify the chromatographic conditions and sample preparation technique used, as at present complex solvent mixtures and extraction proceedures are used.

Much work to-day is done on drugs that alter the levels of amines and metabolites and many workers report increased or decreased levels of amines as alterations in turnover rates. This view of turnover differs from that of Brodie et al, (1966) who provided a kinetical method for the calculation of turnover rates and times. It is hoped that improvements in the analysis of the basic turnover rates and times provided by Brodie et al, (1966) can take place, and that the effects of oxypertine can undergo kinetic analysis.

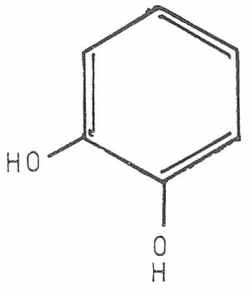
As can be seen, it is hoped to undertake work in three different areas, ie. chromatography, oxypertine in schizophrenia and kinetics. With this in mind the general introduction has been divided into three parts, in an attempt to outline the background of each area of investigation. It is hoped that the different parts will be integrated in the experimental chapters and general discussion.

PART 1 - CATECHOLAMINES

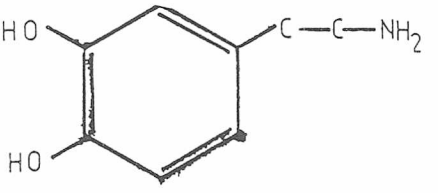
GENERAL ASPECTS

The term "catecholamine" refers, generically, to all organic compounds that contain a catechol nucleus (a benzene ring with two adjacent hydroxyl substituents) and an amine group. (Fig 1.1) In practice the term "catecholamine" usually implies dihydroxyphenylethylamine (dopamine, DA) and its metabolic products, noradrenaline (NA) and adrenaline (A).

Figure 1.1 Catechol and catecholamine structure.



Catechol



Catecholamine

BIOSYNTHESIS

Catecholamines are formed in brain, chromoffin cells, sympathetic nerves and sympathetic ganglia from their amino-acid precursor tyrosine. Blaschko in 1939 first postulated a sequence of enzymatic steps for the biosynthesis of the catecholamines from tyrosine which was finally confirmed by Nagatsu et al (1967) when they showed that tyrosine hydroxylase was involved in the conversion of l-tyrosine to 3,4-dihydroxyphenylalanine (DOPA). The

amino acid percursor, tyrosine, is normally present in the circulation in a concentration of about 5-8 x 10^{-5}M. It is taken up from the bloodstream and concentrated within the brain and presumably also in other sympathetically innervated tissues by an active transport mechanism. In mammals, tyrosine can be derived from the hydrolysis (Phenylalanine hydroxylase) of dietary or tissue phenylalanine. Both phenylalanine and tyrosine are normal constituents of mammalain brain, present in a free form concentration of 5 x 10^{-5}M. However, noradrenaline biosynthesis is usually considered to begin with tyrosine which represents a branch point for many important biosynthetic processes in animal tissue (fig 1.2). It should be emphasized that the percentage of tyrosine utiliszd for catcholamine biosynthesis as opposed to other biochemical pathways is very minimal (2%).

The biosynthetic pathway for the formation of catecholamines is illustrated in Figure 1.3. The conversion of tyrosine to noradrenaline and adrenaline was first demonstrated in the adrenal medulla. With the introduction of highly specific radioactive precursors and chromotographic separation techniques, the above mentioned synthesis pathway has also been confirmed in sympathetic nerves ganglia, heart, arterial and nervous tissue and brain.

Fig 1.2 Metabolism of tyrosine

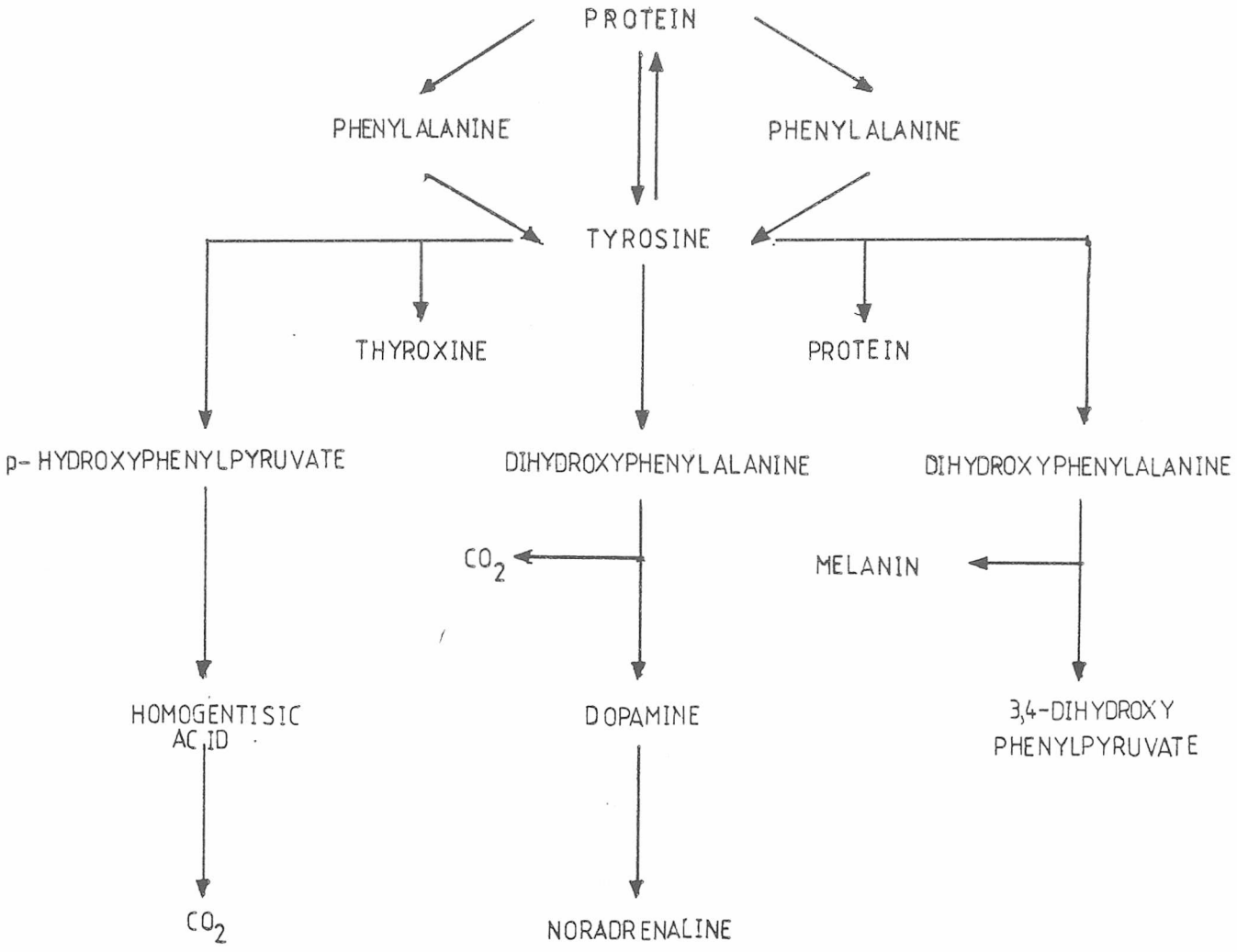
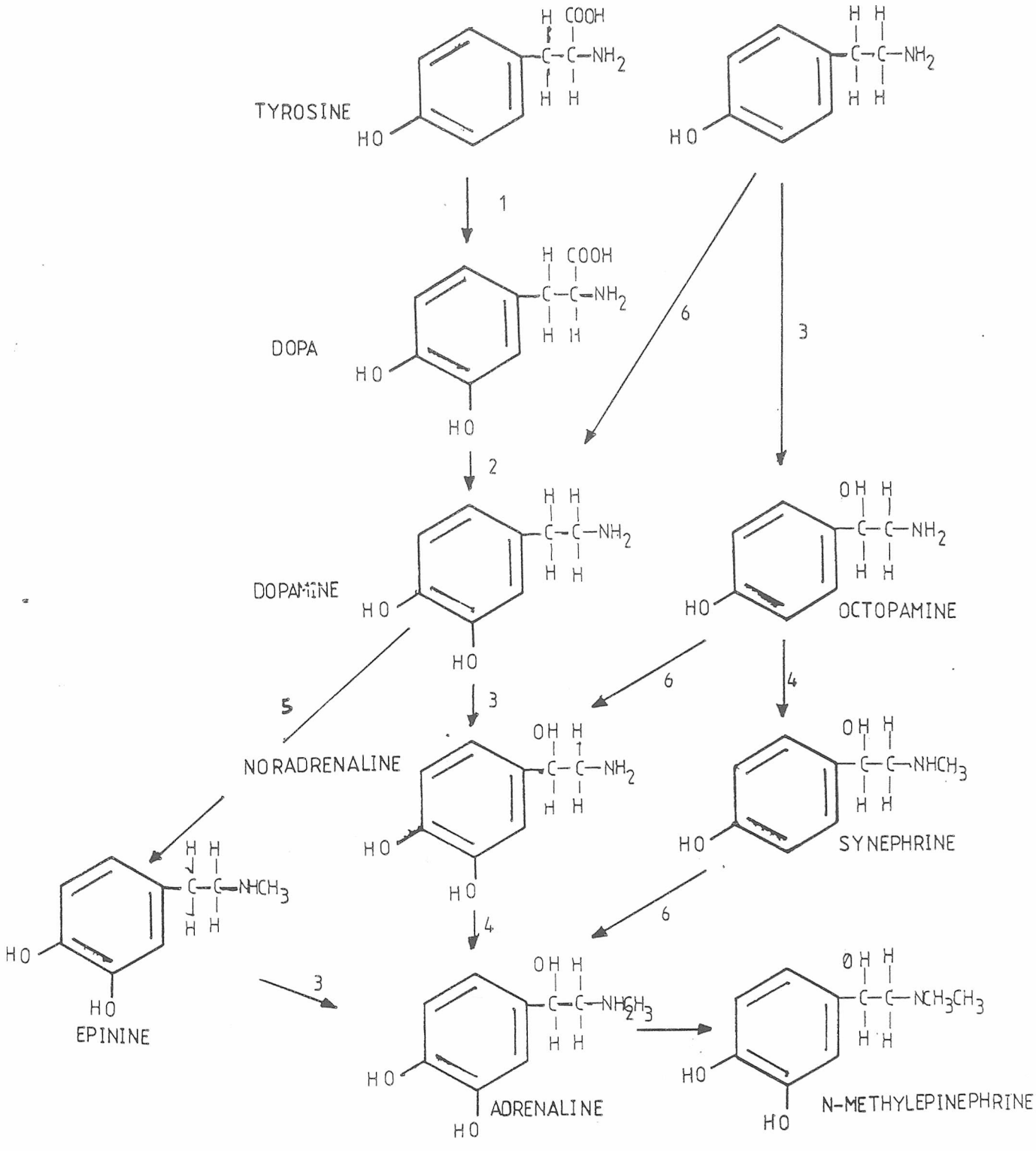


Figure 1.3 Primary and alternative pathways in the formation of catecholamines



1 Tryrosine Hydrosylase
2 Aromatic Amino-acid decarboxylase
3 Dopamine -B-oxidase
4 Phenylethanolamine-N-methyl transferase
5 Nonspecific N-methyl transferase in lung and folate-dependant N-methyl transferase in brain
6 Catechol forming emzyme.

Tyrosine Hydroylase

Tyrosine hydroxylase (TH; L-tyrosine tetrahydropteridine oxygen oxidoreductase, 3-hydroxylating; E.C. 1.14. 16.2) catalyzes the first step in the biosynthesis of catecholamines was the last enzyme in this series to be identified (Nagatsu, 1964). TH has a specific requirement for atmospheric oxygen and an unconjugated pterin as cofactors (Nagatsu et al, 1964(a)). Tetrahydrobiopterin (6, 7 - dihydroxypropyltetrahydropterin) is considered to be the natural cofactor for bovine adrenal tyrosine hydroxylase (Lloyd and Weiner 1971).

The stoichiometry of the TH-catalyzed reaction is formulated in the following reaction (Nagatsu et al 1964(a) (b); Shiman et al 1971).

$$\text{L-Tyrosine} + BH_4 + O_2 \quad \text{dopa} + QH_2B + H_2O.$$

Where BH_4 is tetrahydrobiopterin and QH_2B is the quinonoid form of the dihydrobiopterin. TH is found in all tissues that synthesis catecholamines: adrenal glands, sympathetic nerves and brain. The brain enzymes are concentrated in brain regions which are known to contain high concentrations of catecholamines (Table 1.1)

Table 1.1

Regional Distribution of Tyrosine Hydroxylase in Rat Brain

	Enyzme activity (nmol/g/h)	
	Musacchio et al (1969)	Coyle (1971)
Whole brain	59	77
Cortex	40	14
Hypothalamus	102	125
Mesencephalon-pons-medula	43	-
Medulla-pons	-	21
Midbrain-thalamus	-	27
Caudate nuclei	496	520

From Cooper et al, (1982)

Work on the subcellular distribution of TH has shown the enzyme to be soluble in the adrenal gland (Musacchio 1967, 1968; Wurzburger and Musacchio 1971; Weiner et al 1971). Stjarne and Lishajkg (1967) have demonstrated that TH in sympathetic nerves is exclusively localized in the high-speed supernatant fraction.

A considerable fraction of rat brain TH (60%) is contained in the synaptosomal fraction, but when the synaptosomes are lysed 82% of the enzyme is found in the soluble fraction (Coyle 1972).

Kuczenski and Mandell (1972) studied the subcellular distribution of TH in the rat brain and striatum. The mid brain enzyme is localized mainly in the soluble fraction, while the striatal TH is found mainly in the synaptosomal fraction. This difference in the distribution is expected because the mid brain contains relatively more cell bodies than nerve endings, and the striatum contains dopaminergic nerve endings and no dopaminergic cell bodies. Between 60% and 70% of the enzyme contained in the synaptosomes can be solubilized by hypotonic shock in 2mM phosphate buffer, while the rest remains bound, presumably to synaptosmal membranes (Kuczenski and Mandell 1971). This fraction of TH which remains absorbed to synaptosomal membranes cannot be solubilized by repeated washings. In this respect, the rat striatal enzyme is similar to the aggregated bovine enzyme obtained from the high-speed adrenal gland supernatant and to the adrenal enzyme which absorbs onto subcellular particles.

The km for the enzymatic conversion of tyrosine to DOPA by purified adrenal tyrosine hydroxylase is about 2×10^{-5}M, and in a preparation of brain synaptosomes about 0.4×10^{-5}M. From this it appears that TH is the rate limiting step in the synthesis of noradrenaline in the perepheral nervous system and is likely to the rate-limiting step in the formation of dopamine and noradrenaline in the brain as well.

Dihydroxyphenylalanine Decarboxylase

Dopa decarboxylase (DDC, E.C.7.1.1.28), the second enzyme involved in catecholamine biosynthesis, catalyzes the decarboxylation of several aromatic amino acids and it is widely distributed in several tissues, such as kidney, liver, lung, heart, adrenal medulla and sympathetic nerves in the central nervous system. It was the first catecholamine-synthesizing enzyme described (Holtz et al, 1938) and it is known to be a soluble enzyme (Blaschko et al, 1955). When the brain is homogenised by methods designed to obtain synaptosomes, a large percentage of the enzyme is contained within structures (Udenfriend 1966).

Several aromatic amino acids may serve as substrates for DDC, as indicated by the frequently used name of "aromatic-L-amino acid decarboxylase". However the Vmax for dopa is about tenfold faster than the one for 5-hydroxytryptophan; in decreasing order, lower rates of decarboxylation are shown by phenylalanine, tryptophan and tyrosine (Christensen et al, 1970). The enzyme requires pyridoxal phosphate (Vitamine B_6) as a cofactor and has a km value of 4×10^{-4}M. This high activity might explain why it is difficult to detect endogenous dopa in sympathetically innervated tissue and brain.

Dopamine-B-Hydroxylase

Dopamine-B-hydroxylase (DBH, dopamine -B- monoxygenase. E C.1.14.17.1) is a monooxygenase which catalyzes the final step in noradrenaline biosynthesis. It is a copper-containing protein in which the valence of copper undergoes cyclic changes (Frirdman and Ksufman, 1965). The hydroxylation of dopamine is coupled to a stoichiometrically equivalent oxidation of ascorbate (Levin et al, 1960) according to the following reaction.

Dopamine + ascorbate + 0_2

l-noradrenaline + dehydroscorbate + H_20

The enzyme activity has been found in adrenal gland slices (Hagen, 1956) and homogenates (Goodall and Kirshner, 1957) in the brain (Udenfriend and Creveling, 1959) and in all sympathetically innervated organs. In all organs, DBH is found in the catecholamine containing granules (Kirshner 1957, Livett et al, 1969).

DBH has been found to be a specific and sensitive immunochemical marker for noradrenergic neurones in the central nervous system and in the peripheral sympathetic system (Geffen et al, 1969, Fuxe et al, 1971 (a), (b) Hartman, 1973). In the central nervous system, the enzyme could be localized in neurons which are known to contain noredrenaline such as the cell bodies of the locus coeruleus (Fuxe et al, 1971 (a)). The specificity of the method is demonstrated by the fact that no fluoresence is seen in the cell bodies of the substantia nigra or the mid brain raphe, which are known to be dopaminergic and serotoninergic respectively.

The natural substrate of DBH is dopamine, but the enzyme can catalize the hydroxylation of several amine structures related to dopamine. The km for tyramine ($4x10^{-4}M$) is about tenfold lower than the km for dopamine which is $6 \times 10^{-3}M$ (Creveling, 1962). The following are some of the amines which are substrates for DBH. N-methyl-p-tyramine, methyl-p-tyramine, methyl-m-tyramine, p-hydroxy-N-methylamphetamine, N-methylamphetamine, N-methyldopamine, methyldopamine and mescaline. It is noteworthy that (+)-amphetamine but not (-)amphetamine is a substrate for DBH (Goldstein et al 1964, (a)).

Phenylethanolamine-N-Methytransferase

Phenylethanolamine-N-methytransferase (PNMT, S-adenosyl-methionine: phenylethanolanine-Nmethyltransferase, EC. 2.1. 1.28) is the enzyme that converts noradrenaline to adrenaline. It was reported first by Kirshner and Goodall (1957) to be present in the soluble fraction of homogenates of adrenal medulla and it uses S-adenosylmethionine as the methyl donor, according to the following reaction (Axelrod, 1962 (a)).

Noradrenaline + S-adenosylmethionine

adrenaline + S-adenosylhomocysteine + H^+.

The enzyme is almost exclusively located in the adrenal medulla in mammals, but it has been reported in the heart and brain (Axelrod, 1962, McGeer and McGeer, 1964) and in sympathetic ganglia (Ciarenbello et al, 1973).

The cellular location of PNMT in the rat CNS has been demonstrated with the use of specific antibodies (Hökfelt et al, 1974). Two groups of reticular nerve cells bodies have been localized in the medulla oblongata. The PNMT-containing terminals are mainly found in certain nuclei of the lower brain stem, in the locus coeruleus, in certain nuclei of the hypothalamus and in the periventricular grey matter. It has been postulated that these neurones contain adrenaline and that adrenaline may be a neurotransmitter in the rat brain (Hökfelt et al, 1974).

PNMT catalyzes the N-methylation of a number of primary and secondary B-hydroxyphenylethylamine derivatives. The enzyme from humans (Axelrod and Vessel, 1970) will N-methylate only phenylethylamines with B-hydroxy group. The substrate specificity in decreasing order is the following, (-)-normetanephrine, (±) -phenyl-ethanolamine, (±) -m-hydroxyphenylethanolamine, (-) -noradrenaline (±) - octopamine, (+)-noradrenaline, (-) -norephedrine and (+) adrenaline (Axelrod, 1966). L-Noradrenaline is the most active substrate at low concentration and has the lowest Km indicating that this is the natural substrate (Fuller and Hunt, 1965).

Coupland (1953) suggested that the adrenal glucocorticoids were important for the transformation of noradrenaline into adrenaline. It is noteworthy that there is a portal venous system that drains high concentrations of cortical hormones into the medulla. It was demonstrated by Wurtmann and Axelrod (1966) that hypophysectomy markedly reduced PNMT activity in rat adrenal

glands, and that the enzyme activity could be restored to normal levels by administration of ACTH or high doses of glucocorticoids. The glucocorticoid-mediated increase in PNMT is blocked by inhibitors of protein synthesis. The administration of ACTH or glucocorticoid to normal rats does not produce any increase in adrenal PNMT.

THE METABOLISM OF CATECHOLAMINES

Unlike the synthesis of the catecholamines, which has been well documented and forms a linear stream from tyrosine to adrenaline, the metabolism of the catecholamines involves several enzymes that can act at different stages through the matabolite formation, i.e. they may initiate metabolism or act as the second or third enzyme in a chain of reactions to form one or more metabolites. The formation of metabolites is further complicated through the compound of origin that they are formed from. Metabolites of biochemical interest can be formed from dopamine by the same enzymes that will metabolise both noradenaline and adrenaline. A further complicating factor in the formation of matabolites from noradrenaline and adrenaline is that the same metabolites can be formed from both of these compounds. It must also be remembered that noradrenaline and adrenaline may themself be considered a metabolite of dopamine and adrenaline a metabolite of noradrenaline.

ENZYMES INVOLVED IN CATABOLISM OF CATECHOLAMINES

Two types of enzyme compete for the initial catabolism of the catecholamines. These enzymes are monoamine oxidase (MAO; amine : Oxygenoxidereductase [deaminating] EC. 1.4.3.4.) and catechol-O-methytransferase (COMT; 5-adenosyl -L-methionine : catechol-O-methytransferase, EC 2.1.1.6). The metabolism of an amine by one enzyme does not preclude its further metabolism by the other enzyme. Thus deaminated catecholamines are good substrates for COMT (Guldberg and Marsden, 1975) and O-methylated catecholamines are good substrates for MAO (Housley and Tipton, 1976).

COMT was first described and partially purified by Axelrod and Tomchick (1958) and finally purified from a liver by Assicot and Bohuon (1970). The COMT present in brain is immunologically similar to the enzyme ubiquitously distributed in various peripheral organs (Axelrod, 1971, Borchardt and Cheng, 1978), and little evidence exists for the presence of multiple forms of COMT in a particular species (Borchardt and Cheng, 1978). Initial work by Axelrod et al (1959) showed the highest COMT activity in the brain in the area postrema and lowest in the cerebellum. In the brain, most of the COMT activity is located in the cytoplasm through Borchardt and Cheng (1978); showed that rat brain does contain a significant amount of COMT that is recovered with the microsomal fraction. The cytoplasmic and extraneural location of COMT (Axelrod, 1971, Borchardt and Cheng, 1978) allows for O-methylation of released catecholamines both outside the neuron and after reuptake into the neuron terminal. The membrane-bound

enzyme activity has approximately a threefold lower km for noradrenaline than does the soluble form of the enzyme (Borchardt and Cheng, 1978). Whether the membrane-bound COMT is a physiologically important form of this enzyme has yet to be established. Partially purified COMT from the cytosol of human brain has been found to demonstrate a significantly lower km for dihydroxybenzoic acid compared with the km for dopamine (White and Wu, 1975) and this kinetic behaviour has led to speculation that, within neurons, the deamination of catecholamines by MAO, may normally precede metabolism by COMT. S-Adenosylmethionine (SAM) is an obligatory cosubstrate for the O-methylation of a large number of structurally different catechols by COMT. COMT exhibits a low km for SAM, but S-adenosylthomocysteine (SAH), which is one of the reaction products is a potent inhibitor or COMT activity (Flohe and Schwabe, 1972). Because approximately equal concentrations of SAM and SAH are present in most tissues (Salvatore et al, 1971), COMT activity would be strongly influenced by the ratio of SAM to SAH at any particular time.

Peripherally administered catecholamines are rapidly metabolised by COMT, primarily in the liver (Axelrod, 1971) but controversy still exists regarding the role played by brain COMT in the inactivation of the neuronally released catecholamines. The administration of pyrogallol, a competitive inhibitor of COMT does not increase brain catecholamine levels, but the administration of U-0521, another type of potent COMT inhibition was reported to increase brain catecholamine levels (Crout et al, 1961; Giles and Miller, 1967). Moreover levels of normetanephrine are increased

in brain after inhibition of MAO (Giles and Miller, 1967). Thus COMT activity does play a role in the intraneuronal catecholamine metabolism if the competing enzyme MAO is inhibited (Borchardt and Cheng, 1978). The inability to increase catecholamine levels with the use of certain COMT inhibitors in view may be a consequence of the competition between COMT and MAO for catecholamines within the neuron terminal and of differences between in vitro and in vivo accessibility of inhibitors to COMT located within the catecholamine neuron terminals.

O-Methylation of catecholamines may also occur in the extraneuronal compartments of the brain. Kaplan et al (1979), demonstrated by immunohistochemistry that venticular ependymal cells and the thoroid plexus contain the greatest amounts of COMT in the brain. Glial elements also demonstrated significant amounts of immunoreative COMT, but neurons were shown to contain little or no COMT activity. Although this work does not exclude the presence of small amounts of COMT in neurons, other evidence points to the extraneuronal compartments as the major sites of normetanephrine production and its further metabolism.

The O-methylated derivative of nonadrenaline is a poor substrate for "Uptake $_1$", which is the system responsible for neuronal noradrenaline reuptake (Iversen, 1973). Normetanephrine is, however, rapidly accumulated in brain slices by an extraneuronal uptake system (Hendley et al, 1970), which has characteristics similar to the "Uptake $_2$" system of Inversen (Inversen, 1973). MAO, as well as enzymes that further metabolise

the aldehydes produced from the catecholamines, are present in neuronal and extraneuronal (e.g., glial) compartments in brain (Duncan et al, 1972). Therefore, normetanephrine, whether formed in the neuron or taken up into extraneuronal sites, will be available for further metabolism by MAO and aldehyde dehydrogenases and reductases.

MAO catalyzes the oxidative deamination of catecholamines, serotonin and several other monoamines according to the following equation.

$$R\text{-}CH_2\ NH_2 + O_2 + H_2O \qquad R\text{-}CHO + H_2\ O_2 + NH_3$$

MAO is located on the outer membrane of mitochondria (Tipton, 1967), and Johnson (1968) showed the existance of two forms of the enzyme, which he called Types A and B. These forms are distinguished primarily by their substrate specificity and relative sensitivity to the MAO inhibitors clorgyline and deprenyl. Noradrenaline and normetanephrine are oxidativity deaminated primarily by Type A MAO (Houslay and Tipton 1974) and it is this form of the enzyme that is selectively inhibited by clorgyline in vitro. Dopamine is deaminated by both types of MAO, while type B MAO is inhibitor by deprenyl (i.e. selegiline). The two MAO enzyme forms may, however, not represent distinct proteins, but may represent a single enzyme within different lipid environments in the mitochondrial outer membrane. Houslay and Tipton (1974) have demonstrated that the substrate preferences and inhibitor characteristics of electrophoretically separable form of MAO (Collins et al, 1970) can be eliminated by treating the membrane

bound enzyme with chaotropic agents which disrupt protein-lipid aggregates.

Evidence has been presented to suggest that Types A and B MAO are located within different mitochondrial subtypes (Kroon and Vullstra, 1972; Owen et al, 1977) and that various parts of human brain may have different proportions of Type A and B MAO activity (Owen et al, 1979). The relative distribution of Type A MAO, estimated by in vitro assays, roughly correlates with the anatomical distribution of noradrenaline. Areas containing a high concentration of nonadrenaline, such as the hypothalamus and hippocampus, have been shown to contain high amounts of Type A MAO activity compared with other brain areas, but the human hippocampus also contains a high amount of Type B MAO activity, which preferentially deaminates amines such as phenylethylamine and possibly dopamine (Glover et al, 1977; Owen et al, 1979). Although noradrenaline is one of the preferred substrates for Type A MAO, it is at present premature to conclude that noradrenaline is deaminated in vitro or vivo by only Type A MAO and this the functional significance of the various types of MAO in vivo has yet to be fully clarified. It is, however, clear that normetanephrine and metanephrine are converted by the mitochondrial MAO to a single product i.e. the aldehyde derivative of these amines, 3-methoxy-4-hydroxyphenylglycolaldhyde. The action of MAO and COMT in catecholamines is to produce an aldehyde intermediate product. This unstable intermediate can be reduced or oxidised depending on enzyme availability. Therefore the deamination of noradrenaline, adrenaline or normetanephrine by MAO plays little role in

determining whether the final product of the metabolism of a biogenic amine will be an acid or alcohol (eg. MHPG or VMA). Dopamine metabolism differs slightly as the major metabolites of interest are both acid. (Reason for this will be explained latter).

The finding by Eccleston et al, (1966); and Feldstein and Williamson (1968) that NADPH, rather than NADH was the preferred cofactor for the reduction of "biogenic aldehydes" to their alcohol derivatives by brain tissue casted doubt on the earlier assumption that the formation of alhohol derivatives (e.g MHPG) of the biogenic amines was catalysed by an alcohol dehydrogenase similar to that found in the liver. NADH is the preferred cofactor for alcohol dehydrogenase from the liver, and pyrazole is a potent inhibitor of alcohol dehydrogenase. Anderson et al (1976) showed that the inclusion of pyrazole into reaction mixtures containing rat brain homogenate did not affect the production of MHPG by these homogenates. Furthermore, although human liver alcohol dehydrogenase will metabolize the aldehyde derivative of noradrenaline (Wermuth and Munch, 1979), the alcohol dehydrogenase derived from the liver of other species (eg horse) does not seem to catalyze readily the reduction of 3, -4-dihydroxyphenylglycol aldehyde (Duncan, 1975). Thus the small amount of alcohol dehydrogenase present in brain (Ranskin and Sokoloff, 1972) does not play a major role in production of MHPG, although it may be of possible importance in an as yet - undetermined biological function.

Tabakoff and Erwin (1970) have isolated a NADPH dependant enzyme from bovine brain that reduces a wide variety of aromatic and aliphatic aldehydes to their alcohol derivatives. The hypothalamus and brain stem area are the areas containing the highest amount of this activity and the aldehyde derivatives of catecholamines and indolamines were found to be some of the substrates metabolised by the enzymes (Tabakoff et al, 1973). Further studies into the nature of this enzyme have shown that its action is inhibited by barbiturates (Erwin et al, 1971) but was not inhibited by pyrazole (Tabakoff and Erwin, 1970). In the course of these studies in bovine brain, a second enzyme that metabolised aldehydes to alcohols, using either NADH or NADPH as a cofactor was recorded. This enzyme activity is separable from the NADPH - dependant enzyme and was partially purified and characterised by Erwin et al (1972). Studies by other workers in pig brain (Turner and Tipton, 1972) and in rat brain (Ris and Von Warburg, 1973) also showed the presence of two enzyme forms with the ability to reduce biogenic aldehydes. These brain enzymes have been classified as alcohol - NAD(P) oxidoreductase (EC. 1.1.1.2) (aldehyde reductases) Tabakoff and Erwin, 1970). Turner and Tipton (1972), after establishing the one form of aldehyde reductase exhibited much lower Michaelis constants (Km valves) for various aldehydes than the second form, designated the two enzyme forms as the "low Km" and "high Km" enzymes. The two enzymes forms isolated from rat brain by Ris and Von Wartburgh, 1973) and labeled as enzyme 4.1 and 4.2 have, strong similarities with the high Km and low Km forms isolated pig brain by Turner and Tiptor (1972). The brain "aldose

reductase" isolated by Dons and Doughty (1976) also have many properties in common with the previously mentioned enzymes and may be equivalent to the aldehyde reductase. From now on the "NADPH-dependant aldehyde reductase", the high Km enzyme and rat brain enzyme form 4.1, will be referred to as "Form 1", as these forms of aldehyde reductase seem to be equivalent. "Form 2" shall be used in reference to the low Km enzyme and the rat brain enzyme form 4.2 due their many similarities (e.g. their ability to use either NADH or NADPH as a cofactor). However it should be noted that Ris and Von Wartburg (1973) showed that human brain has four separable forms of aldehyde reductase. One form, labeled AR-3 by Ris and Von Wartburg (1973), closely resembles Form 1 derived from other mammalian species, whereas a second form of the human brain reductase, AR-2, has certain properties similar with Form 2 found in rat and pig brain. The remaining two forms of human brain aldehyde reductases correspond to an aromatic before reductase found in various tissues of mammals (Sawada and Hara, 1978) and a reductase of narrow substrate specificity, which reduces succinic semialdehyde to hydroxybutyrate (Anderson et, 1977; Hoffman et al, 1980).

Form 1 and Form 2 aldehyde reductases are those primarily responsible for reduction of aldehydes derived from noradrenaline, but Forms 1 and 2 aldehyde reductases do differ in substrate and cofactor specificities and sensitivity to various inhibitors. Form 1 enzyme isolated from bovine, rat and pig brain and the AR-3 form (Form 1) of human brain reductase are relatively specific in their use of NADPH as a cofactor. Form 2 brain aldehyde reductase can

utilize NADH as well as NADPH. Both forms of aldehyde reductase possess a broad spectrum of specificity, which include aromatic and aliphatic aldehydes (Tabakoff and Erwin, 1970; Turner and Tipton 1972; Ris and Von Wartburg, 1973). The enzymes have been shown to catalyze the reduction of a number of aldehyde derivatives of the biogenic animes, and the aldehydes with a hydroxy group are preferred substrates (Tabakoff et al, 1973).

Investigations into the subcellular distribution of Form 1 and Form 2 aldehyde reductase in rat brain showed that Form 1 is found primarily in the cytosol, whereas Form 2 is found in the mitochondrial fraction (Anderson et al, 1976). Using selective inhibitors, Anderson et al (1976) demonstrated that, in rat brain Form 2 aldehyde reductase was responsible for the production of MHPG from the aldehyde derived from normetanephrine. From this one may assume that both the production of biogenic aldehydes by MAO and their subsequent metabolism to their alcohol derivatives will take place on or within the mitochondrion. When considering the differences between CNS and the peripheral metabolism of noradrenaline, the tissue distribution of Form 1 and Form 2 aldehyde reductase becomes an important factor. In several studies in the livers of humans (Wermuth et al, 1977) and other animals (Feldsted et al, 1977; Tulsiani and Touster, 1977) no evidence was found of Form 2 in the liver, while Form 1 was clearly shown to be there. Under normal physiological conditions, there is a high NAD.NADH ratio in the liver (Schulman et al, 1974) which prevents the reduction of various aldehydes present in low concentrations in the organ. The absence of Form 2 from the liver and the

presence of aldehyde dehydrogenase in this tissue which also compete for aldehyde substrates are important features in determining the pathway of biogenic aldehyde metabolism in the periphery.

The oxidation of aldehydes in the brain and other tissues primarily depends on the activity of NAD-dependent aldehyde dehydroxgenases (aldehyde : NAD oxidoreductases EC1.2.1.3) Deitrich, 1966; Racher, 1949). The aldehyde dehydrogenases that catalize the oxidation of biogenic aldehydes to the acid excretion products possess a broad substrate specificity and occur in various tissues in multiple molecular forms, which are selectively distributed in the cytosol or mitochondria (Siew et al, 1976; Tottmar and Marchner, 1976). Two forms of aldehyde dehydrogenase may be present in mitochondria derived from liver (Siew et al, 1978). The enzyme form found in the mitochondrial matrix in the rat exhibits a particularly low km value for a variety of aldehydes and is quite sensitive to inhibition by disulfiram and cyanamide (Deitrich et al, 1976; Siew et al, 1976). Although there is growing evidence that liver and brain aldehyde dehydrogenases are not identical (Deitrich et al, 1976) and also brain mitochondria also contains an aldehyde dehydrogenase that has a high affinity for a variety of aldehyde substrates (Erwin and Deitrich, 1966; Duncan et al 1971). Brain aldehyde dehydrogenase, however, displays a substantially higher km for hydroxy-substituted aldehydes compaired with aldehydes lacking the hydroxy substitution (Duncan and Sourkes, 1974). From this fact the aldehyde derivative of dopamine is therefore a significantly better substrate for brain mitochondrial aldehyde dehydrogenase than the

aldehyde derivative of normetanephrine (Duncan and Sourkes, 1974). As previously stated, Form 2 aldehyde reductase, which is also located in the brain mitochondria, exhibits a lower km and a substantially higher maximum velocity with the hydroxy-substituted aldehydes derived from normetanephrine, as compared with the aldehyde derivative of dopamine. The ability of Form 2 aldehyde reductase to compete effectively with brain aldehyde dehydrogenases for the aldehyde derivative normetanephrine may be the determining feature why noradrenaline is primary metabolised to its alcohol derivative in brain.

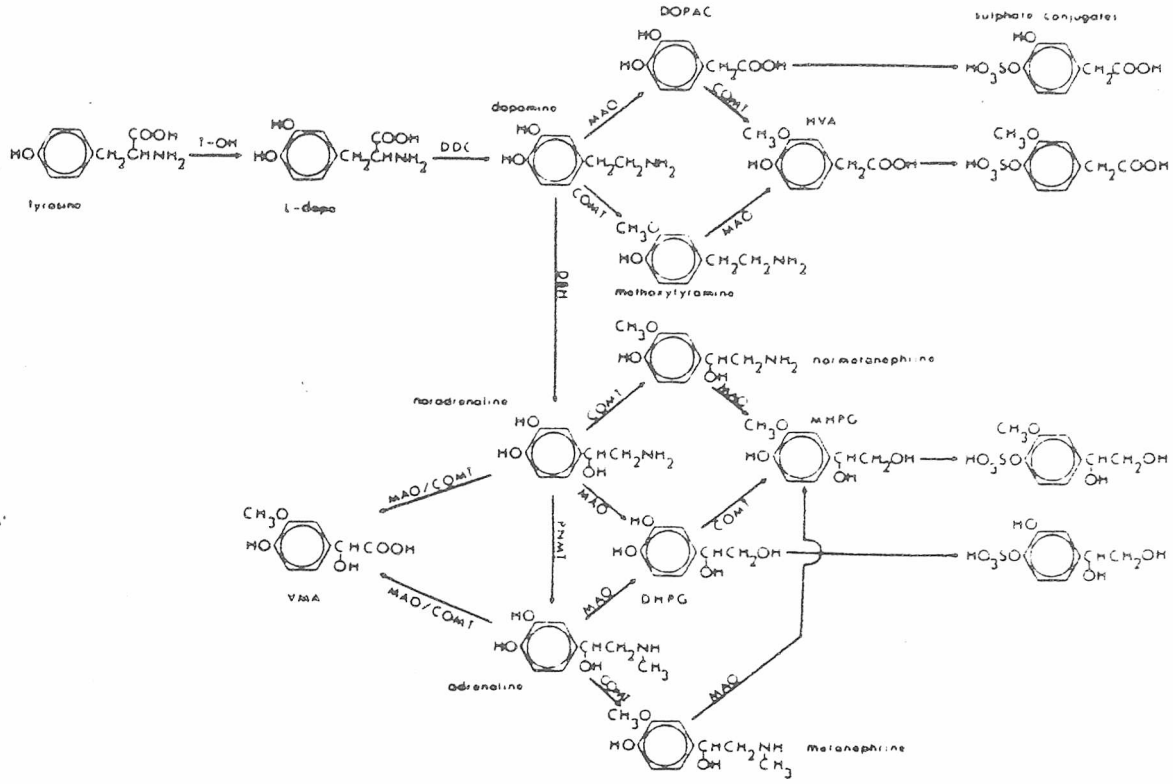
Aldehyde dehydrogenase activity is found in all brain areas, but a greater amount of enzyme activity has been noted in areas such as the striatum (Erwin and Deitrich, 1966). This anatomical distribution of brain aldehyde dehydrogenase may be responsible for the greater amounts of VMA found in the rat striatum, as compared to other brain areas (Karoum et al, 1976) even though the actual source of brain VMA remains an enigma (Ader et al, 1978). VMA is distributed in brain in a manner that does not correspond to the distribution of noradrenaline (Karoum et al, 1976) and stimulation or destruction of nonadrenaline neurons in the brain does not produce changes in brain VMA levels (Ader et al, 1978).

The final stage in the metabolism of the catecholamines is in the formation of sulphate or glucuronate conjugates of the acids and the alcohol. The extent of conjugate formation varies widely from species to species, from component to component and the type

of conjugation taking place varies in the peripheral and central nervous system. Little if any MHPG has been found conjugated with glucuronide in the brains of several animal species (Karoum et al, 1977, Maas et al, 1976) and therefore, glucuronide conjugation is most probably a feature of peripheral MHPG metabolism. In rat brain nearly 100% of the MHPG found is in the sulphate ester form (Karoum et al, 1976, Schanberg et al, 1968) while in human brain less than 25% of the MPHG is found in the sulphate ester form. The enzyme responsible for sulphate conjugation is phenol sulfo-transferase (PST; 3-phosphoadenylsulphatophosphate : phenol sulfotransferase EC.2.8.2.1). This enzyme which uses adenosine 3-phosphate-5 -solfophosphate as a sulphate donor (Pennings et al, 1977) is located in the cytosol of cells (Jansen et al, 1974) and is unevenly distributed in various parts of the brain, with the hypothalamus having a substantially greater amount of enzyme than other parts of brain (Foldes and Meek, 1974). Denervation studies indicate that PST is not localized primarily in noradrenergic neurons. There is, however, good correlation between the absolute levels of PST present in brain of a particular animal species and the proportion of MHPG appearing in the free or sulphate-conjugated form in brain. Thus human brain has substantially less enzyme activity per brain than rat brain (Foldes and Meek, 1974), and as previously mentioned, the major proportion of MHPG in human brain appears in the free form (Karoun et al, 1977). The formation of the sulphate conjugate of MHPG in brain allows for the active transport out of the CNS by the probenecid-sensitive organic acid transport system (Meek and Neff, 1972), whereas free MHPG is probably removed by passive diffusion into the plasma. Brain

levels of free MHPG are little affected by pretreatment of animals with probenecid. The MHPG appearing in the plasma can be sulfate-conjugated by PST that is present in organs other than brain (Foldes and Meek, 1974), or it may be converted to the glucuronide conjugate.

Figure 1.4 gives a detailed account of the metabolism of the catecholamines by the enzymes previously discussed.



Abbreviations

Enzymes

T-OH	Tyrosine hydroxylase
DDC	DOPA-decarboxylase
DBH	Dopamine-β-hydroxylase
PNMT	Phenylethanolamine-N-methyl transferase
MAO	Monoamine oxidase (Where the final product is an acid the second enzyme responsible for the transformation is aldehyde dehydrogenase; where it is an alcohol, the second enzyme is aldehyde reductase).
COMT	Catechol-O-methyl transferase

Substances

L-DOPA	L-3,4-dihydroxyphenylalanine
DOPAC	3,4-dihydroxyphenylacetic acid
DHPG	1-(3,4-dihydroxyphenyl)ethan-1,2-diol
HVA	4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid)
MHPG	1-(4-hydroxy-3-methoxyphenyl)ethan-1,2-diol
VMA	(4-hydroxy-3-methoxyphenyl)glycollic acid (vanillylmandelic acid)

5-HYDROXYTRYPTAMINE

General Aspects

Since the mid-nineteenth century, scientists have been aware that a substance found in the serum can cause a powerful contraction of smooth muscle organs. It was over one hundred years before a group of American scientists succeeded in isolating this substance and showed it as a possible cause of high blood pressure. At the same time workers in Italy were characterizing a substance found in high concentrations in chromaffin cells of the intestinal mucosa. They showed that this material could also constrict smooth muscle elements, especially those of the gut. The material isolated from the bloodstream was named "serotonin" while that from the intestinal tract was called "enteramine". Subsequently, both materials were purified, crystallized, and identified as 5-hydroxytryptamine (5HT).

Biosynthesis of 5HT

5HT is found in many cells that are not neurones, such as platelets, mast cells, and enterochromaffin cell. In total the brain contains only 1-2% of the 5HT found in the body. 5HT cannot cross the blood brain barrier, therefore it is clear that brain cells must synthesize their own. The biosynthesis of 5HT in the brain occurs in two enzymatic steps with the amino acid L-tryptophan being the primary substrate for synthesis. Plasma tryptophan arises primarily from the diet and an active uptake

process is known to facilitate the entry of tryptophan into cells (Cooper et al, 1983). Because plasma tryptophan has a daily rhythmic variation in its concentration, it seems likely that this concentration variation could profoundly influence the rate and synthesis of brain 5HT.

Tryptophan Hydroxylase

Tryptophan hydroxylase is the first enzyme in the synthesis of 5HT and it converts L-tryptophan to L-5-hydroxytryptophan (5HTP). The distribution of tryptophan hydroxylase in the brain has been studied in detail in various animals; the dog (Grahame-Smith, 1967) the guinea pig (Ichiyama et al, 1968), the cat (Peters et al, 1968) and the rat (Deguchi and Barchas, 1972; Renson, 1973). In general, the brain stem, hypothalamus, caudate nucleus and the amygdala have a relatively higher activity than that of the brain.

Grahame-Smith (1967) showed that when nerve ending synaptosomes are formed, between 40% and 75% of the brain hydroxylase activity would be recovered in the crude mitochondrial fraction and that the enzyme does not rely on added exogenous cofactors for activity. Rupture of the synaptosomes leads to loss of activity, which can be restored by adding the cofactor pterine. The regulation of substrate concentration, through the membrane active transport system (Grahame-Smith and Parfait, 1970), within synaptosomes and the endogenous availability of the natural cofactor make any true assessment of the relative properties of

the 'soluble' and 'particulate' enzyme activity in brain tissue extremely difficult. The fact that hypotonic shock can release all the enzyme activity from the crude mitochondrial or synaptosomal fractions suggests that in brain tissue the enzyme is soluble (Lovenberg et al, 1968). It therefore suggests that the enzyme is cytoplasmic.

Tryptophan hydroxylase is known to be a mixed-function oxidase with many essential properties similar to those of phenylalanine-4-hydroxylase, (Kaufman, 1971). The enzyme has 3 substrates molecular oxygen, a reduced pterine cofactor (perhaps tetrahydrobiopterin, but not positively identified in brain) and l-tryptophan. The situation regarding the pterine cofactor is complicated. Some workers have used 2-amino-4-hydroxy-6, 7-dimethyl 5, 6, 7, 8-tetrahydropteridine ($DMPH_4$) while others have used tetrahydrobiopterin as the cofactor. Both Jequier et al, (1968) in rat brain and Freedman et al (1972) in rabbit brain found that the km for L-tryptophan with $DMPH_4$ was about 300um, and with tetrahydrobiopterin about 50um. These differences in km values have a great relevance to the possibility that the availability of tryptophan is a factor in controlling the rate of synthesis of brain 5HT.

There is little doubt that tryptophan hydroxylase is the rate-limiting step in 5HT synthesis in the brain. Renson (1973) showed that in the interpeduncular nucleus the rate of tryptophan

hydroxylation is about 1.6 ug /g/h while Grahame-Smith (1967) showed that the rate of decarboxylation of 5HTP in the hypothalamus is 117 ug/g/h.

Renson (1973) also suggests that 5HT has no inhibitive feedback mechanism on tryptophan hydroxylase and that increased levels of tryptophan increase 5HT biosynthesis by just providing more substrate rather than directly affecting enzyme activity.

5-Hydroxytryptophan Decarboxylase

The formation of dopamine from L-dopa and 5HT from 5HTP requires decarboxylation. Early work on this enzyme was carried out by Clark et al (1954) in the guinea-pig kidney and at that time it was considered that there were two different enzymes, 5-hydroxytryptophan decarboxylase (5-HTP-DC, E.C.4.1.1.28) and dopa decarboxylase (dopa-DC, E.C.1.1.26). Latter through improved purification techniques and comparisons between the ratio of activities of dopa-DC and 5-HTP-DC in various brain regions (which proved constant (Kuntzman et al, 1961)), it was concluded that there was only one enzyme rather than two. The enzyme was therefore renamed, "aromatic-L-amino acid decarboxylase". Sims and Bloom (1971) again raised the possibility that there may infact be two separate enzymes involved in the decarboxylation which has lead to such speculation between groups of workers.

In 1973, Sims and Bloom showed that following intracisternal 6-hydroxydopamine and intraperitoneal pargyline treatment there are marked decreases in dopa-DC activity in the rat brain stem areas

and the cortical areas. This agrees well with previous studies (Bloom et al, 1967, Uretsky and Iversen, 1970) although the time course of the enzyme activity decrease did not coincide with the ultrastructural damage and catecholamine depletion. 5-HTP-DC activity determined in the same regions did not decrease, thus suggesting two decarboxylase activities in the rat brain catalyzed by distinct proteins.

Christenson et al (1972) using an immunological approach, titrated dopa-DC and 5-HTP-DC with antiserum to hog kidney decarboxylase and showed that the activity of both enzymes was neutralized proportionately. Concluding that, unless the two enzymes were so structurally closely related, they were identical by immunological criteria, thus providing further evidence for the single-enzyme hypothesis.

Sims et al (1973) in turn suggested that the immunological data were indeed not specific enough for the two enzyme hypothesis. They also presented further evidence showing difference optimum conditions which the enzymes worked under, eg, changes in pH and temperature. Other work suggests that dopa-DC is easily denatured, the two enzymes are stiumlated differently by cofactors and exhibit different solubilities. Finally they could not confirm the earlier findings of parallel distribution in brain of the two enzymes (Kuntzman et al, 1961), instead finding a 2:1 ratio of dopa-DC to 5-HTP-DC in the cerebellum and in 8:1 ratio in the corpus striatum. Bender and Coulson (1972) also noted the difference in pH optima and in an attempt to reconcile the

immunological data of Christenson et al (1972) and the work of Sims and Bloom (1971) they have suggested the enzyme has a complex active site with separate binding sites for the two substrates ajacent to a single catalytic site. Clearly further investigations into the nature of the decarboxylation enzyme are in order. Figure 1.5 outlines the synthesis of 5HT.

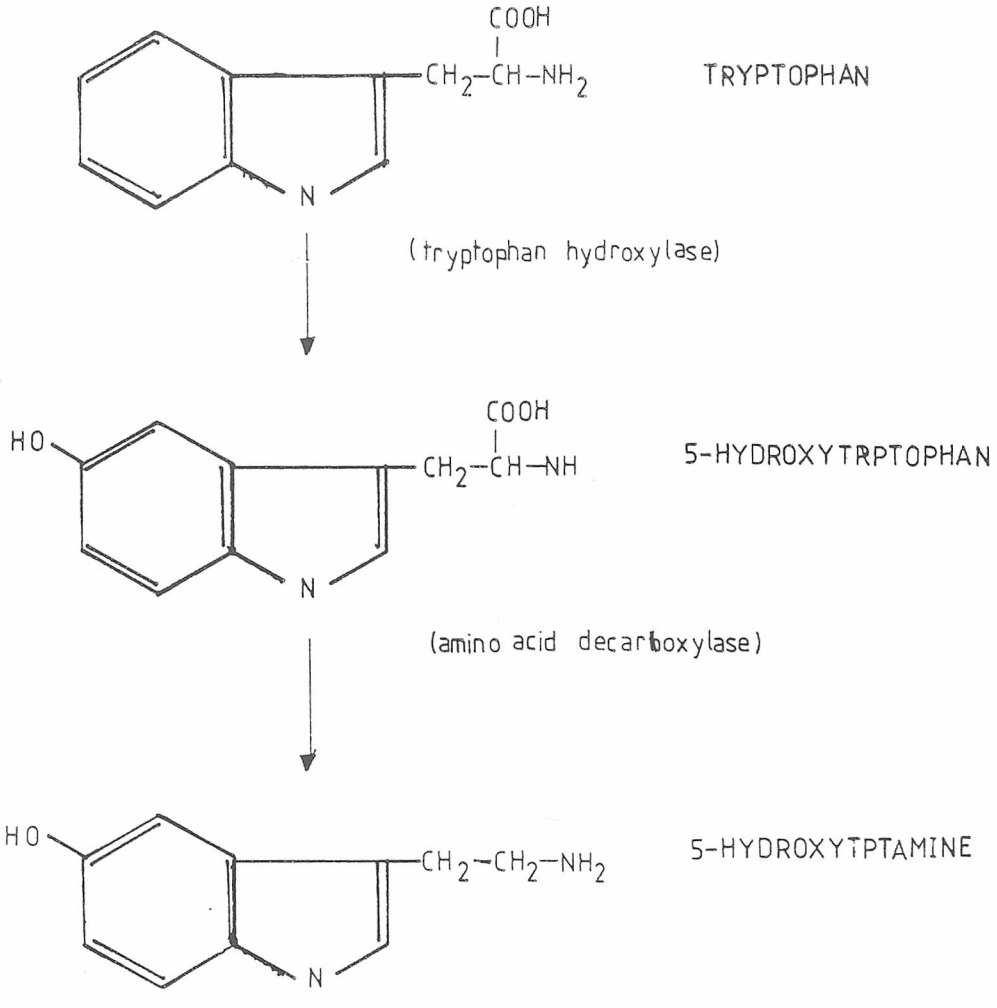


Figure 1.5 The synthesis of 5HT

METABOLISM OF 5HT

The first step in the degradation of 5HT is its oxidative deamination by monoamine oxidase (E.C. 14.3.4) to 5-hydroxyindole-acetaldehyde. Monoamine oxidase has a wide spread substrate specificity, being able to deaminate many amines with the formula $R-CH_2-NH_2$. A detailed description of the distribution and activity of the enzyme has been given earlier, during the discussion on catecholamine metabolism.

5-Hydroxyindole acetaldehyde may be metabolized in two ways. It can be either dehydrogenated to the acid 5-hydroxyindole acetic acid (5HIAA) or reduced to the alcohol 5-hydroxytryptophol (5HTOH). Both of these compounds are found in the liver, and ethanol injection will shift the metabolism of the acetaldehyde so that 5HTOH is produced rather than 5HIAA (Feldstein et al, 1967, Tyce et al, 1968). Eccleston et al (1966) demonstrated that both rat and human brains are capable of forming the alcohol. In examining the cofactor requirements of the enzymes it is noted that the aldehyde reductase requires $NADPH_2$ and the dehydrogenase requires NAD or NADP, although the latter is preferred. In the presence of excess NADP, 5HTOH is converted back to the aldehyde, wherease 5HIAA formation from the aldehyde is irreversible Eccleston et al (1969) attempted to shift the metabolism of the aldehyde to the alcohol in the brain but were unsuccessful. They remarked that although they had shown the brains ability to form

5HTOH, they doubted whether this occurred physiologically. Tyce et al (1968) came to a similar conclusion working with [^{14}C] -5-HT in the caudate nucleus.

The possibility that 5HIAA undergoes other degradative pathways has also been examined. Splitting of the side chain, formation of unidentified compounds, and binding to protein will occur (Keglevic et al, 1968) but quantitatively these pathways are very minor and little work has been done on them. 5HTOH cannot normally be detected in brain owing to its instability, but its presence can be demonstrated by the use of trapping agents (Udenfriend et al, 1956, Weissbach et al, 1957).

The formation of 5-HT-O sulfate, undoubtedly takes place as it is found in human urine (Davies et al, 1967) but is importance in inactivating 5-HT in the brain is uncertain. The existance of an enzyme system 5-HT-sulfotransferase using the sulfate-donating properties of 3'-phosphoadenosine-5'-phosphosulfate (PAPS) has been demonstrated in various organs (Hidaka et al, 1969,a) and in brain (Hidaka et al 1969,b).

Hidaka et al (1969b) observed that rabbit brain has a relatively high activity, with lower activity found in the dog, with even less in the rat. Korf and Sebens (1970), after hydrolysis of rat brains extracts could not detect 5-HT-O-SO_3 either in normal brains or after 3 days of treatment with pargyline. Hidaka and Austin (1972) accounted for this by

pointing out that the rat brain has low sulfotransferase activity. This however is not an entirely satisfactory explanation, for it does not explain the absence of 5-HT-O-SO_3 after MAO inhibition with pargyline. Gal (1972) has given more convincing reasons for Korf and Seben's failure to detect 5-HT-O-SO_3. He demonstrated that 5-HT-O-SO_3 labelled with ^{35}S is rapidly eliminated when administered intracerebrally and pargyline has no effect on the retention. Gal (1972) attempted to find 5-HT-O-SO_3 in rat brains through several radio isotope methods and he found that about 8% of 5HT is conjugated 2 hours after MAO inhibition. If this figure is accurate Neff and Tozer (1968) postulated that one might expect to see differences in turnover rates measured by MAO inhibition compared to those measured by accumulation of 5HIAA following probenecid. Since no differences were observed the importance of the O-sulfate conjugation pathway for 5HT remains to be clarified.

Distribution of the Biogenic Amines within the CNS

Detailed analysis of the biogenic pathways within the CNS has been made possible with the development of such techniques as fluorescent histochemistry, immunocytochemical localization of catecholamine synthesizing enzymes and direct methods for observing noradrenergic terminals with the electron microscope.

Noradrenergic Systems

There are two major clusters of noradrenaline cell bodies in the CNS from which axonal systems arise to innovate areas throughout the entire neuraxis.

Locus ceruleus : This is a compact group of cells found within the caudal pontine central grey and is named after the pigment found in the cells. The axons of these neurons form five major noradrenergic tracts (Figure 1.6); the central tegmental tract (or dorsal bundle), a central gray dorsal longitudinal fasciculus tract, and a ventral tegmental-medial forebrain bundle tract. These three ascending tracts follow major vascular and fascicular routes to innervate all cortices, specific thalamic and hypothalamic nuclei and the olfactory bulb. The fourth major tract ascends in the superior cerebellar peduncle to innervate the cerebellar cortex and the fifth major tract descends into the mesencephalon and spinal cord, where the fibres course in the ventral-lateral column. (Cooper et al, 1983).

The Lateral Tegmental Noradrenergic Neurons : A large number of noradrenergic neurons lie outside of the locus coeruleus, where they are more loosely scattered throughout the lateral tegmental fields. In general, these fibres intermingle with those arising from the locus coeruleus, with the more posterior tegmental levels as descending fiberes within the mesencephalon and spinal cord, and those from the more anterior tegmental levels which innervate the forebrain and diencephalon. The neurones of the lateral tegmental system may also be the primary source of the noradrenergic fibers observed in the basal forebrain, such as amygdala and septum.

Dopaminergic System

The central dopamine systems are considerably more complex in their organisation than the noradrenergic systems. Not only are there more dopamine containing cells, there are also several major dopamine-containing nuclei as well as specialized dopamine neurones that make extremely localized connections within the retina and olfactory bulb.

Based on recent progress in detailing the anatomy of the dopaminergic system (Figure 1.6), it is convenient to consider the system under three major categories according to the length of the different dopamine fibres.

1) Ultrashort Systems. Among the ultrashort systems are the interplexiform amacrine like neurons, which link inner and outer plexiform layers of the retina, and the periglomerular dopamine

cells of the olfactory bulb, which link together mitral cell dendrites in separate adjacent glomeruli.

2) Intermediate-Length Systems. These include (A) the tuberohypophysial dopamine cells, which project from arcuate and periventricular nuclei into the intermediate lobe of the pituitary and into the mediam eminence.

3) Long-Length Systems. These projections link the ventral tegmental and substantia nigra dopamine cells with principally three areas, the neostriatum (principally the caudate and putamen); the limbic cortex (medial prefrontal, cingulate and entorhinal areas); and other limbic structures (the regions of the septi, olfactory tubercle, nucleus accumbens septic, amygdaloid complex, and piriform cortex). These latter two groups have frequently been termed the mesocortical and mesolimbic dopamine projections, respectively.

Adrenergic System

Adrenergic neurones are the least abundant of the biogenic amines neurons found in the C.N.S. They are found mainly in two groups called C1 and C2 by Hokfelt et al (1978). The C1 neurons are intermingled with the noradrenergic cells of the lateral tegmental system and the C2 neurons are found in the regions which the noradrenergic cells of the dorsal medulla are found. The axons of these two systems ascend to the hypothalamus with the central tegmental track, then via the periventricular system into the hypothalamus. Within the mesencephalon, the adrenaline-containing

fibers innervate the nuclei of visceral efferent and afferent systems, expecially the dorsal motor nucleus of the vagus nerve. In addition, adrenaline fibers also innervate the locus coeruleus, the intermediolateral cell columns of the spinal cord, and the periventricular regions of the fourth ventricle. (Figure 1.6)

5-Hydroxytryptamine

When considering the distribution of 5-HT within the C.N.S. special attention should be awarded to the pineal organ, a small gland contained within the connective tissue extensions of the dorsal surface of the thalamus. While physically connected to the brain, the pineal organ is cytologically isolated for all intents, since, as one of the circumventricular organs, it is on the "peripheral" side of the blood permeability barriers and its innervation arises from the superior cervical sympathetic ganglion. The pineal gland is of interest because it contains fifty times as much 5HT (per gramme) as the whole brain, thus making it a useful site to examine the physiological function of 5HT.

Within the brain, 5HT neurones are known to be restricted to clusters of cells lying in or near the middle or raphe regions of the pons and upper brain stem (Figure 1.6). In addition to the nine 5HT nuclei (B_1-B_9), 5HT neurones have also been detected in the area postrema, the caudal locus coeruleus, and in and around the interpeduncular nucleus. The caudal groups have neuronal projections to the medulla and spinal cord. The rostral neurons (raphe dorsalis, raphe medianus, and centralis superior, or

B_7 - B_9) are thought to provide extensive innervation of the telencephalon and diencephalon. There is also on extensive innervation of the cerebral cortex, but unlike noradrenergic fibers, the patern of innervation seems random. Conclusions about specific areas of 5HT innervation are poor in general as most raphe neurons appear to innervate overlapping terminal fields, and thus are more noradrenaline like than dopamine like. Exceptions to the generalization are that the B_8 group (raphe medianus) appears to account for most of the 5HT innervation of the limbic system, while B_7 (or dorsal raphe) neurons innervate with greater density to the neostriatum, cerebral and cerebellar cortices and thalamus.

Figure 1.6

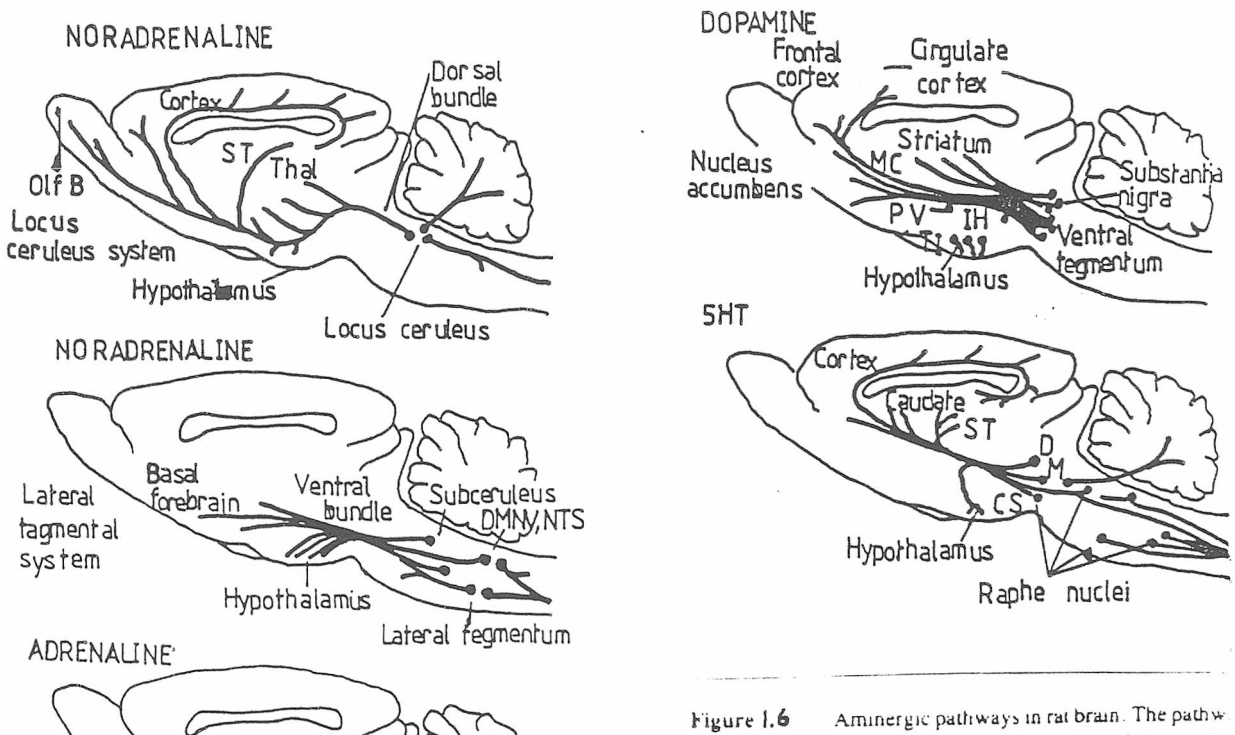


Figure 1.6 Aminergic pathways in rat brain. The pathw humans are similar. The 2 principal noradrenergic systems (ceruleus and lateral tegmental) are shown separately. C olfactory bulb; Thal, thalamus; ST, stria terminalis; DM dorsal motor nucleus of vagus; NTS, nucleus of tractus solit PG, periaqueductal gray; NS, nigrostriatal system; MC, mes tical system, PV, periventricular system; IH, t tohypothalamic system, TI, tuberoinfundibular system; D and CS, dorsal, medial, and central superior raphe nuclei

DETERMINATION OF MONOAMINE SYNTHESIS TURNOVER RATES

Ever since it was established that tyrosine hydroxylase is the rate-limiting enzyme in the biosynthesis of noradrenaline (Levitt et al, 1965) and its activity can be inhibited by 3, 4 dihydroxyphenylalamine (DOPA) and catecholamines (Ikeda et al, 1966; Spector et al, 1967) much interest has been shown in attempting to calculate the synthesis and turnover rates of the biogenic amines. The intense interest shown in these studies is, because, the biogenic amine containing neurones of the C.N.S. control many physiological conditions, including, hypertension, atherosclerosis, stress, hormone release, temperature regulation, motivation, depression, hallucinations, schizophrenia, and narcotic dependence. Since most of these phenomena can only be examined in intact organisms, in vivo methods for the study of biogenic amine metabolism and aminergic neuronal activity have been developed. Development of these methods has also increased overall understanding of many of these neuronal systems.

Initial estimates of in vivo noradrenaline turnover rates were conducted by Udenfriend and Zaltzman-Nirenberg (1963) and Burack and Draskoczy (1964), who followed the rates of synthesis and turnover of noradrenaline from ^{3}H-dihydroxyphenylalnine in different tissues. Montenari et al (1963) examined the turnover of noradrenaline by injecting ^{3}H-noradrenaline in trace amounts and following its rate of decline from the heart. The first in vivo method using enzyme inhibition then following the rate of decline of an endogenous compound was introduced by Brodie et al (1966).

They used methyl-para-tyrosine to inhibit the synthesis of the catecholamines. Today there are many methods available for the estimation of biogenic amine turnover in vivo. The methods can be classifiifed as "steady state" or 'nonsteady state". Each method has particular advantages but also, significant theoretical disadvantages, which should be taken into consideration when choosing a method of investigation.

A. Steady State Methods. These offer the theoretical advantage over the nonsteady state methods, in that the analysis does not necessarily involve disruption of the system.

1) ^{3}H-Noradrenaline tracer techniques.

In this technique, trace amounts of ^{3}H-noradrenaline are injected into animals and the rate of decline of the specific activity is determined. Most of the studies carried out with this method conclude that the rate of decline follows first order kinetics (Brodie et al, 1966, Costa, 1970). However Neff et al (1968) showed that the rate of decline in fact could be more complex than this, depending on the amout of tracer used, or in the manner in which the relationship of the ^{3}H-noradrenaline specific activity decay with time is interpreted.

In using this method it is assumed that the ^{3}H-noradrenaline is distributed evenly though all the endogenous noradrenaline compartments and that newly syntheisised amines equilibrates rapidly with all amine pools and the ^{3}H-amine (Weiner, 1974 b).

Thus, as with all steady state methods, it assumes that the amines in the tissue behave kinetically as if it were fully equilibrated in a single homogenous compartment. Several groups of workers have suggested that ^{3}H-noradrenaline in fact does not fully equilibrate with endogenous noradrenaline (Crout et al, 1962; Weiner, 1970; Thierry et al, 1971, 1973). There have also been reports that indicate that newly synthesised noradrenaline is preferentially released from the isolated spleen during nerve stimulation (Kopin et al, 1968; Gewirtz and Kopin, 1970) and Thoa et al (1971) showed similar results with the vas deferens preparation. Bove et al (1973) and Weiner et al (1973) could only show this preferential release of newly synthesized noradrenaline at high frequencies of nerve stimulation or in the presence of phenoxybenzamine but overall this work suggests that the amines themselves may not be stored within one compartment within neurones.

There is also considerable evidence that noradrenaline is compartmentalized within the C.N.S. and turns over at different rates in different brain regions. (Iversen and Glowinski, 1966). Furthermore work by Thierry et al (1973) suggests that there may be a preferential release of the newly synthesized amines which turn over much more rapidly than the bulk of noradrenaline in the brain.

2) Rate of Formation of Labelled amine after injection of the lablled precursor amino acid.

In this method, labelled tyrosine is administered by either pulse injection or intravenous infusion. The rate of formation of labelled biogenic amine is determined in the tissue and the specific activity of the labelled precursor is monitored either in the plasma or the tissue. From these two sets of data, the rate of synthesis and, therefore, at steady state, the turnover of the amine can be deduced (Gordon et al, 1966; Sedvall et al, 1968; Neff et al, 1969; Costa, 1970). This technique assumes that the system is not upset by the administration of a tracer amount of the amino acid, which holds when there is large endgenous levels of tyrosine (or tryptophan). However, this assumption is invalid when the hydroxylated amino acids (DOPA or 5-hydroxytryptophan) are used as "tracer" precursors as the endogenous pool of these compounds is normally extremely small.

It is also assumed that the newly synthesised amine is not lost or metabolised in the tissue. This assumption is hardly likely particularly if newly synthesised amine is preferentially released from the tissue (Kopin et al, 1968) or preferentially metabolized (Thierry et al., 1973, Weiner et al., 1973). To the degree that the loss or metabolism of newly synthesised transmitter is not considered, the rate of synthesis and turnover will be underestimated by this procedure.

3. Combined mass Fragmentography-gas chromatography analysis of the rate of conversion of labelled dopamine to noradrenaline after pulse injection of labeled tyrosine.

In this elaborate procedure, ^{3}H-tyrosine in injected into an animal and the changes in specific activities of dopamine and noradrenaline are followed over a short period of time (30 minutes). This method allows calculation of the rate of efflux of noradrenaline from a tissue, and at a steady state, the turnover rate of the amine.

This procedure assumes that endogenous dopamine levels are negligible and that dopamine is quantitatively converted into noradrenaline. This method cannot therefore be applied to brain tissue, since dopamine containing neurons are present in the tissue and their processes innervate most brain regions. The presence of dopamine-containing cells in sympathetic ganglia (Schumann, 1956, Bjorklund et al, 1970) and in other tissues suggests that the method may have limited application in the periphery also. It is also not likely that dopamine is quantitively converted to noradrenaline, as oxidative deamination and O-methylation of the amine also occurs. Finally, this, method, like all methods which involve estimation of noradrenaline synthesis from tyrosine in vivo, assume that the labeled noradrenaline formed will not be lost from, or metabolized in, the tissue. As noted above, there are serious questions about the validity of this assumption.

B. Nonsteady State Methods.

All the following methods possess the major theoretical disadvantage of disturbing the system in a profound way. Disturbing the system might be expected to modify the behaviour of and distort steady state metabolism.

1) Inhibition of amine synthesis to determine the rate of decline, and therefore turnover of the endogenous amine.

To calculate the turnover of dopamine and noradrenaline, methyl-para-tyrosine is used to block synthesis, and the rate of decline of the endogenous amine is followed. (Brodie et al, 1966; Spector, 1966). This method assumes complete inhibition of catecholamine synthesis, a requirement which is adequately satisfied if high doses of the amino acid are given at sufficiently short intervals (Brodie et al, 1966, Spector et al, 1966). The method must presume that the blockade of synthesis does not affect the activity of the neuronal systems deprived of their ability to synthesise amines. In view of the profound pharmacological effects of methyl-para-tyrosine and of the servomechanisms that may come into play to modify neural activity when normal functions are compromised (Weiner, 1974,a), it seems that this assumption is of dubious validility. An additional complication might arise because the loss of endogenous noradrenaline without impairment of the catecholamine storage site might be expected to facilitate the storage of the remaining amine and slow turnover correspondingly.

2) Blockade of amine degradation and estimation of synthesis rate from the rate of accumulation of the amine.

In this method, a monamine oxidase inhibitor is employed and the initial rate of accumulation of the biogenic amine is measured. Neff and Tozer (1963) suggest that this method is most appropriate for estimates of 5-hydroxytryptamine accumulation, since the major pathway of matabolism of this amine is via deamination. With the catecholamines, O-methylation is a second major pathway of metabolism (Axelrod, 1959), and neglecting the formation of this product may introduce a significant underestimation of catecholamine turnover.

The major problem with this method is the possible effects which accumulating amines may have on the tunrover of endogenous amines. It is possible that the accumulating amine may reduce its own biosynthesis by end-product feedback inhibition (Alousi and Weiner, 1966, Ikeda et al., 1966). Dopamine may serve as the precursor of noradrenaline or it may be directly denaminated to dihydroxyphenylacetaldelyde. Blocking the action of monoamine oxidase could distrub the system by favouring B-hydroxylation of dopamine to a degree that does not pertain under normal circumstances (Weiner, 1970). Even amino acid precursor availability may be affected by block of monoamine oxidase as Mandel (1974) has shown that tyrosine amino transferase is inhibited by noradrenaline.

3) Inhibit aromatic -L-amino acid decarboxylase and follow rate of accumulation of hydroxylated amino acid.

This method involves the injection of large doses of aromatic -L- amino acid decarboxylase inhibitors, such as [N-CDL-seryl)-N'-(2. 3. 4 - trihydroxybenzyl)] hydrazine (Ro - 4 - 4602) or 3-hydroxybenzylhydrazine (NSD - 1015) (Carlsson et al, 1972). When given in sufficiently high doses, these inhibitors appear to block completely the decarboxylase enzyme. The initial accumulation of either 5-hydroxytryptophan or DOPA, which are normally not detectable in tissues, is followed. since the rate of accumulation of the hydroxylated amino acid is linear with time for a few hours, it is assumed that this provides an accurate estimate of amine synthesis. Like the preceding method with monoamine oxidase inhibitors, this method has the theoretical defect of disturbing the system by allowing a normal substance to accumulate in abnormal amounts. In the case of DOPA this catechol would be expected in inhibit tyrosine hydroxylase by end product feedback inhibition. Sourkes (1966) showed that the hydrazines can inhibit a variety of amino transferase and decarboxylase enzymes and a DOPA transaminase has been demonstrated in the brain (Fonnum and Larsen 1965). Thus the use of some hydrazines might eliminate alternative pathways for the metabolism of DOPA resulting in an overestimation of turnover and synthesis rates.

The administration of the decarboxylase inhibitor will also lead to a reduction in any rapidly turning over pools of noradrenaline, dopamine, or 5-HT which may exist in the tissue. Such pools may be very important from a functional standpoint and may directly or indirectly influence amine synthesis and turnover. Since neither DOPA or 5-hydroxytyptophan is known to be stored in reserves, it is possible that the amino acid will leave the tissue in proportion to its accumulation, causing an underestimation of synthesis proportional to the degree of loss.

4. Accumulation of acid and of glycolmetabolites in cerebrospinal fluid often probenecid.

In this procedure, the removal of the major metabolites of catecholamine or indoleamine metabolism from the central nervous system is blocked by a inhibitor of the organic acid secretion mechanism (Neff and Tozer, 1968). this method assumes that the acids or glycols are the major or exclusive metabolites of the biogenic amines in the brain. Although approximately correct for 5HT, normetanephrine and methoxytyramine may be significant metabolites of noradrenaline and dopamine, respectively, in the brain (Rutledge and Jonason, 1967; Schildkraut et al, 1971). It also assumes that the removal of the glycols and acids from the brain is entirely by the organic acid secretion mechanism. Further the dose of probenecid is assumed to be adequate for complete blockade of the secretion process. High doses are required

to produce maximal block of this process, and such doses are difficult to achieve in men without producing serious side effects.

As can be seen with all the methods used in the estimation of biogenic amine synthesis and turnover rates, there are either real or potential shortcomings that have not been excluded. The methods owe their popularity to the ease with which most of the procedures can be performed and because they have been found to be useful. In spite of their inherent problems, the methods all seen to provide an estimate of relative rates of biogenic amine synthesis and turnover when two or more experimental situations are being compared (Weiner, 1974, b).

PART 2 - METHODS OF BIOGENIC AMINE MEASUREMENT

Advances in the understanding of the biochemistry, physiology and pharmacology of the biogenic amines has resulted from the development of new assay techniques, and through methods for visualizing biogenic amines and their metabolic enzymes or receptors in, in vivo and in vitro preparations.

Bioassay

Historically the classic organ bath techniques of pharmacology used to be one of the most sensitive methods for the estimation of noradrenaline and adrenaline from tissue extracts or biological fluids (Cooper et al, 1983). Despite the recent development and availability of more sensitive methods (e.g. fluorometry, HPLC/ECD) the use of a bioassay may still be indicated either under certain specialized conditions or when adequate equipment or resources are not availiable. Table 1.2 outlines some of the various bioassay technique used and their relative sensitivities. Coupling a bioassay technique to a superfusion method, removes any purification and extraction procedures required, so it is possible to measure the release and fate of vasoactive hormones directly from the circulation. Even the combining of the above two techniques fails to overcome the primary disadvantage of lack of specificity found with these assay procedures. Vane (1969) showed the specificity of a bioassay can be increased by the use of

specific antagonists and the employment of parallel assay systems in which two or more tissues with different sensitivities to the desired agents are employed.

Table 1.2 Biological assay preparations for noradrenaline and adrenaline.

Assay Preparation	Response Measured	Noradrenaline	Adrenaline
Chick rectal caecum	Relaxation	100 ng/ml	1ng/ml
Blood pressure of pithed rat	Increase in blood pressure	0.1-1 ng	5-10 ng
Rat uterus treated stilboersterol and contracted with carbachol	Relaxation	50 ng/ml	0.5 ng/ml
Perfused rabbit ear	Increase in Perfusion pressure	1-5 ng	1-5 ng
Rat stomach strip in presence of serotonin	Relaxation	5-20 ng	1-10 ng

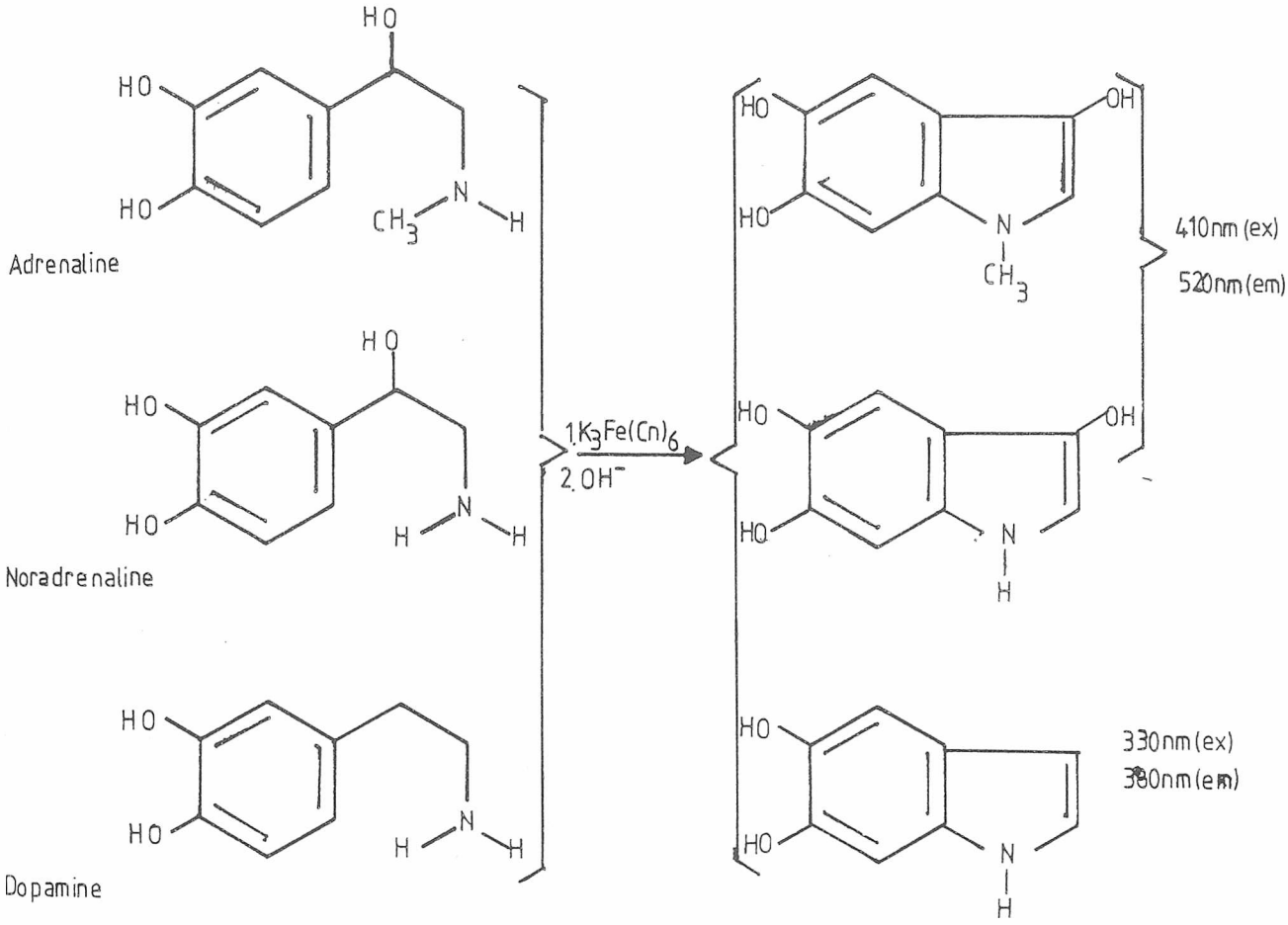
From Cooper et al (1983)

With the wide spread technical development of assay procedures in the late 60's and early 70's, there are now many different methods for the measurement of biogenic amines from different tissues. A list of these techniques includes, gas chromatography - electron capture detection (Arnold and Ford, 1973; Lovelady and Foster, 1975) gas chromatography - mass spectrometry (Koslow et al, 1972; Lhuguenot and Maume, 1974; Curtius et al., 1974), high performance liquid chromatography - ultraviolet detection (Mell and Gustafson, 1977) high performance liquid chromotography - fluorometric detection (Imai et al, 1977), high performance liquid chromotography - electrochemical detection (Refshauge et al, 1974); Kissinger et al, 1977) and the radio enzymatic assay method (Engelman and Portnoy, 1970; Peuler and Johnson, 1977). Fluorometry is another method and is the most commonly used (Schellenberger and Gordon, 1971; Cox Jnr. and Perchard, Jnr., 1973).

Fluorometric Assays.

There are at present two widely used chemical procedures for the estimation of catecholamines after their conversion into fluorescent derivatives; the ethylene diamine condensation method and the trihydroxindole method. The trihydroxindole method was the first method described in the literature (Harrison, 1963). This method involved the oxidation of catecholamines to their intermediate quinone products, which then reacts with ethylene diamine to form intensely fluorescent products. (Figure 2.1).

Figure 2.1. Trihydroxyindole method for catecholamines.

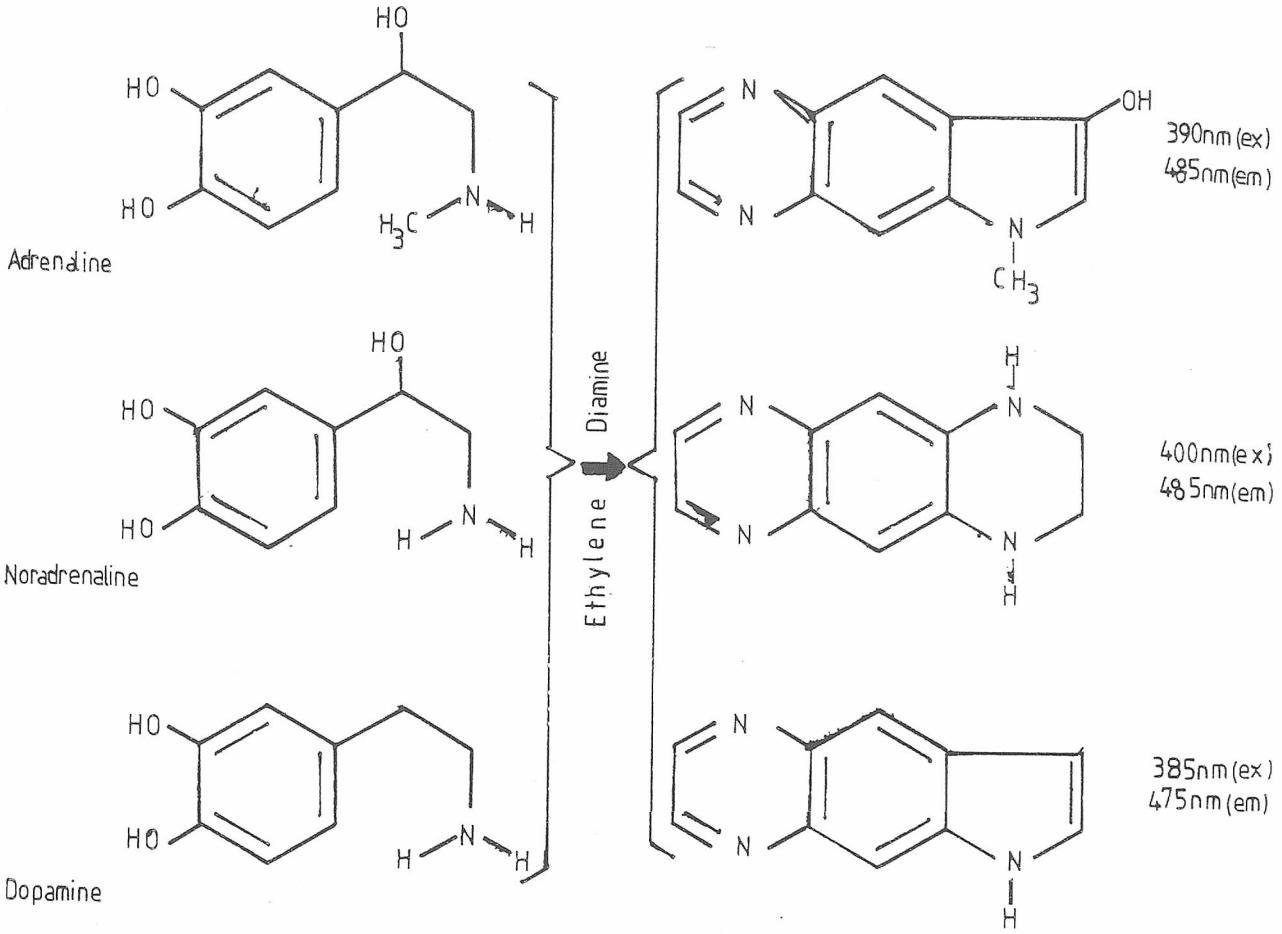


These products can be distinguished from each other using reaction rates and by varying the pH of medium (Vochten et al, 1968). From the figure 2.1 it can by seen that dopamine is converted into dehydroxyindole, and it needs a stronger oxidizing reagent to cyclize and epimerize it in order to yield a fluorophore (Anton and Sayre, 1964). Thus it is not easy to detect all three catecholamines.

Ethylene diamine condenses with the 3 catecholamines to produce flourescent compounds. (Figure 2.2) (Natelson et al,

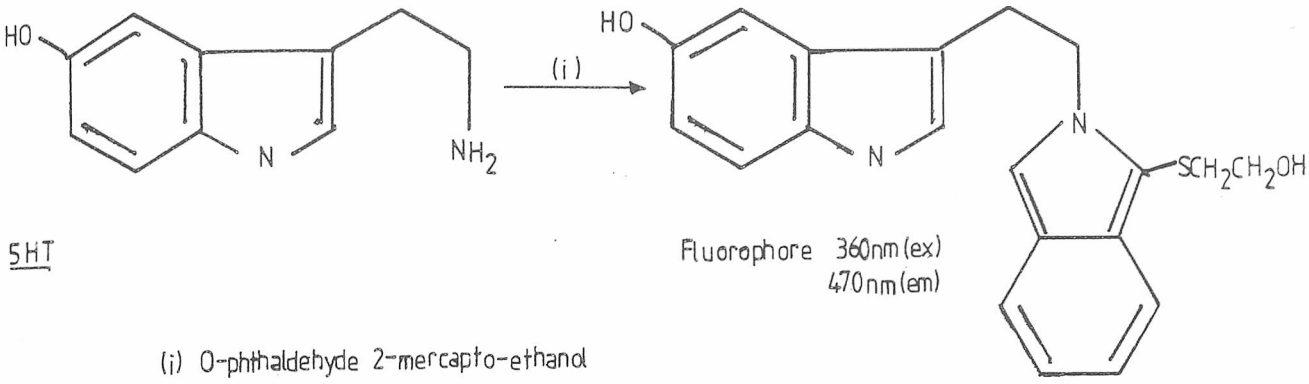
1949). One problem with this reaction however is that the fluorescent intensity of the dopamine derived is about one tenth that of the intensities of the noradrenaline and adrenaline fluophores.

Figure 2.2. Ethlene diamine condensation method for catecholamines.



5HT in neutral pH, fluoresces at 330nm with excetation at 295nm while in a strongly acid solution it fluorescent at 550nm. This phenomenon has been used until recently for the determination of 5HT. Now 5HT is reacted with o-phthaldehyde to give a fluoraphore. (Maickel and Miller, 1966) Figure 2.3.

Figure 2.3 Reaction of 5HT with o-phthaldehyde.



Although many fluorogenic reaction specific for biogenic amines have been developed for years, it is difficult to measure correctly the amount of biogenic amines in biolocial samples by the use of fluoresence analysis mainly becuase of the presence of many interfering substances.

HPLC - Fluorometric Detection.

As shown above many fluorogenic reactions can take place, thus chromatography combined with fluorometry can provide a useful means of biogenic amine analysis. Paper and thin layer chromotography can be used for qualatative analysis, while combination with HPLC provides quantitation of biogenic amines from biogenic specimens.

HPLC with its high efficiency in separating objective substances from interfering compounds means a low specific fluorescene (or reaction) for substances may be used. The fluororganic reaction can be carried out in two ways.

A) pre-column : off-line ; the reaction is carried out separately before the chromotography; on line; the reaction is carried out between the injector and the column inlet.

B) post-column ; off line ; derivatization is carried out on collected fractions, after the chromatography; on line : derivation is carried out in a specifically designed reactor cell placed between the column outlet and the detector.

Generally speaking, pre-column, off-line derivation is often used since it does not pose any restrictions on the mobile phase composition, duration and temperature of reaction and product stabiltiy, which is not the case with the on line derivation. Post-column, on line derivation is carried out on separate chromatographic bands. Therefore, the mobile phase composition must be compatible with the reaction medium. In addition, the reaction detector must be carefully designed in order to minimize dispersion.

Since a low specific fluorescence may be used, the native fluorescence of the biogenic amines, ascribed to the skeletons of the amines can be used for their detection. For instance, 5HT and its metabolites can be separated and detected in fluorescence at 345 nm with excitation at 280 nm (Graffeo and Karger, 1976). Catecholamines and their metabolites are also separated and detected in fluorescence at 316 nm with excitation at 284 nm (Mell and Gustafson, 1977).

The use of o-phthaldehyde can also be used in the detection of 5HT and its metabolites (Korf et al, 1973), dopamine and noradrenaline (Okamoto et al, 1978). Mixtures of these compounds may also be seperated by HPLC (Davis et al 1979).

In conclusion the combination of fluorometric analysis with HPLC has provided a sensitive, selective and separative tool for the analysis of biogenic amines with some workers claiming as much

sensitivity as HPLC-electrochemical detection (Yui and Kawai, 1981). The main disadvantages compared to HPLC-electrochemical detection are the sample handling in the preparation of the fluorophores and the amount of information available for a single sample. Since particular fluorogenic agents only react with a limited number of biogenic amines and metabolites this limits the information available from a single sample. Electrochemical detection following HPLC, in contrast, is capable of detecting the full range of biogenic amines and metabolites present in a single sample.

Radioenzymatic Assay.

The development of radioenzymatic assays for catecholamines in the early 1970's provided another highly sensitive method of analysis for researchers. The radioenzymatic assay of noradrenaline and adrenaline was first described by Engleman and Portnoy (1970) and soon extended to include dopamine (Coyle and Henry, 1973). Further development of the method by Passon and Peuler (1973) on the sensitivity and specificity of the assay provided the basic techniques and working procedures for the commercially available kits today. These radioenzymatic methods have proven to be precise, reproducable, and for the most part relatively simple to perform for the researcher with enzyme preparation experience.

The basis of radio enzymatic assays entails the conversion of the catecholamines to their o-methylated derivatives by catechol-o-methyl transferase or epinephrine by

phenylethanolamine-N- methyl transferase. Incorporation of the radioactive group into the reaction product is through the methyl donor for the reaction. The two most popular compounds used are S-adenosyl -L-[methyl-^{3}H] methionine and S-adenosyl -L-[Methyl-^{14}C] methionine. Since the enzymes used in the reactions are relatively unspecific in substrate selectivity, additional separation steps are required to obtain the products under investigation. These steps include a series of organic extractions and thin layer chromatography. A second method (non enzymatic) uses tritiated acetic anhydride to produce stable radioactive acetyl derivatives.

In both methods, the addition of tracer amounts of [^{3}H] or [^{14}C] labelled catecholamines to the tissue or biological fluid prior to the extraction and analysis serves to correct for any losses incurred throughout the procedure. Determination of the endogenous levels of catecholamines is based on the [^{14}C]:[^{3}H] ratios of the purified derivatives. One of the advantages of this technique is that the calculation of the amount of amine present is based on a ratio rather than an absolute amount, with the result obtained independant of losses incurred in the purification procedure. Results obtained using radio enzymatic techniques are in general highly specific and at least 50 times more sensitive that fluorometric assays (Cooper et al, 1983).

Radioimmunoassay

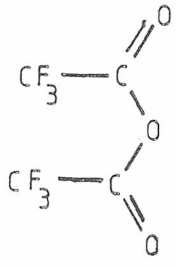
The technique of radioimmunoassay, which has found widespread application in clinical chemistry and endocrinology during the last 10-15 years, has scarcely been used for the determination of catecholamines (Knoll and Wisser, 1984). Despite many efforts only one working group (Miwa et al, 1976, 1977, 1978,a, 1978,b) has reported the successful production of antibodies to catecholamines but they did not develop a radioimmunoassay. In contrast, antibodies to some of the metabolites have been developed (Knoll and Wisser, 1984), whose specificity were high enough to permit the development of sensitive radioimmunoassays.

Gas Chromatography

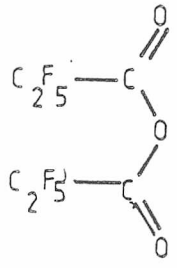
Gas chromatography, when introduced, provided a noticeable advance in the field of anlalytical instrumentation, simultaneously providing a suitable means of separation, characterization and quantitation of the complex organic mixtures encountered in tissue extracts. Gas chromotography has been used for the identification and quantitation of catecholamines and especially their matabolites in biological fluids and in tissue extracts. Measurement of the catecholamines by gas chromatography requires the formation of halogenated catechol derivatives. There are a wide range of halogen derivatives, for catecholamines. The two most popular for analytical applications are the trifluoroacetyl derivatives and the pentafluoropropionyl derivatives. These derivatives are easily

prepaired in a high yield with one step reactions by treating the catecholamines with trifluoracetic anhydride or pentofluoropropionic anhydride in either ethyl acetate or acetonitrile.

Figure 2.4



Trifluoroacetic anhydride



Pentafluoroproprionic anhydride

Initially one of the main disadvantages in gas chromotography was the limited resolving power of the columns used for catecholamine analysis. Now most workers prefer to use glass columns although Karoum et al (1975, 1976) have shown metal column to be just as effective. The complex nature of catecholamine sample has resulted in some workers having to use columns of 12 feet, rather than the standard 6 feet columns. (Karoum et al, 1975, Arnold and Ford, 1973).

Another disadvantage of packed gas chromatography columns is that they have chemically active surfaces which can absorb and chemically decompose injected compounds. Active surfaces are also present in the transfer lines connecting the end of the gas

chomatograph column to the detector being used. These active surfaces can result in partial or complete loss of the injected sample producing a decrease or total loss of sensitivity. This problem has been reported by at least two groups working with catecholamines (Goodwin et al 1973; Curtius et al, 1974). Furthermore, displacement of compounds adsorbed on the active surfaces can produce false positive results. Initial work with gas chromatography used either a flame ionisation detector system (Lovelady and Foster, 1975) or an electron capture detection system (Lhuguenot and Maume, 1974). These techniques, however have not found widespread acceptance due to the inherent problems of electron capture analysis and the easy contamination of the sensitive detector systems. (Cooper et al, 1983).

Gas Chromatography - Mass Fragmentography (GCMF)

The technique of gas chromatography-mass fragmentography (GCMF) involves the combined instrumentation of gas chromatography and mass spectrometry. This combination provides the most specific method for catecholamine measurement, with measurement in the 10 to 50 x 1075 range possible (Cattabeni et al, 1972; Costa et al, 1972; Karoum et al, 1975, 1979a). This is accomplished through the separation properties of gas chromatography and the ability of the mass spectrometer to offer structural information on compounds.

Mass fragmentography is attained by modifiying the function of a mass spectrometer, to detect and measure the intensity of

fragments with preselected atomic mass units (a.m.u.). Separation of mixtures occurs exclusively in the gas chromatography after which they pass to the mass spectrometer. Organic compound are then ionised through collision with electrons accelerated across a system called the ion source. Through these collisions organic molecules either aquire or lose an additional electron to give a negative or positively charged ion. The positively charged ions are unstable and break up into a number of positive, neutral and negative fragments. These positively charged fragments are collected and separated according to their a.m.u. or mass-to-charge ratio. The relative abundance of each group of ions of a particular mass-to-charge ratio is the output or the mass spectrum of the mass spectrometer.

One useful advantage of GCMF is in cases when the amount compound under investigation is not sufficient to produce a total mass spectrum. Its identity can be confirmed by the technique of multiple ion detection, (Karoum, 1983). In this technique the mass spectrometer is focused to detect and measure the intensity of more than one fragment. If the ratio of the intensities of fragments from a compound in a biological medium to that of a standard are similar and the two substances have the same retention times, then the possibility that the two are the same is strongly suggested. Similarly, if the ratio of ions recorded fails to match that of a standard, then one or more of the fragments selected are probably generated by contaminating materials in the biological sample. These properties demonstrate that GCMF is extremely useful in qualitative examination of biological sample when looking for

specific compounds. For all the advantages and high specificity of GCMF, the technique is still not in common use due to the high costs of the equipment, its high running costs, and technical expertise required to operate the equipment.

BASIS OF HIGH PERFORMANCE LIQUID CHROMATOGRAPHY FOR THE ANALYSIS OF BIOGENIC AMINES AND THEIR METABOLITES

During the last decade several techniques have been used in the analysis of catecholamines and related compounds (as mentioned previously). Since 1973 the number of papers describing the analysis of biogenic amines by high performance liquid chromatography (HPLC) has increased considerably. The proliferation in the number of papers published has resulted from technological improvements which make this method reliable in routine analysis. These improvements, outwith the reliability and variety of new hardware available, have occured in two areas.

1st. The stationary phases which allow sufficiently efficient HPLC columns.

2nd. The development of an electrochemical detector sensitive enough to determine trace levels of biongenic amines and their metabolites (i.e. a few picogrammes, Kissinger et al, 1973).

Except for the composition of the mobile phases which are used for elution, the most important part of a HPLC system is the column. There are several different types of packing material available for columns, but not all are suitable for use in the analysis of biogenic amines. Therefore only those methods that are directly involved in the analysis of biogenic amines will be considered.

Figure 2.5

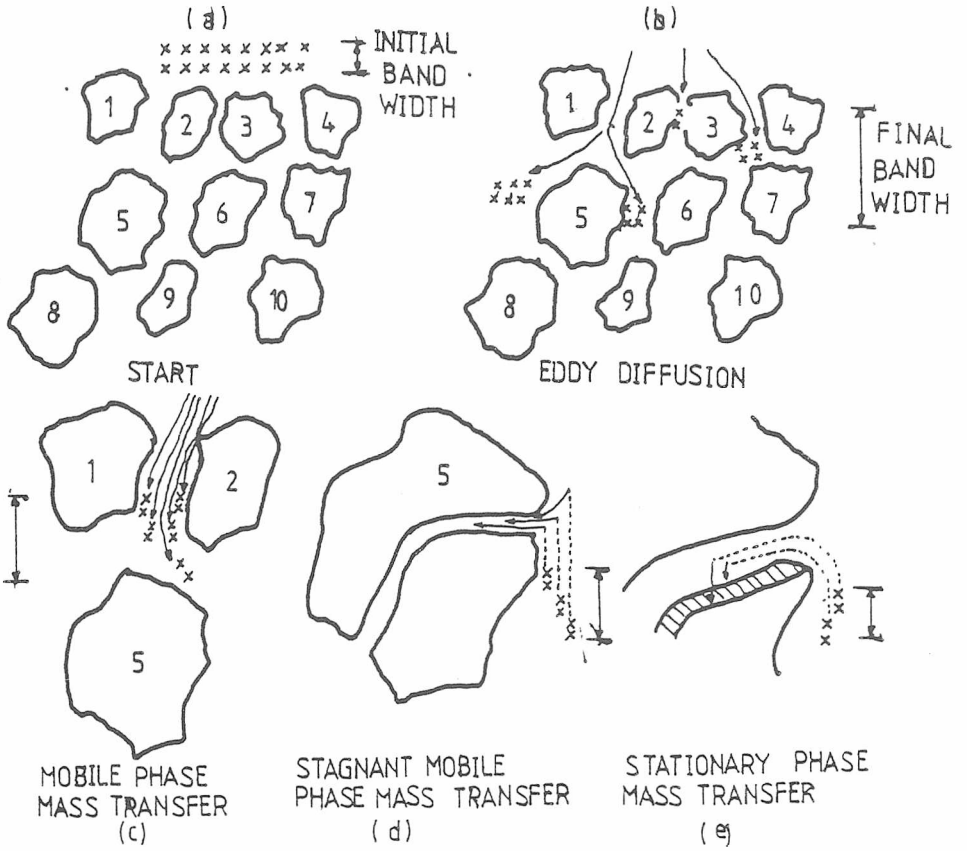


Fig.2.5 Contributions to molecular spreading in LC. From Snyder & Kirkland (1979)

Figure 2.5.(a) shows the cross section of a column top (with individual particles numbered from 1 to 10). Sample molecules are shown as X's at the top of the column, just after injection. At this point they form a narrow band (called the "initial band width") Figure 2.5.(b). shows eddy diffusion or multiple flowpaths. As can be seen the molecules can take different flow paths through the phase bed. Thus some molecules will travel a shorter distance than other through the phase bed and some will have a greater velocity than others as they pass through the wider gaps between the packing material. Both of these phenomena lead to an increase in the band

width. Reduction in the eddy diffusion effect can be achieved through the use of spherical particles with a small dispersion of the mean diameter. Figure 2.5.(c). Mass transfer in the mobile phase. This refers to different flow rates of molecules in different parts of the flowstream or path between surrounding particles. As shown in the diagram, liquid adjacent to a particle moves slowly or not at all, whereas liquid in the centre of the flow stream moves fastest. As a result molecules at the side of the flow, move slowly thus increasing band width. Figure 2.5.(d). Mass transfer in the mobile stagnant phase. When the column packing material is porous, one part of the mobile phase is stored inside the pore and is therefore still. Solute molecules move in and out of the pores through diffusion and the time spent within the pore varies. This decreases the molecules migration velocity and cause band broadening. Figure 2.5.(e). Mass transfer in the stationary phase; Once a molecule has diffused into a pore, they may penetrate the stationary phase or become attached to it in some fashion. The deeper the stationary phase is penetrated, the slower the molecule passes down the column resulting in another way to increase the band width.

Radical diffusion of the molecules also occurs, the molecules have a natural tendency to diffuse randomly in all directions. This does not enter substantially into the broadening of peaks, but may take on more importance when low flow rates of the mobile phase are used with columns packed with particles of small diameter.

Finally, under ideal conditions, each compound leaves the column as a band or a peak which may be symmetrical and Gaussian.

Each peak emerges at a time that can serve to characterize the chemical nature of the compound if the other parameters are the same. This is called the "retention time" (tr) and is measured from the time of injection. The seperation of two compounds is characterized by the difference in their retention times. Each peak is also characterized by its width (tw) Consequently, a good seperation will imply.

1st. That different constituents of the mixture are sufficiently retained to appear after an elution volume larger than the intersticial volume of the column. From this follows a sufficiently great affinity for the stationary phase.

2nd. That the peaks corresponding to the different compounds are well separated (Figure 2.6.)

3rd The analysis takes as little time as possible.

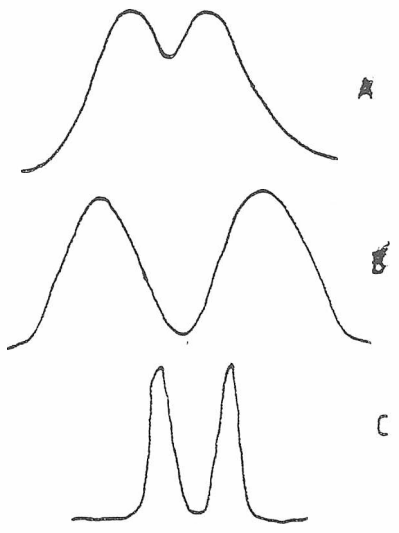


Fig.2.6 Example of a chromatographic separation. A, bad separation; B, separation with equal peak width; C, separation achieved by reducing the width of the peaks (the elution times being equal).
From Parvey et al (1983)

In practice, it is necessary to compromise between these different sometimes contradictory parameters. Figure 2.6 shows good and poor seperation of two peaks.

Retention parameters in Liquid Chromatograph (LC)

Up to this point the discussion has been purely qualitative, rather than quantitative. In LC it is possible to reduce many of the foregoing concepts to precise mathematical relationships.

If the flow rate of the mobile phase is kept constant, the volume of retention, V_R is defined by

$$V_R = t_R d = t_R . v . s .$$

where, v = linear velocity of the mobile phase, s = column section, d = flow rate, t_R= retention time.

V_R represents the volume of the mobile phase which is necessary for the elution of a given compounds, not counting the broadening of the peak. Compounds that are washed through the column on the solvent front appear after a time corresponding to the interstitial volume or column dead volume V_M. In the case of a linear elution (assuming that K (distribution constant) is independent of the concentration of the solute in the mobile phase):

$$V_R = V_M + KV_S$$

V_S being the volume of the stationary phase, or, depending on the

unity which expresses K, the mass or specific area of the support. Taking into account the geometric parameter of the column, the factor of capacity k can be used to characterize the retention of a compound.

$$k' = \frac{C_S V_S}{C_M V_M} = K \frac{V_S}{V_m} = \frac{V_R - V_M}{V_M}.$$

It therefore follows that

$$k' = \frac{R - t_o}{t_o} \quad \ldots\ldots\ldots\ldots\ldots\ldots [I(1)]$$

$$t_R = t_o (1 + k') \quad \ldots\ldots\ldots\ldots\ldots\ldots [I(2)]$$

It is important that the terms 'factor of capacity' and 'capacity' should not be confused, the latter term defining the maximum quantity of a sample beyond which the column loses it efficiency by overloading. The volume of k' is a function of the temperature (the reason why many HPLC systems have a temperature control fitted to the column).

Indeed, the transfer enthalpy of the solution from the mobile phase to the stationary phase is a function of the temperature, and k' follows Von't Hoff's equation;

$$\frac{d \log k'}{dt} = \frac{\Delta H(m\ s)}{RT^2}$$

Peak Broadening, efficiency and number of theoretical plates

The band width (tw) phenomenon in LC is commonly expressed in terms of the 'theoretical plate number', N, of the column. This

theory elaborates on a static model saying, that the eluted peaks could be assimilated within, the curve of Gauss where the variance, is a function of the number of theoretical plates.

$$\sigma^2 = \frac{t_R^2}{N}$$

From the geometrical properties of the curve of Gauss, one finds;

$$N = 16 \left(\frac{tR}{tw}\right)^2$$

In practice, to achieve greater accuracy in measurement it is preferable to use the width of the peak at half of the light,

$$N = 5.54 \left(\frac{t_R}{\delta}\right) \qquad \text{.....[I(3)]}$$

The quantity N is approximately constant for difference bands in a chromatogram for a given set of operating conditions. Therefore, N is a useful measure of column efficiency; the relative ability of a given column to provide narrow bands (small values of t_W) .

Equation I(3) predicts that the band width of peaks will increase proportionately with t_R. Because of the widening of LC bands as retention times increase, late-eluting bands show a corresponding reduction in peak height and eventually disappear into the baseline. The quantity N is proportional to the volume length L, so that, other factors being equal, an increase in L results in an increase in N and better separation. The proportionality of N and L can be expressed in terms of the equation.

$$N = \frac{L}{H}$$

Where H is the so called "height equivalent of theoretical plate (HETP). The quantity H measures the efficiency of a given column per unit length of column, so H can be used to compare the efficiency of columns of different lengths (operated under the same set of operating conditions). The smaller the H value, the more efficient the column is for HPLC. The observed values for H run from 0.03 to 1mm. In general H is smaller for small particules of column packing, for less viscous mobile phases, for higher separation temperature and H is smaller for small sample molecules.

The dependance of column H values on the mobile phase velocity (V) is of great practical importance. A knowledge of the

theoretical relationship is the key to understanding and controlling separation after a particular column packing has been selected for a given LC separation or application. The relationship between H and μ can be expressed through the following equation.

$$H = A\mu^{0.33} + \frac{B}{\mu} + C\mu$$

The constant A, B and C vary from column to column, and depend to some extent on the sample, mobile-phase, and separation temperature. For a good column A = 1, B = 2, C 0'1. It is useful to plot a graph of H versus v for every new column as it can be used as a diagnostic tool to check the columns performance after being in use.

There are two factors which increase peak broadening which are independant of the column parameters. These are (1) The presence of dead volumes between the point of injection and the detector. (2) An increased volume of sample injected.

Notion of selectivity

The factor of selectivity , characterizes the distance between two peaks,

$$= \frac{t_R^2 - t_o}{t_{R1} - t_o} = \frac{K_2}{K_1}\frac{1}{1}$$

Consequently it is a measure of the differences of

thermodynamical distributions of two compounds.

$$\log \alpha = \frac{(\Delta(\Delta G^{o})}{(RT)}$$

Where, $(\Delta G^{o}) = \Delta G^{o} - \Delta G^{o}$, and ΔG^{o} is the free energies of the distribution of the two compounds.

Resolution and Analysis Time

The aim of LC is to achieve adequate separation of a given sample mixture. In approaching this there must be some quantitative measure of the relative separation or resolution achieved. Resolution is defined as the distance between the center of two peaks, divided by the average width of the peaks.

$$R_S = \frac{t_2 - t_1}{1/2(tw_2 - tw_1)} \qquad R_S = \text{resolution.....[I(5)]}$$

The quantities t_1 and t_2 refer to the t_R values of the bands, and tw_1 and the tw_2 to their two values. The larger the R_S values, the better the separation between two peaks, the lower the R_S value, the poorer the separation.

To control the separation of two peaks, it is essential to know how R_S varies with experimental parameters such as k' and N. For two overlapping peaks, it is possible to derive such a relationship. Equation I(2) gives $t_1 = t_o (1 + k',)$ and $t_2 = t_o (1+ k_2)$, where k_1 and k'_2 are the values of peaks 1 and 2. Since t_1 and t_2 are approximately equal, from equation I(3) we have tw_1 and tw_2 (N assumed constant for peaks 1 and 2).

Inserting these relationships into equation I(5) gives.

$$R_S = \frac{t_o(k'_2 - k'_1)}{tw_1} \quad \text{.....[I(6)]}$$

Similarly, Eq. I(3) for peak 1 gives

$$tw_1 = 4t_1/\sqrt{N} = 4t_o(1+k'_1)/\sqrt{N}$$

Substituting this expression of tw_1 into Eq I(6)gives

$$R_S = \frac{(k'_2 - k'_1)\sqrt{N}}{4(1 + K'_1)}$$

$$= (1/4)(k'_2/k'_1 - 1)\sqrt{N}\ (k'_1 + k'_1) \quad [I(7)]$$

The factor of selectivity has been defined previously and recognizing that k'_1 k'_2, which equals the average value k' for the two peaks, then Eq. I(7) becomes

$$R_S = (1/4)(\alpha - 1)\sqrt{N}\ (k'/1+k') \text{ ..... } [I(8)]$$

(i)(ii) (iii)

This relationship is fundamental to LC as it allows the control of the resolution (hence the chromatographic separation), by varying each one of the three terms, α, N or k'.

These terms are roughly independent.

(i) The selectivity measured by can be varied by changing the composition of the mobile phase and/or the stationary phase

(ii) The efficiency measured by N can be modified by varying the length of the column or the flow rate of the mobile phase. One can also use the multidimensional methods by a commutation during analysis of one column to another.

(iii) The last term involving the factor of capacity can be modified by varying the elution strength of the mobile phase.

The total duration of an analysis is roughly equal to the retention time of the last compound eluted. This duration is related to the resolution as the previous equation [I (7)] shows,

$$tmax = t_o (1 + k' max)$$

k'max being the capacity factor from the last compound eluted. For most separations, k' varies roughly between 3 and 20. This signifies a duration of analysis between 4 and 21 times the elution time of an unretained compound.

Liquid solid chromatography, or adsorption chromatography

Liquid solid or adsorption chromatography (LSC) is the oldest form of LC that is in use today. In its classical form, so called open column chromatography was first introduced at the turn of the century. In the 1950's, development of thin-layer chromatography (TLC) the open bed version of adsorption chromatography took place. It was not until 1967 that Snyder introduced modern LSC featuring automated operation, plus, fast, high-resolution separation.

The stationary phases used in LSC are either, silica, alumina or carbon, with silica being the one most commonly used. The silica packing material has active sites present on it. These active sites are silanyl groups that are either isolated or hydrogen bonded. Separation by LSC relies on the selective adsorption of different compounds to the active sites of the surface adsorbant. This selective adsorption leads to competion for the active site between molecules of the solvent and molecules of the solute during a separation.

i.e. $s + nS_{ads} \rightleftharpoons s_{ad} + nS$ S = solvent, s = solute.

LSC is still commonly used but unfortunately it has few applications in the analysis of biogenic amines. This is because of the nature of the compound, that are almost insoluble in the solvents used in LSC eg. pentane, chloroform, ethyl acetate, methanol. Also, the biological samples are aqueous and the presence of water is very important in this method. It is therefore impossible to inject a biological sample into a column. This, therefore implies that samples must be pretreated. This involves an extraction followed by concentrating and dissolving the sample in a small amount of solvent which is either identical to the mobile phase, or analogous to it. Ghebregzabher et al (1981) used a LSC method for the analysis of the urinary metabolites of tryptophan and phenylalanine. For this it was necessary to use successive extractions of the sample which proved to be an advantage as it fractionated the complex sample into several groups of neighbouring chemical affinities.

Through the extraction processes it is sometimes necessary to make a derivation of the compounds under examination. Compounds are commonly dansylated which makes it possible to use fluorometric detection. This method was used by Yamada et al (1981) for the determination of urinary vanillylmandelic acid (VMA).

Ion-Exchange Chromatography

Ion-exchange chromatography (IEC) was the first of the various LC methods to be widely used under modern LC conditions, but until recently, separations by IEC have been carried out by classical, low-pressure LC procedures. For various practical reasons, however, IEC has remained less popular than the other LC methods, especially since the introduction of the new technique of ion-pairing chromatography. Nevertheless, for certain separation problems, IEC is still uniquely useful.

IEC is carried out on stationary phases containing acid groups (sulphonic acid or carboxylic acid) for the separation of cations, and basic groups (amines or quaternary amines) for the separation of anions. Therefore, an ion-exchange process may be explained by the following equilibrium.

$$R\text{-}N^{+}, X^{-} + S^{-} \rightleftharpoons R\text{-}N^{+}, S^{-} + X^{-}$$

$R\text{-}N^{+}$, representing an active site bounded to the matrix; X^{-} being the counter ion in the mobile phase; S^{-} the molecule of solute in the ionized form. The counter ion will be displaced from

the active site by the solute ion, S^- giving the ion pair $R\text{-}N,^+S^-$. In IEC chromatography we are dealing with a displacement reaction in which ion displaces another ion which has a stronger but neighbouring affinity for the active site. For a chromatographic process, there must be an equilibrium.

$$K_{IE} = \frac{[R\text{-}N^+, S^-][X^-]}{[R\text{-}N^+, X^-][S^-]}$$

k' being proportional to the distribution factor D, (C_s/C_m)

$$k' \propto \frac{[R\text{-}N^+, S^-]}{S^-} = K_{IE} \frac{[R\text{-}N^+, X^-]}{X^-}$$

For a given stationary phase $[R\text{-}N,^+ X^-]$ is constant and it follows:

$$k' \propto \frac{1}{[X^-]}$$

k' is then inversely proportional to the concentration of the counter ion in the mobile phase.

As only the ionized part of the solute is attracted to the support, the distribution of the solute between its ionized and non-ionized portions is important. Therefore, indirectly, k' is affected by the pH. Let us consider the case of an acid

$$\text{Ionized fraction} = \frac{[RCOO^-]}{[RCOO^-] + [RCOOH]} = \frac{Ka}{[H_3O^+] + K_a}$$

If the pH is sufficiently maintained below the pKa value, with a good approximation, one can write that,

$$\text{Ionized fraction} = \frac{1}{[H_3O^+]}$$

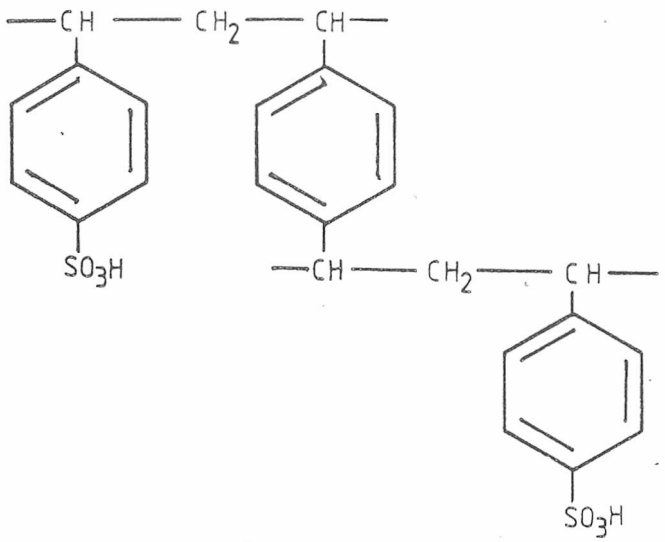
consequently; k' is inversely proportional to $[H_3O^+]$, as demonstrated by Knox and Jurand (1973).

Column packings used in ion-exchange chromatographhy

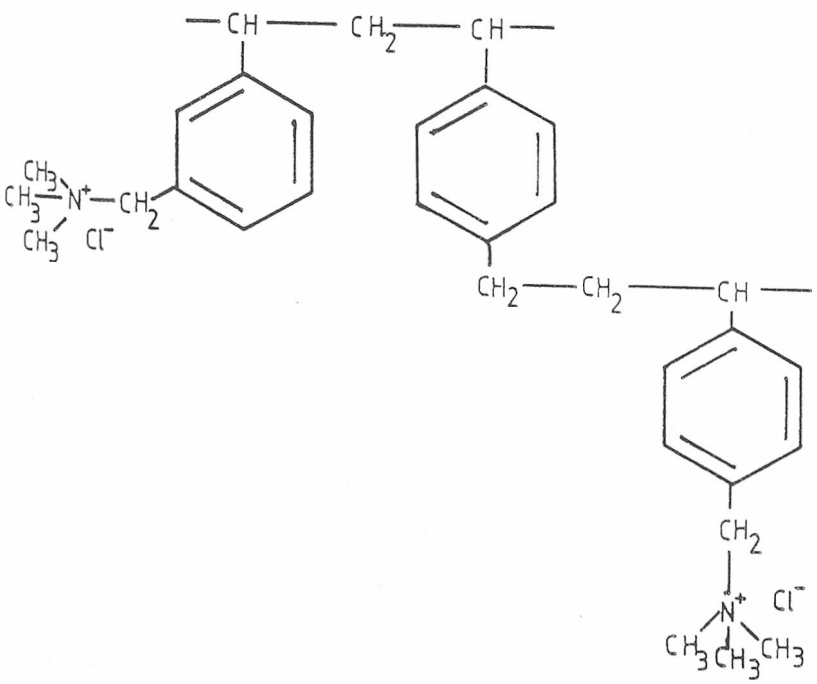
Many different ion-exchange packing have been used in LC, ranging from soft gels, to very rigid gels. Classically the ion exchange resins are styrene divinylbenzene copolymers with acid or basic functional groups.

Figure 2.7

Strongly acidic cation exchange resins.



Strongly basic anion exchange resins



These types of resins are not suitable for HPLC because they are compressible under high pressure and their mass transfer characteristics are modest. To overcome these problems, pellicular ion exchange supports have been used. These are glass beads loaded with a thin layer of ion exchange resin. However, the capacity of these supports still remains low.

At present the best results are obtained with materials in which the ion exchange groups are chemically bonded onto a porous matrix of microparticles of silica. These strong cationic exchanges and strong anionic exchangers often carry the lable SCX or SAX, respectively. Weak cationic and ionic exchangers, based on either carboxylate ($-COO^-$) or amine ($-NH_3^+$) groups, have also been used occasionally. A very important property of ion exchangers is the exchange capacity. This is a measure of the number of ionogenic groups per unit of weight (per gramme usually), able to enter into an exchange process.

The mobile phases generally used with ion exchangers are buffered salt solutions. Sometimes small quantities of organic solvents that are miscible with water are added. The elution strength and the selectivety of the mobile phase depend upon several factors; the nature of and concentration of ions which are used for the buffer and other ions which are in solution; the pH; the nature and concentration of the organic solvents added.

IEC has been used by many investigators interested in biogenic amines, who have used the ionizable nature of these compounds. (Adlakha and Villanueva, 1980; Adler et al, 1977; Froelich and Cunningham, 1978, Garnier, et al 1979,).

Through a variety of chromatographic conditions the above investigators succeeded in separating many different compounds from a single sample injection.

Liquid-Liquid Partition Chromatography and Bonded-Phase Chromatography

Liquid-liquid chromatography (LLC), sometimes called liquid-partition chromatography was introduced in the early 1940's. The basis of modern LLC is slightly different from the original method. In LLC the solute molecules are distributed between two immiscible liquids according to their relative solubilities. One liquid is the mobile phase (sometimes called the carrier) and the other is the stationary phase which is dispersed onto a finely divided, usually inert support. In conventional LLC, the stationary phase is a bulk liquid that is held to the support by adsorption. In recent years development of organic phases that are chemically bonded to the support have been developed, leading to another LC method called bonded-phase chromatography (BPC). LLC still remains an important separation technique and is useful to introduce the concept of reverse phase chromatography.

Advantages of LLC are

A) The stationary phase can be easily stripped of the support and renewed to increase column life.

B) High loadability, the pores of the support can be completely filled with a low-viscosity stationary phase, thus providing a maximum stationary-phase volume and allowing maximum sample sizes with highest column efficiencies.

C) A broad range of selectivity, resulting from the large number of stationary phases available.

D) Good reproduceability between columns, since the support itself plays little part in the retention characteristics of the column.

The choice of stationary phases available has led to two types of LLC being possible, based on the relative polarities of the mobile and stationary phases. In "normal-phase" LLC the support is coated with a polar stationary phase, whereas the mobile phase is a relatively nonpolar solvent. Normal-phase LLC is used for more polar, water samples and the solute elution order is similar to that observed in adsorption chromatography.

The two LLC phases can be interchanged so that the less polar liquid becomes the stationary phase and the polar liquid the mobile phase. This form of separation is referred to as reverse-phase LLC and is generally used to separate samples with poor water

solubility. Usually the elution order of solutes is the reverse of that observed in normal phase, i.e. compounds elute essentially in the reverse order of their water solubility.

With the introduction of chemically bonded supports ionic as well as polar compound can be efficiently separated. The use of bonded supports has also resulted in a larger choice of eluents that can be used and also the use of gradient chromatography without removing the stationary phase from its support.

It is possible for severaly different groups to be bonded to the silica support in a variety of chemical reactions.but not all are suitable for use in HPLC.

(i) $-Si-OH + HO-R \rightleftharpoons Si-OR + H_2O$

These compounds are unstable at high temperatures and easily hydrolyzed.

ii) $Si-^{C} + Br-(CH_2)_3-CH_3 \xrightarrow{Na} Si-(CH_2)_3-CH_3$

Although more stable than the -Si-O bond, the -Si-C bond is sensitive to electrophilic or nucleophilic agents.

(iii)

$$-Si-OH + X-\underset{R3}{\overset{R1}{Si}}-R_2 \rightarrow -Si-O-\underset{R3}{\overset{R1}{Si}}-R_2 + HX$$

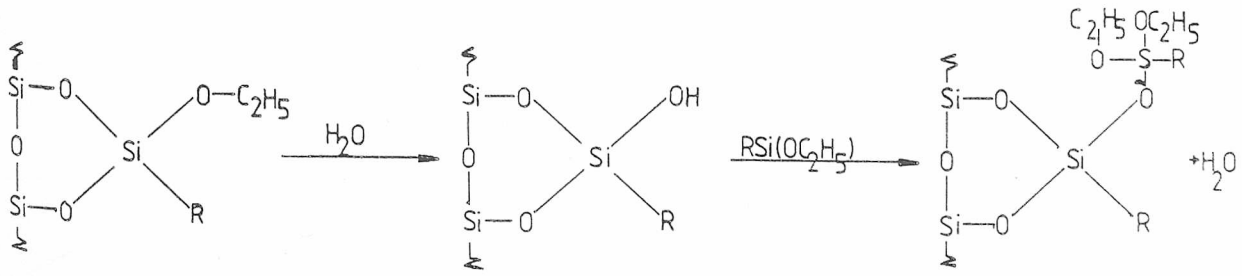
(X= Cl, $-OCH_3$, $-OC_2H_5$)

These supports allows the use of highly polar-mobile phases like equeous or alchololic solutions, which work very well in reversed phase chromatography. For the separation of biogenic amines, only the types of supports obtained by a silicone bonding reaction as shown above can be used. These types of supports are also suitable for ion paired chromatography or soap chromatography which will be discussed later.

Properties of the support materials

It is known that silica contains an average number of hydroxyl groups per nm^3 varying between 0 and 5. For reasons of steric hindrance, only 3 siland groups per nm^3 are capable of reacting. With only 3 siland groups per nm^3 reacting it suggests that the percentage of carbon loaded is indicative of the surface coverage, but this is not the case. (The percentage of carbon loaded is the ratio between the weight of carbon introduced by the bonded groups and the total weight of the packing). Figure 2.8 shows the silanization achieved by a trialkoxysilane in the presence of water. (Parvey et al, 1973)

Figure 2.8



As can be seen this leads to a polymeric layer, with a high percentage of carbon loaded for the silanization of two OH sites of silica. This polymerization of silane at the surface of the stationary phase results in a decrease in the column efficiency.

When a constant surface coverage is achieved, the logarithm of k' varies linearly with the length of the bonded carbon chains. This shows that the bonded chains interact with the molecules of the solute along their entire length (Unger et al, 1976). This fact favours the predominantly hydrophobic effect of reverse phase partition chromatography. In practice, carbon chains of up to 18 atoms are bonded because beyond this size of chains, steric interferance reduces the surface coverage of the packing material.

For the analysis of biogenic amines, most workers prefer to use techniques derived from this mode of chromatography, i.e. ion-pair chromatography or soap chromatography. However, Molnor and Horvath (1976, 1978) were the first worker to use 'pure' reversed phase chromatography for the measurement of cathecholamines and related compounds. They used an octadecyl-bonded phase and phosphate buffer O.1M, pH2.1 for the mobile phase. Many workers have used this method for the separation of biogenic amines with success. (Anderson et al, 1979, 1981; Fornstedt, 1978; Hefti, 1979; Riggin and Kissinger, 1977; Schewalt, 1977; Schewdt and Bassemas, 1977; and Sjut, 1981).

Ion Pair Chromatography

Ion pair chromatography was first described by Shill and his co-workers in 1973 (Eksborg et al, 1973). Since then various names have been given to this form of chromatography, i.e. chromatography with a liquid ion exchanger, extraction chromatography, paired ion chromatography, and soap chromatography. All these techniques are similar and the term 'ion pair chromatography' (IPC) is now used for all of them.

The development of IPC arose out of the problems encountered in IEC. In IEC the working life of the column is relatively short, reproducibility between batches of columns is not guaranteed and the choice of packing material is limited.

IPC can be used equally with normal phase or reversal phase, with reversed phase being most frequently used. Other advantages of IPC are, aqueous samples can be injected directly onto the column, and gradent elution systems as well as isocratic systems can be used. The bleeding of the counter ion off the stationary phase is eliminated and biological samples require less purification prior to injection. The elution strengths of the solvents used are easily modified and their selectivity can be easily altered by modification of pH. Through the numerous examples in the literature, IPC is now a major method in the analysis of biogenic amines.

Mechanism

The mechanism of separation for reversed phase will be described as this is the most commonly used.

The mobile phase in IPC is an aqueous buffer (to which an organic co-solvent can be added), with a counter ion of opposite charge to that of the solute molecule. For example, let us examine the separation of a group of carboxylic acids (RCOOH) using a mobile phase buffered at pH.7, so that all the samples are in the form $RCOO^-$. Using tetrabutylammonium as the counter ion in the mobile phase (Bu_4N^+) is the simplist case of IPC, it can be assumed that the sample and counter-ions are only soluble in the aqueous mobile phase, and the ion pair formed by these ions is soluble only in the organic stationary phase. The equilibrium between these two phases can be expressed by:

$$RCOO^-_{aq} + Bu_4N^+_{aq} \rightleftharpoons RCOO^-, Bu_4N^+_{org}$$

aq and org referring respectively to aqueous and organic phases. The extraction constant can therefore be expressed by:

$$E = \frac{[RCOO^-, Bu_4N^+]_{org}}{[RCOO^-_{aq}][Bu_4^+{}_{aq}]}$$

E is a constant for a given system but varies with the pH and ionic strength of the mobile phase, with concentration and nature of an eventual organic co-solvent, and with temperature.

$$k' = \frac{V_S}{V_M} = \frac{[RCOO^-, Bu_4 N^+] \text{ org}}{[RCOO^-] \text{ aq}}$$

$$k' = \frac{V_S}{V_M} = E[Bu_4 N^+] \text{ aq}$$

Thus, k' is therefore proportional to the concentration of the counter ion. The variation of the counter ion allows control of the elution strength of the solvent maintaining the selectivity more or less constant.

In order to describe the mechanism of separation it is assumed that the counter ion is not soluble in the stationary organic phase. Work carried out in the mid 1970's has suggested that this might not be the case and that the counter ion is infact adsorbed on the surface of the stationary organic phase and that the retention of the ionic species follows the law of a simple ion-exchange process. Scott and Kucera (1977) suggested that the above relationship does occur for normal ion pair chromatography and the counter ion is not adsorbed as long as it is sufficiently small. However, in cases where a counter ion possessing a sufficiently large hydrophobic part is used, retention of the counter ion occurs and one is dealing with a mechanism similar to that of IEC for the solute molecules. This method of separation was called 'soap chromatography' and was described in detail by Knox and Laird (1976). The technique uses a classical reversed phase support, generally an octadecyl bonded, and an hydrophilic mobile phase containing an organic modifier (up to 30%) to which is added a weak concentration of detergent (0.1% or less). The difference between this technique and classical ion pairing being

the nature of the counter ion. Since the solute molecules form an ion pair only in their ionized form strict control of the pH is essential so the compounds appear in their correct state. For amines an anionic detergent such as sodium dodecylsulfate (SDS) is often chosen.

The packing materials used in the separation of biogenic amines are porous particles of 5 to 10 m mean diameter with a polar octadecyl bonded phases (as described in the discussion of liquid partition chromatography).

In reversed phase the elution strength of the solvent may be modified by changing the nature of the counter ion or its concentration. For anionic samples.

$$K' = \frac{V_S}{V_M} E [C^+]$$

and for cationic samples

$$k' = \frac{V_S}{V_M} E[C^-]$$

$[C^+]$ and $[C^-]$ being the concentrations of the counter ion, cationic and anionic respectively, and E the extraction constant for given conditions. When ionized species are bivalent or trivalent, k' then varies as $[C\pm]^2$ and $[C\pm]^3$. In reversed phase, k' increases with the size of the counter ion. In soap chromatography the influences described above remain valid, but the relationship between k' and $[C\pm]$ is no longer linear (Knox and Jurand, 1976).

Water is the principal solvent used in reversed phase, with methanol or acetonitrile commonly being added. The k' decrease as the percentage of the organic modifier is added. Changes in pH also alter the k' values. In reversed phase k' is maximum for the values of pH which ensure total ioinization of the solute molecules, and hence a maximal formation of ion pairs. Changes in pH generally result in important modifications of selectivity and therefore are rarely used to modify the elution strength of a solvent.

Many workers have reported separations of biogenic amines by IPC (Persson and Karger, 1974; Asmus and Freed, 1979; Biddard and Cronenberger, 1979; Crombeen et al, 1978; Jenner et al, 1981; Knox and Jurard, 1976; Magnusson et al 1980; Moyer and Jiang, 1978, Scratchley et al, 1979; Seiler and Knodgen, 1980: Wagner et al, 1979). Wagner et al (1979) used this method in the routine analysis of several biogenic amines from different tissues. The optimization of the method allowed for the analysis of up to 60 samples a day. It should be noted that the accuracy of quantitative results depend on a repeated calibration of the system at regular intervals. Most works use an internal standard method of calibration. Magnusson et al (1980) have defined a way of choosing a column for each particular problem, of separation and how separation conditions can be optimized. Sulfonic acids are widely used in ion pair chromatography but Asmus and Freed (1979) demonstrated it is possible to use simple acids as counter ions. Applying this to biogenic amines, they showed that the retention times of amines are generally greater with a SDS mobile phase, but

the use of HNO_3 or TCA as a counter ion also provides satisfactory results. Also the column equilibrium is reached quicker. The use of HNO_3 causes a loss of column efficiency with time, but the replacement of HNO_3 by TCA recovers a great deal of the efficiency by increasing the retention.

ELECTROCHEMICAL DETECTION

The principles of the electrochemical detection (ECD) method are based on electrochemical reactions on an electrode surface which are quite different from those of other spectroscopic detection methods using physical phenomena, such as light absorbance and fluorescence emission as the basis of detection. Because the method works on the principle of electrochemical reactions of intact molecules there is no need for tedious and time-consuming derivatization before or after separation by high performance liquid chromatography (HPLC). Quantification of catecholamines in picogramme quantities is usually possible (Hashimoto and Maruyama, 1983).

Initial work with ECD took place in the early 1970's, but is was not until the mid 70's that reliable equipment for every day use was developed. It was in 1970 that Koen et al first reported the development of an EC detector, utilizing a dropping mercury electrode as the working electrode. However, analysis of compounds with this type of electrode is limited to reducible compounds because the ionisation of metal mercury is the primary process at positive potentials (Koen et al, 1970). Since biogenic amines and

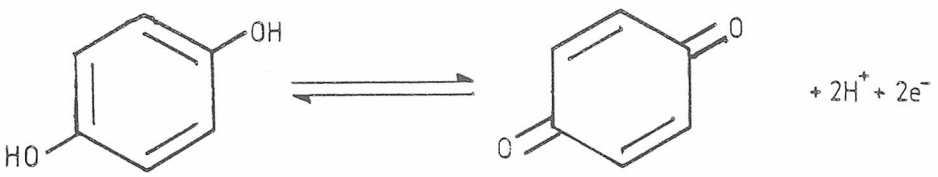
their metabolites are oxidisible, this detector system could not be used in the analysis of these compounds. Joynes and Maggs (1970) working independantly from Koen's group also produced an EC detector, which utilized either a dropping mercury electrode or a carbon-impregnated silicone rubber membrane as the working electrode. In testing the different types of electrode they concluded that the carbon-impregnated silicone rubber membrane electrode had superior, sensitivity, strength and reliability. They also demonstrated that it was possible to combine this electrode with liquid chromatography. However, again only reducible compounds such as metal ions and some nitro compounds could be detected. It should be remembered that at the time of development of these first EC detector prototypes, high performance liquid chromatography was still a new and relatively primitive method of analysis. With the development and improvement of high performance liquid chromatographic systems and equipment in the 1970's (see earlier part of introduction) it then became possible to develop new EC detectors to use in combination with high performance liquid chromatographic systems.

Work on a new type of EC detector compatable with modern high performance liquid chromatography by Prof. R.N. Adams group (Kissinger et al, 1973) showed that the EC detector could detect the oxidation of biogenic amines. They used a carbon paste electrode as the working electrode which made the detection of pmol amounts of biogenic amines possible.

Electrochemical processes on the surface of the electrode

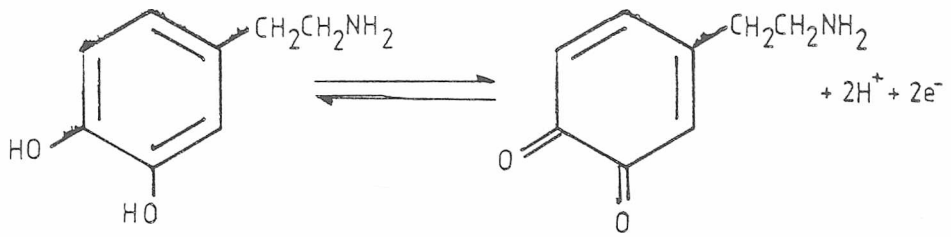
Oxidation of hydroquinone (a well-known reducing agent) produces benzoquinone. The reaction is represented in Equation 1. Catecholamines like hydroquinone, can also be oxidized to their corresponding o-quinones. A typical example is that of dopamine in Equation 2.

Equation - 1



Hydroquinone Benzoquinone

Equation - 2



Dopamine An "O-quinone"

This type of redox reaction can take place since only a relatively simple electron transfer occurs. In the chemical oxidation of catecholamines, oxidizing agents act as acceptors of electrons. In

electrochemical oxidation, where the reaction takes place on the surface of electrode, an electrical source such as batteries withdraws the electrons away from the electrode surface. Therefore if oxidizable substances are contained in the medium passing over an electrode, a change in the current being recorded through the circuit will occur as they pass over the electrode. It is on this principle that all detector and electrochemical analytical methods have been developed, such as polarography and voltammetry. What makes EC detection different from the other electro-analytical methods is that the applied potential through the electrode is kept constant. In voltammetry, current changes are monitored when the potential is swept. In EC detection, changes in the current, i.e. changes in the concentration of substances passing over the electrode, are simply monitored.

Consider the oxidation of dopamine, dopamine is oxidized on the surface of the electrode according to equation 2 shown above, resulting in the release of 2 electrons and 2 protons. The release of these electrons, reaction current, is amplified and the signals are recorded on an appropriate device, such as a strip chart recorder, as responses to the oxidation reaction. The size of the response recorded is proportional to the concentration of the electrochemically active substance on the electrode surface. By keeping the parameters which affect the reaction rate and degree of reaction rate constant e.g. the mobile phase, the temperature and pH of the reaction medium and the ionic strength of the solution it is possible to carry out quantitative analysis.

Applied potentials and redox reactions

Since the applied potential to the electrode is kept at a constant voltage throughout an experiment, it is important to know what effect change the potential might have on the reaction properties. Lewis and Johnson (1978) carried out a series of experiments studying the effects of applied potentials on the degree of reaction. Voltammograms for four hypothetical compounds (A,B,C and D) are presented in Figure 2.9.

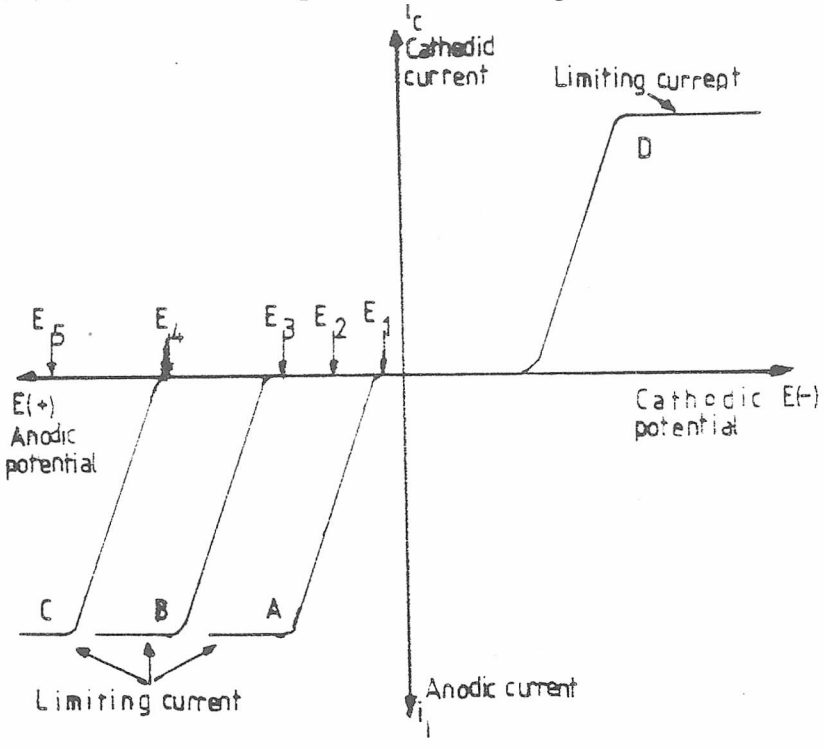


Fig.2.9 Relationship between applied potentials and reaction currents. Compounds A, B and C are oxidizable and compound D is reducible. The limiting currents for compounds A, B, C and D are equal in this figure, but are dependent on the concentration of the target compound.
From Hashimoto & Maruyama (1983)

Although the voltammograms are hypothetical, they are useful in the explanation of the selectivity of the EC detection method. Three of the compounds (A,B and C) are oxidizable while one (D) is reducible. Setting the potential at E_1 against the reference electrode (typical reference electrodes are either silver-silver chloride (Ag/AgCl), or saturated calomel electrodes (SCE)), no

change in the reaction current will be observed because no compounds undergo electrochemical reactions at this potential. A gradual increase in the positive charge been applied, results in a current increase. This current increase is caused by the oxidation of compound A and is roughly proportional to the increase in the applied potential. This increased current will be recorded on a chromatogram as an increase in peak height. Point E_2 in Figure 2.9 is at the centre of the sigmoid curve for A. In electrochemistry this point is referred to as the "half-wave potential", which is the measure of the feasibility of electrochemical reaction and is also used for identification because the potentials are characteristic values for electrochemical active species under identical conditions. Increasing the applied potential to point E_3 will cause a further increase in the measureable current, but increasing the potential past point E_3 will have no effect on the measured current of compound A. This current is referred to as the "limiting current". At this point the oxidation of A is controlled only by diffusion of A to the electrode surface. Fixing the applied potential at E_3 results in only compound A being recorded in a chromatogram, even when other electrochemically active species are present in the sample. In other words, selective detection of compound A can be achieved by selecting the proper applied potential. Increasing the applied potential to E_4 will result in the oxidation of compound B as well as compound A and further increasing the potential to E_5 will result in the oxidation of compound C also. Therefore at E_5 all the oxidizable compounds are oxidized to their maximum. Compound D being a reducable compound is not affected by any positive increase in the applied

potential. So from points E_1 to E_5 compound D is electrochemically inactive.

Sensitive detection of compound A requires that the applied potential is adjusted to the limiting current for A (potentials higher than E_3). Fixing the potential at E_4 will give sensitive detection of compound A as well as compound B. This can cause problems in the identification of compounds especially when the half wave potentials of A and B are close and B interferes with A because of poor selectivity. There are two ways in overcoming this problem, (1) lowering of the applied potential to increase the selectivity of compound A at the expense of sensitivity. (2) Develop a chromatographic method that seperates compound A from B.

A list of half-wave potentials and peak potentials for some biogenic amines and their metabolites are presented in Table 1.3 (Felice and Kissinger, 1976, Ponchon et al, 1979 and Richards, 1979). When the half-wave potentials are determined by EC detection (hydrodynamic voltammograms) the values obtained are usually higher than those listed in Table 3. This is because voltammetric determinations of half-wave potentials are normally conducted under 'static' conditions while EC determinations are 'dynamic' because of the rapid transfer of column effluent through the EC detection cell (Hashimoto and Maruyama, 1983). This forced introduction of solutions probably causes disturbances in the elctrical double layer which is produced in the vicinity of the electrode surface. This changes the reaction mechanism from that of static solutions, with the resultant shift in half-wave

potentials. These changes though are academic since optimisation of chromatographic conditions are established prior to routine determinations.

Table 1.3

HALF-WAVE POTENTIALS OF SELECTED BIOGENIC AMINES AND THEIR METABOLITES

COMPOUNDS	Half-wave potentials	
	Ponchon et al (1979)	Kissinger et al (1979)
DOPA	0.450	-
3-METHOXYTYRAMINE	0.460	-
DOPAMINE	0.200	-
HOMOVANILLIC ACID	0.530	0.63
NORMETANEPHRINE	0.500	-
NORADRENALINE	0.240	-
VANILMANDELIC ACID	0.570	0.63
ADRENALINE	0.380	-
5 HYDROXYTRYPTAMINE	0.340	-
5-HYDROXYINDOLE ACETIC ACID	0.500	0.55
3,4-DIHYDROXYPHENYL ACETIC ACID	-	0.51
	(vs. SCE)	PEAK POTENTIAL

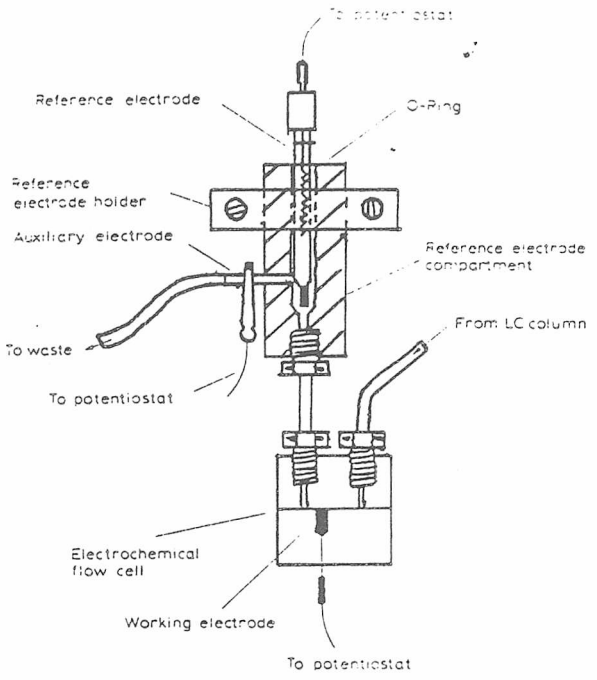


Fig.2.10 Structure of an electrochemical detector flow cell. (Reprinted from LC-4 amperometric controller operations/maintenance manual, Bioanalytical Systems Inc., West Lafayette, Indiana).

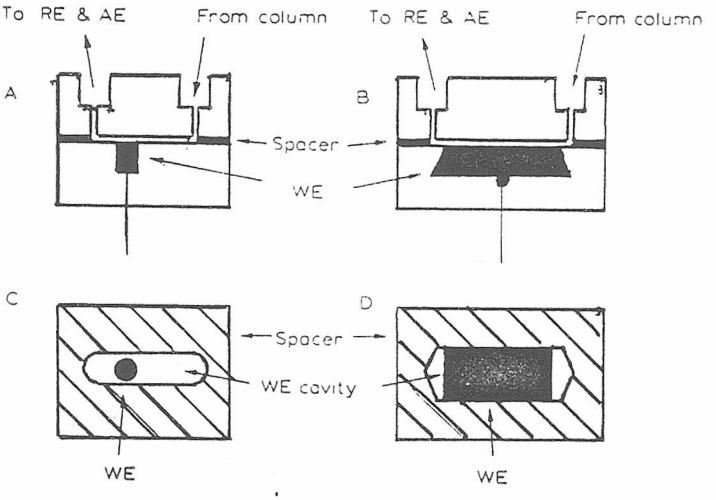


Fig.2.11. Cross sections of an amperometric detector cell (A and C) and a coulometric detector cell (B and D). (Abbreviations: AE = auxiliary electrode, RE = reference electrode, WE = working electrode.)

Design and function of the electrochemical detector

EC detectors can be divided into two types according to their cell design and electrolytic efficiencies; amperometric and coulometric, though there are no fundamental differences in the principles of current change associated with electrochemical redox reactions. Figure 2.10 is a diagram of a typical amperometric detector which is the basis of all cell designs to date. The surface area of the working electrode in the flow cell is relatively small ($-0.5cm^2$) and their electrolytic efficiency is (= coulometric yield) is low, usually in the range of 1-10%. In contrast to their low electrolytic efficiencies, compensation of background current, which flows steadily through the circuit is feasible because the background current is very low. Since the surface area of the working electrode is small it is possible to situate it centrally in the flow cell where the solvent flow is continuous and steady. As a result, the detectors have minimal noise induced by fluctuations in solvent flow in the cell despite the fact that the detectors are very flow sensitive. Figure 2.11 shows cross sections (vertical and horizontal) of amperometric and coulometric detectors.

Examination of the design of coulometric detectors shows that a continuous and steady flow of column effluent is not possible in the vicinty of the inlet and outlet ports. The tubular flow from the column suddenly changes to a thin, film-like flow just after the inlet port and reverts to a tubular flow after the outlet port. Noises arising from these changes in flow characteristics

are probably the major drawbacks in using coulometric detectors. (Takata and Muto, 1973, Johnson and Larchelle, 1973). These detectors were designed to improve coulometric yield and this efficiency is much higher than in amperometric detectors. To achieve this the surface area of the working electrode had to be increased. This larger surface area account for the flow noise generated as well as creating a large dead volume in the detector cell and an increased background current. This can lead to a reduction in the resolution of the chromatographic peaks and difficulties is steadying the background noise. Overall there is no significant difference in the performance of the two types of detectors, although amperometric detectors are in more general use since operation and maintenance is easier.

The choice of material for the working electrode depends on the type of analysis being carried out. Metal mercury is the most useful working electrode material for the voltammetry, and especially for polarography. However mercury electrodes are not suitable for the detection of biogenic amines primarily because at positive potentials where biogenic amines are detected, the main electrode process is the oxidation of mercury to mercurous (Hg_2^{2+}) or mercuric (Hg^{2+}) ions. Therefore, an electrode material which is resistant to oxidation and physically stable is necessary for the EC detection of biogenic amines. The most frequently used materials are carbon paste, glassy carbon and platinum, with glassy carbon and carbon paste making the best electrodes. Carbon paste, prepared by kneading carbon powder with an oil such as liquid paraffin or silicone grease, is highly sensitive and has a low

background current when carefully prepared, packed, polished and conditioned before analysis. The main disadvantage of carbon paste electrodes is their relatively short working life (several months), and susceptibility to damage from air bubbles and sudden changes in flow rate. Glassy carbon electrodes are more durable and stable, but their performance is inferior to that of carefully prepared carbon paste electrodes. Ultimately the choise of detector system used depends on the analysis to be performed.

Silver-silver chloride electrodes and saturated calomel electrodes (SCE) are commonly used as reference electrodes while stainless steel and platinum are used for the auxiliary electrodes. The dead volume of the electrochemical cell is determined by the thickness of teflon spacer, the length and width of the working electrode cavity, which is relatively small and does not exceed 50 (the typical dead volume for amperometric detector is less than 10).

PART 3 - SCHIZOPHRENIA

The Condition of Schizophrenia

In the eye of the general public, schizophrenia is a form of mental illness in which the patient suffers from a split personality i.e. the patient thinks he is somebody else (a famous historical character) or the patient has two or more different personalities sharing the one body. In clinical reality, the observation of such cases is rare and the diagnosis of schizophrenia covers a wider range of abnormal behavioural states.

What is schizophrenia? Answering this question is difficult because as Kendell noted in 1975, in the absence of an identified etiology, the clinical definition of the syndrome must be the starting point. This is further complicated as considerable disparities exist in the literature among investigators as to the diagnosis of the disorder and their conceptualization of the essential defining symptoms. This situation is best understood from a historical perspective of the syndrome. One school of thought followed Kraepelin (1896) and his description of the condition. Kraepelin was a careful observer and classifier of abnormal mental phenomena and distinguished between patients with manic-depressive illness and patients with dementia praecox (schizophrenia). This classification was mainly on the basis of the chronicity and progressive deterioration of the latter group. He also recorded importance to the presence of hallucinations, delusions, affective abnormalities and stereotypes. Kraepelin

regarded the illness to be found mainly in early adulthood and, despite an intact sensorium, reflected the presence of an organic cerebral pathology.

A second school of thought followed the observations of Bleuler (1930, 1950) who first introduced the name 'schizophrenia'. His observations on the condition considerably broadened it by including a heterogeneous group of disorders (e.g. alcoholic psychoses). Bleuler regarded the diagnosis of schizophrenia as quite variable and he also suggested that typical manic-depressive symptoms were also part of the syndrome. Rather than diagnosing schizophrenia on the basis of its natural history, Bleuler emphasized two types of cross-sectional symptomatic criteria. In one section of his book (Bleuler, 1950) he described nine "basic symptoms and signs" which he believed to be present only and always in schizophrenia during both the actute and chronic phases of the disorder. These nine basic symptoms have been reformulated as "the four A's of schizophrenia", ambivalence, autism, associated loosening, and affective inappropriateness. Also listed were seven "accessory" symptoms which he said were not unique to schizophrenia nor necessarily present in parties with the condition. These symptoms included delusions and hallucinations.

Bleuler's basic symptoms were used as the bases for diagnosis of schizophrenia between the 1930's and the 1960's, when psychiatry was dominated by a psychoanalytic approach. Thus prior to the

1960's in the USA this lead to an overly inclusive use of diagnostic entity schizophrenia based on the unreliable basic symptoms of Bleuler.

In the 1960's a study was set up to determine why the rate of diagnoses of schizophrenia was higher in the United States than in Britain (Cooper et al, 1972). This showed that psychiatrists from the United States often diagnosed a person as schizophrenia whereas in Britain they would have been diagnosed as suffering from conditions such as neurosis, personality disorders, mania and depression. The results of this study lead to the development of some reliable, phenomenologically based criteria for the diagnosis of schizophrenia and other major psychiatric disorders.

Critical symptoms used in the diagnoses of schizophrenia

Schizophrenic symptoms are classified as "negative or positive". The negative symptoms are produced by the disease process itself, while the positive symptoms originate from an adaptive evolution of the remaining, healthy parts of the nervous system. Under this discription, negative symptoms include affective flattening, poverty of speech and the loss of motivation, while positive symptoms take the form of hallucinations, delusions or thought disorder. Schneider in 1957 and 1959 identified a number of symptoms which he thought were the most representative positive symptoms found in schizophrenic patients. These symptoms, called the first rank Schneiderian symptoms, were stated by that worker to be of primary importance for the diagnosis of

schizophrenia when they occur in the absence of coarse brain disease. It should be emphasized though that schizophrenia can not be diagnosed on the basis of delusions or hallucinations alone. The advantage of the first-rank symptoms is that they are positive manifestations of psychosis which are reliably identified by clinicians (Mellor, 1970). For this reason, first-rank symptoms often form the core of categorical diagnostic rating instruments for schizophrenia.

The manner in which the diagnostic criteria for a condition is constructed, has implications in the attempt to find a pathological correlation for the syndrome. Specifically, what is gained in diagnostic reliability by heavy reliance on positive symptoms or on even the more restricted Schneiderian first-rank symptoms may lead to a loss of validity. This loss of validity is a serious problem in the diagnosis of schizophrenia because it does not have an established constant validity in terms of independant confirmation by physical examination or known laboratory tests.

Most currently used diagnostic instruments tend to include both cross-sectional (e.g. acute symptomatic manifestations) and longitudinal features, but with greater emphasis on the former (Endicott and Spitzer, 1979). An example of this combined approach is the Diagnostic and Statistical Manual (DSM -III) used by psychiatrists in North America. In most diagnostic systems there is a tendency to downplay the negative symptoms of schizophrenia. This problem of diagnostic emphasis in part stems

from the fundamental difficulty of rating the absence of normal phenomena as opposed to the presence of abnormal ones.

One of the problems associated with the identification of first-rank symptoms and other positive symptoms is that they have also been reported to occur in conditions, as, major affective disorders, delirious states and in organic brain disease. Pope and Lipinski (1978) concluded that first-rank symptoms alone are not diagnostic of schizophrenia. Even when they are present, they have little predictive value with respect to the course and outcome of the disease, (Carpenter and Strauss 1973, Carpenter et al, 1981). Studies, during the last two decades have generally supported the original findings of Stephens et al (1966) that the acute onset and florid positive symptoms are associated with a much better long-term prognosis of schizophrenia.

The classical Kraepelinian markers of the clinical course of schizophrenia e.g., chronicity and irreversible deterioration, do not correlate closely with the presence of first-rank symptoms and are likely to have a different clinical significance. The progressive characteristics of the condition are sometimes confusingly referred to as "negative features" and should be distinquished from the negative symptoms referred to above. The Krapelinian features can infact still be considered an important part of schizophrenia (Mackay, 1980), both that they remain stable despite the waxing and waning of positive symptoms, and that when considered together with the negative symptoms, they represent a major aspect of the morbidity of the disorder.

In summary it can be seen that firstly there has been an evolution in the criteria used in the diagnosis of schizophrenia, especially since the 1960's with the more recent formulations resulting in a more homogeneous group of patients with presumably less contamination by patients suffering from primary affective disturbances. Secondly, there has been an increasing appreciation from a large number of cross-sectional and longitudinal studies that the first-rank and other positive symptoms, which are present during the acute psychotic episodes accompanying schizophrenia, have poor diagnostic reliability when taken alone and little predictive ability to the course of the disorder. These findings raise serious questions concerning the intrinsic or etiological association of these symptoms with the disorder. Finally, growing evidence supports the prognostic validity of the Kraepelinian primary symptoms and negative symptoms seen in schizophrenia, although difficult to evaluate in an objective fashion, which may represent a more stable and fundamental manifestation of the underlying pathology.

Schizophrenia - theories of causes

The dopamine theory of schizophrenia, first postulated in the early 1970's is still one of the most important conceptualizations pertinent to the biological basis of behaviour. The theory arose out of the use of neuroleptics in the treatment of schizophrenia (Mathysse, 1973; Randrup and Munkvad, 1972; Snyder et al, 1974) and of stimulants known to potentiate dopaminergic neuronal function. (Connell, 1958; Ellinwood, 1967). The theory in its

original form, postulated that dysfunction of the dopaminergic neuronal system is fundamental in the symptomatic manifestations of schizophrenia. Improvements in the clinical understanding of schizophrenia and in the biochemical analysis of the dopaminergic system over the last decade has lead to a reappraisal of the dopamine theory.

Three main questions arise out of the dopamine theory.

1. Is dopamine an etiological factor? The most widely stated version of the theory is that dopamine hyperactivity is an etiological factor in schizophrenia. If this were the case, reductions in dopamine activity would eliminate the symptoms.
2. Is dopamine a symptom factor? In this case, dopamine activity would be required for symptoms to occur regardless of whether it has a role in etiology. If this is so, dopamine activity will be entirely normal in schizophrenics but will be essential for the mediation of some other fault, that is in turn the etiological factor.
3. Is dopamine reduction a necessary and/or sufficient condition for symptom remission? With schizophrenia being such a complex condition it is hard to assume that it is caused by a single process for which the neuroleptics are a kind of "magic bullet", i.e. that dopamine reduction is both necessary and sufficient for symptom remission.

Dopamine as an etiological factor.

Data supporting the theory at this level can be from biochemical or pharmacological studies. If there is a biochemical abnormality in schizophrenics causing dopamine hyperactivity, the neuroleptics would produce their symptom remission by reducing the dopamine activity. A variety of biochemical indices have been used in attempts to differentiate schizophrenics from normal subjects. These include dopamine itself, homovanillic acid (HVA, a major metabolite of dopamine), prolactin levels (prolactin release is inhibited by dopamine (Boyd and Reichlin, 1978)), dopamine-B-hydroxylase (DBH), and the number of dopamine receptors in brain.

The analysis of dopamine and HVA in post-mortem brain material gave no significant difference between schizophrenics and normals. (Bird et al, 1979, Crow et al, 1979). Similarly, baseline levels of HVA in cerebrospinal fluid showed no difference between schizophrenics and normals (Post et al, 1975, Carlson, 1978). Prolactin levels also fail to differentiate schizophrenics from normals (Kleinmann et al, 1979, Meltzer and Fang, 1976).

If the number of dopamine receptors in the brains of schizophrenics is increased, this could produce a functional hyperactivity of dopamine in the absence of an elevation of the amine itself. Lee et al (1978) and Seeman (1979) reported an increase in dopamine receptors in post-mortem brains of schizophrenics but Snyder (1981) has suggested this increase could be due to the long term treatment with neuroleptics. More recent

studies by Lee and Seeman (1980, 1982) in studies of brains from drug free patients have shown that an increase in receptor numbers does occur in the caudate, putamen and the nucleus accumbens. However, there is still some doubt whether these receptors are necessarily the functionally relevent binding sites for dopamine. Overall the best that can be said for these data is that they are promising but controversial and have not yet linked dopamine to the etiology of schizophrenia.

Pharmacological studies have used a number of compounds known to potentiate dopaminergic neruonal function. Amphetamine is the most commonly used compound, in these studies. Other compounds examined include, cocaine, benztropine, amantadine, phencyclidine, levodopa, bromocriptine and apomorphine. The discussion here will be limited to amphetamine, but for greater details about the other compounds see Carlton and Manowitz (1983).

Randrup and Munkvad (1967) and Ellinwood (1969) showed that prolonged and excessive use of amphetamine can produce a toxic state that mimics the symptoms of paranoid schizophrenia. These early studies suggested that the amphetamine action was associated with dopamine neurons as opposed to noradrenaline neurons. Unfortunately these studies have not proven to be entirely reproducable and it is now thought that the action on noradrenaline neurons may be more critical (Hornykiewicz, 1978, Segal, 1975). Furthermore abnormalities in noradrenaline levels characterise paranoid, but not other schizophrenics (Bird et al, 1978; Farley et al, 1978). One theory in the action of amphetamine is that it increases dopamine receptor numbers. Howlett and Nahorski (1978,

1979) did show that acute amphetamine administration in laboratory animals does increase receptor numbers in striatal structures, but this increase wanes with prolonged treatment.

The fact that amphetamine treatment mimics paranoid symptomatology and not the broad range of symptoms is of interest. Davis and Cole (1975) showed that the paranoid symptoms in schizophrenics are generally less succeptable to neuroleptic treatment than the other signs. Thus the relevance of amphetamine psychosis to schizophrenia as a broadly defind clinical entity would appear to be limited. It might be expected that treatment of schizophrenics with amphetamine would exacerbate the symptoms. There have been conflicting reports when this is done and Kornetsky, (1976) reported a hyporesponsive response. Furthermore, in the minority of patients who do exhibit increased schizophrenic symptoms, pretreatment with neuroleptics has no effect (Van Kammen et al, 1982).

As can be seen there is an array of information that provides little support for the idea that dopamine hyperactivity is an etiological factor in schizophrenia as a broadly defined clinical entity. This lack of support may be a reflection of inadequate experimentation to date. It could be that the data is generally negative because the experiments have been trying to examine too broad a target as far as the symptoms are concerned. Also the emphasis on a single amine (dopamine) to the exclusion of others, results in the omission of consistant and reliable information.

Dopamine as a symptom factor : Laboratory evidence

There are a number of measures that have been widely used to try to elucidate the relationship between reductions in dopamine activity and symptom control. The four main ones are behavioural stereotyping, apomorphine emesis, receptor binding, prolactin studies.

Amphetamine and apomorphine (a dopamine agonist) can induce similar, but not identical repetitive, stereotyped patterns of movement in laboratory animals (Fray et at, 1980) which is antagonised by the neuroleptics (Cresse et al, 1976). This antagonism is not unique to neuroleptics, and antihallucinogenics attenuate the effect of neuroleptics (Berger et al, 1978). There is no lag phase on the onset of action of neuroleptics, while chronic treatment reduces the antagonistic effect on most of the components of the stereotypic patterns. The antagonism of stereotyping produced by neuroleptics is characterized by features that link it more closely to "extrapyramidal symptoms" than to symptom control, (Exrapyramidal symptoms are movement disturbances that are similar to known extrapyramidal dystfunctions eg Parkinson's disease).

Apomorphine also produces vomiting and again this action is antagonised by the neuroleptics as well as other compounds (Freedman and Giarman, 1956). It is known that this emetic effect is mediated by dopamine sensitive medullary structures in the brain. From a theoretical point of view, it is not clear just how

these primitive brain loci are to be implicated in the symptoms of schizophrenia.

Dopamine shows a high affinity when binding to membranes, and Seeman (1980) showed that the neuroleptics compete for occupation of these membrane bound receptor sites. Thus different neuroleptics can be categorized in terms of their affinities for dopamine receptors, but these affinities correlate very poorly with their clinical potencies. This could be taken as indicating that dopamine receptor binding is unrelated to clinical efficacy of the neuroleptics. Creese et al (1976) have also shown that many neuroleptics compete with haloperidol for the dopamine binding sites and their potencies in doing so are highly correlated with their clinical potencies. It has been suggested that the dopamine receptor has two freely interconvertible confirmations, an against mode and an antagonistic mode, but if this were the case there would be no difference in a drugs affinity for either form of the receptor. Thus the difference in binding affinities for the dopamine receptor remains unexplained.

Prolactin release is inhibited by dopamine and Boyd and Reichlin (1978) showed that the neuroleptics promote it. It has generally been assumed that elevated prolactin levels are maintained in the course of chronic medication in man and that the phenomenon thus parallels symptom management. Other groups have suggested that tolerance may occur (Bowers et al, 1982, Brown and Laughren, 1981). Elevation of the prolactin levels occurs rapidly after the initation of medication and the levels fall quickly on

the withdrawal of treatment, whereas the efficacy of neuroleptics continues for a longer time. (Meltzer and Fang, 1976, Ohman et al, 1980). Thus the prolactin response fails to show a parallel response to symptom remission.

As can be seen the, work done presents a discouraging picture of the theoretical issue of dopamine as a symptom factor in schizophrenia. The lack of pertinent data is due in part to the questionable relevance the various laboratory measures of reduced dopamine activity have to the core phenomenon of clinical symptom management.

Is reduced dopamine activity necessary and/or sufficient?

As noted earlier, it is scarcely credible that such a complex disorder as schizophrenia can be managed solely by reducing dopamine activity. With virtually all the research being carried out on the neuroleptics it implies this is a tacit assumption on behalf of investigators. (Carton and Manowitz, 1983). This being so it can obscure important theoretical issues. In investigating these theoretical issues it is advantageous to call all drugs that produce symptom control as neuroleptics and all other drugs as nonneuroleptics.

Experimental attention has focused almost exclusively on neuroleptics that reduce dopamine activity, but as has been shown there is much evidence that shows that dopamine reduction does not parallel symptom management. Also, use of these drugs ignores the

question of whether dopamine reduction may be sufficient but not necessary for symptom control i.e. are there neuroleptics that do not oreduce dopamine activity.

Propranolol is a B-adrenoceptor blocking drug that has been reported to reduce symptoms, (Roberts and Amacher, 1978, Yorkston et al, 1981), and several groups have suggested that it does not reduce dopamine activity (Belmaker et al, 1979; Ebstein et al, 1979, Sheppard, 1979). Therefore research into the action of propranolol could answer two crucial questions relevant to the theory. Firstly, is propranolol truly a neuroleptic, that secondly fails to reduce dopamine activity. If so it would indicate that dopamine reduction is sufficient, but not necessary in the control of schizophrenic symptoms.

If all drugs were to be either neuroleptics that reduced dopamine activity or nonneuroleptics without any dopamine reducing action, then a reduction in dopamine activity would be both necessary and sufficient. If this were the case it would be a logically impossible task because proving all nonneuroleptics have no dopamine reduction activity amounts to confirming a universal negative (Carlton and Manowitz, 1983). It is therefore reasonable to assume that some nonneuroleptics can reduce dopamine activity. Drugs capable of this type of action would suggest that a reduction in dopamine activity is necessary but not sufficient for the control of symptoms. Bulbocapnine falls into this category. It shares most of the actions of neuroleptics, but is widely cited as having no clinical neuroleptic activity. (Costall and Naylor, 1973

(a), (b), Papeschi, 1972, Tseng and Walasyek, 1972). This in turn may be due to its side effects limiting realistic clinical evaluation. Other drugs of this type may be -methyl dopa and -methyl tyrosine. Both of these drugs reduce dopamine activity but have no clinical efficacy (Meltzer and Stahl, 1976). This result suggests that neuroleptics reduce dopamine activity but also have another action not shared by -methyl dopa or -methyl tyrosine. The significant theoretical question raised by this is, not what other factor, to the exclusion of dopamine is involved, rather what factor in addition to the action on dopamine activity is shared by the neuroleptics, but not shared by the nonneuroleptics (Carlton and Manowitz, 1983).

As has been shown the dopamine theory of schizophrenia still remains important in the study of schizophrenia but it has been both inadequately tested and formulated. The laboratory tests of the theory do not faithfully mirror clinical reality and the theory itself has been questioned because it is too broad based. It is reasonable to assume that dopamine activity is related to only a factor of the phenomena observed in schizophrenia. So in the majority of investigations into dopamine activity will be inconclusive because the experiments have been inappropriately formulated. Overall, a more refined statement of the theoretical relationship is needed. Csernansky, et al (1983) have proposed that the dopamine hypothesis of schizophrenia should be though of as a dynamic process rather than a static one. The increased dopaminergic activity may represent a stage of a dopamine shcizophrenic process rather than its cause. Dopamine as well as

other neurotransmitters, may respond in an adaptive fashion to stimuli that upset the equilibrium of the brain. One such stimuli could be an epileptic focus in the temporal lobe. The above formulation of the dopamine theory is only one of several that could be suggested. Furthermore, it and others like it will probably be proven to be inadequate. Still, the dopamine theory remains the main route of investigation into the biological determinants of schizophrenia.

Recent interest in the role of noradrenaline in schizophrenia has raised much speculation about the development of new antischizophrenic drugs acting on the noradrenergic system. Two phases of research have lead to the connection of noradrenergic system and schizophrenia. Wise and Stein (1973) first reported a deficit of dopamine-B-hydroxylase activity in schizophrenic post-mortem brain tissue. This enzyme synthesis noradrenaline and hence, a reduction in its activity, would imply a reduction in noradrenaline synthesis in schizophrenics. However, Wyatt et al (1975) failed to confirm these findings. Interest in the noradrenaline theory was rekindled by Farley et al (1978) who reported an increase in noradrenaline levels in the limbic areas of out patients with paranoid schizophrenia at post-mortem. These findings were confirmed and expanded by Hornykiewicz (1978). So a possible dysfunction in the noradrenergic system as well as the dopaminergic system should be taken into consideration when examining the causes of schizophrenia.

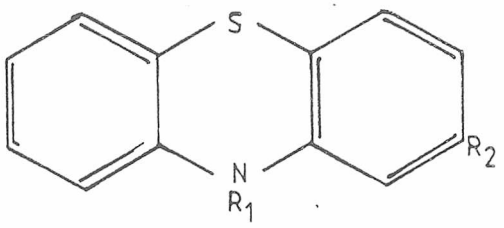
TREATMENT OF SCHIZOPHRENIA

Historically, the drugs used in the treatment of schizophrenia patients were referred to as neuroleptic compounds because of their ability to produce a state of 'neurolepsis' or calm indifference without the loss of consciousness. The neuroleptics are usually classified according to their chemical configuration. These groupings include phenothiazines, thioxanthenes, indole derivatives, butyrophenones, diphenylbutypiperidines and benzamide derivatives.

Phenothiazines

The phenothiazines are the most commonly used group of compounds in the treatment of schizophrenia, with chlorpromazine being the major drug of choice. Chlorpromazine was first synthesised by the French pharmaceutical firm, Rhone-Poulenc in 1950 and within 3 months they were testing it on patients. Today this quick transfer from the chemistry laboratory to the hospital would be impossible. Chlorpramazine was soon found to have pronounced calmative effects on disturbed pyschotic patients. Since then, chlorpramazine has become the standard by which all other neuroleptic compounds are measured, (Silverston and Turner, 1982). The phenothiazine group of compounds have a large number of pharmacological properties and small changes in the side groups of the nucleus varies the therapeutic activity and the severity of

therapeutic activity and the severity of side effects. The common nucleus of the phenothiazines looks thus.



The phenothiazine nucleus.

Substitution on the nucleus usually occurs in positions R_1 and R_2. R_1 side chains can be classified into three groups (1) dimethylaminopropyl (aliphatic) (ii) piperazine (iii) piperidine. Substitutions of the R_2 side chain include halogens, methoxy, acetyl, thiomethyl and other organic radicals. Compounds that are unsubstituted in the R_2 position have a reduced pharmacological action compared to compounds substituted with chloro-or trifluroro-methyl groups. Snyder (1976) suggests that the best acting compounds have been 'sculpted' to fit the dopamine receptor where they act by blocking dopamine transmittion. Also the more active phenothiazines are the most lipid soluble, thus facilitating their transport into the brain.

Carlson (1974) suggested that the phenothiazine drugs might act as dopamine receptors blockers. Subsequent work has shown that the phenothiazines block dopamine receptors in all four dopaminergic systems of the brain (Silverston and Turner, 1982). The post-synaptic block of the dopamine receptors induces a feedback mechanisim in which stimulation of presynaptic receptors causes the release of more dopamine to overcome the blockade. So the use of phenothiazines indirectly increase the release of dopamine and a subsequent increase in metabolites. Reduction in

this compensatory dopamine synthesis i.e. by ∝-methylparatyrosine, enhances the anti-psychotic activity of chlorpromazine.

The relative potencies of the various phenothiazine compounds is not the same at all sites. Thoridazine, which has an aliphatic side chain, increases dopamine metabolism in the nigro-striatal system. Chlorpromazine in turn is less effective than fluphenazine, which has a piperazine side chain, which is in keeping with the greater tendency of the piperazine phenothiazines to produce extrapyramidal symptoms. Drugs such as thioridazine, which are less likely to produce extrapyramidal symptoms are thought to have an inbuilt anticholinergic action (Silverston and Turner, 1982). It is thought that dopamine neurones in the striatum inhibit acetylcholine neurones, which normally activate gamma aminobutyric acid (GABA) neurones. These GABA neurones in turn inhibit the dopamine neurones. If the action of acetylcholine is blocked at the same time as the dopamine receptors then the effects on the motor system are cancelled out, but if the acetylcholine system is stimulated while the dopaminergic system is inhibited, extrapyramidal symptoms emerge.

The phenothiazines are also potent antiemetic drugs, preventing the vomiting produced by other drugs such as apomorphine, which stimulates the dopaminergic chemoreceptor trigger zone (Freedman and Giarman, 1956). An action on the neighbouring medullary areas might be responsible for their usefulness in the control of persistent hiccough.

The phenothiazines also act on the hypothalamic pituitary portal pathway which controls the release of prolactin from the pituitary. Dopamine inhibits the release of prolactin so the phenothiazines increase the serum levels of prolactin through dopamine receptor blockade. Kolakowska et al (1979) have shown that a sudden increase in prolactin levels after chlorpromazine treatment results in the appearance of extrapyramidal symptoms.

Thioxanthenes In these compounds the nitrogen in the central ring of the phenothiazine is replaced by carbon. The analogues thus formed appear to be no more effective than their phenothiazines progenitors.

Butyrophenones Although the butyrophenone compounds are structurally distinct from the phenothiazines they share many of their pharmacological properties. Like the phenothiazines they cause accumulation of O-methylated metabolites of dopamine and noradrenaline within the brain, suggesting that they also block dopamine and noradrenaline receptors. Butyrophenones like haloperidol and trifluoperidol selectively block dopamine receptors in the peripheral autonomic nervous system, and it could be that a similar action occurs in the central nervous system (Davis and Garvier, 1978). They are also potent antiemetic compounds and produce extrapyramidal disorders similar to those produced by phenothiazine treatment.

Diphenylbutylpiperidines These compounds are derived from the butyrophenones and have a longer duration of action. It also appears that they have a more specific dopamine receptor blocking action.

Benzamide derivatives Sulpiride and metoclopromide, two compounds belonging to this group of compounds have been shown to be useful in the treatment of acute and chronic schizophrenia. Pharmacologically, these compounds differ from the phenothiazines and butyrophenones in that they have a greater activity in blocking D_2 dopamine receptors than D_1 dopamine receptors. Theoretically this might result in this group of compounds producing fewer unwanted extrapyramidal symptoms (Jenner and Marsden, 1979).

Other Drugs Tetrabenazine is a synthetic benzoquinolizine which retains some of the structural features of reserpine and has a similar pharmacology. It depletes the brain of monoaminerigic stores and this is probably the basis of its sedative and antipsychotic effects. Some of the side effects of this compound though are mental depression and extrapyramidal symptoms. Its dopamine depleting property is thought to be useful in the management of tardive dyskinesia (Silverston & Turner, 1982). Certain indole derivatives, such as oxypertine and molindene appear to possess antipsychotic properties. (Davis and Gravier, 1978), while other dibenzyl structures including clozapine and metrapine have similar effects with less tendency to produce Parkinsonian symptoms.

Unwanted effects of neuroleptic drugs

Extrapyramidal symptoms

Since the introduction of the phenothiazines in the 1950's the minor side effects of the compounds have been easily observed. With the use of these drugs in the long term maintenance of schizophrenia the emergence of serious extrapyrimidal Parkinsonian like symptoms has occurred. This casts doubts on the current use of the phenothiazines and their use in the future. The most serious of these extrapyramidal symptoms is tardive dyskinesia which has been investigated and still is being investigated by many workers (Crane, 1978, Kidger et al, 1980, Klawans et al, 1980, Mackay and Sheppard, 1979, Marsden and Jenner, 1980, Marsden and Schachter, 1981).

Overall the extrapyramidal symptoms can be considered under four headings. Such adverse reactions occur in up to 40% of patients treated with phenothiazines and are equally frequent with the other classes of antipsychotic drugs.

1) Acute dystonia. This is an involuntary contraction of skeletal muscles most frequently occurring in the head and neck, giving rise to what is frequently referred to as an oculogyric crisis. It begins with a fixed stare, followed by an upwards turn of the eys, with hyperextension of the neck and opening of the mouth. The attack may last for several hours before subsiding

spontaneously. The reactions can also involve the trunk and limbs, producing grotesque postures and writhing movements which are extremely distressing.

2) Pseudoparkinsonism. This clinically observed condition mimics idiopathic Parkinsonism very closely, with a stiffening of limbs, lack of facial expression, a characteristic coarse tremor of the hands and head at rest, plus sialorrhea and seborrhoea. It may progress to a complete seizing up with virtual absence of movement. It is observed in up to 40% of patients treated, being especially common in elderly female patients.

3) Akathesia. This condition which is characterised by minor restlessness and tension causes great distress to the patient who finds that he cannot keep still. This can lead to a characteristic fidgetiness, with the patient continuously placing his weight first on one foot and then another. The subjective sense of restlessness can be so persistent and pervasive that the patient finds it impossible to get to sleep.

The three conditions are relatively acute and are also dose-dependent and disappear when medication ceases.

4) Tardive dyskinesia. This term relates to a chronic syndrome of hyperkinetic involuntary movements which are most frequently limited to the face, lips, tongue, jaw and neck, but can also involve the trunk, hands and arms. There would appear to be two distinquishable sub-type of the syndrome. The first, and by far

the most common, is referred to as the 'bucco-linguo-masticatory' (BLM) syndrome. It ranges from infrequent lateral movement of the jaw together with puckering and pouting of the lips and slight tongue movements which distend the cheek, to a clinical picture dominated by unceasing movements of the lower face associated with frequent mouth opening and protrusions of the tongue. The other sub-type includes movements of the whole trunk in which body-rocking, shoulder shrugging, back arching and even pelvic gyrations appear, the limbs display monoclonic jerks and respiratory arrythmias are also described. Some workers have classified this second group separate from tardive dyskinesia and catagorise them as 'drug-related encephalopathies'.

Tardive dyskinesia, mainly of the BLM type, has been reported in 10 to 30% of patients suffering from chronic schizophrenia. It is generally associated with long term treatment with anti-psychotic drugs and is very rarely seen before such treatment has lasted at least 6 months, and is uncommon before 4 or 5 years. Interestingly there are a number of well documentated patients who have never taken or been given any antipsychotic drugs and yet who show unequivocal tardive dyskinesia.

Although the symptoms are generally suspected as being a consequence of prolonged antipsychotic drug treatment, stopping treatment may well lead to a sharp exacerbation of the symptoms, and in some patients they are observed for the first time on stopping antipsychotic drugs. It is thought that tardive dyskinesia is caused by an increase in sensitivity of dopamine receptors. Stopping the

blockade of these supersensitive dopamine receptors by stopping the drug only further increases the action of dopamine, giving rise to abnormal movements. It appears that this dopamine super-sensitivity is confirmed largely to the dopamine receptors in the neostriatum. The fact that the syndrome has been observed in patients who have never received antipsychotic drugs, and the repeated observation that it is much commoner in elderly patients has lead to the suggestion that antipsychotic drugs merely accelerate a naturally occuring degnerative process within the extrapyramidal system leading to dopamine receptor sensitivity. In other words, it occurs as a result of both morphological and pharmacological denervation, and it is perhaps because of the morphological element that it often persists for years after all antipsychotic drugs have been stopped.

Overall it can be seen that the drugs used today for the treatment of schizophrenia only deal with a few of the obvious manifestation of a complex disease condition. It can also be seen that the drugs of choice used result in disturbing side-effects for many of the patients. To these ends it is obvious why continuous research is underway to try to find better drugs for the treatment of schizophrenia.

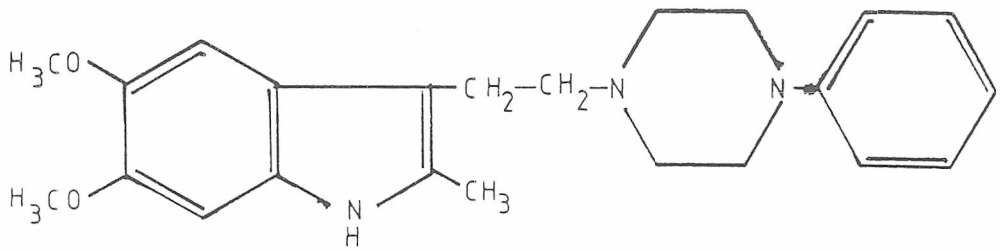
OXYPERTINE

Introduction

Oxypertine is an example of a compound whose structure was rationally deduced from available knowledge and concepts. In the late 1950's there was a great interest in the indoles and their relation to mental disease. Structurally, oxypertine is an indole derivative.

(See figure 3.1)

Figure 3.1



Structure of Oxypertine.

Oxypertine was formed by adding phenylpiperazine to 5HT, to increase the lipid solubility of 5HT, and enable it to pass across the blood brain barrier. The phenylpiperazine molecule also has some adrenoceptor antogonistic action (Penn, 1972) and it was postulated that this might be of value in the treatment of mental disease. Unfortunately, there are no specific animal test for the effectiveness of compounds in the treatment of mental disease. However, tests can be made for a drugs potential effectiveness, by comparing it with a drug of known action. Some of these tests carried out by Wylie and Archer (1962) are briefly described.

Potentiation of Hexobarbitone Anaesthesia

Initially mice were given the test drug, then later a sub-hypnotic dose of intraperitoneal hexobarbitone. The number of mice losing their righting reflex for one minute or more over a period of 20 minutes was counted and the ED_{50} calculated. The ED_{50} is the dose effective in 50% of the test animals, and is a common way of expressing this type of result. Oxypertine behaved very similar to chlorpromazine.

Adrenergic Blockade

Intravenous adrenaline and noradrenaline are toxic to the rat, so test substances were given simultaneously with a lethal dose of adrenaline or noradrenaline. The amount of protection given by a compound was assessed by comparing the death rate in the test group of animals with control. Again oxypertine behaved similar to chlorpromazine.

Mouse Head-Withdrawal Reflex

The effect of drugs on conditioned reflexes is informative, and a simple one was used to test oxypertine. Mice withdraw their heads when their vibrissae are touched. This reflex was blocked both by oxypertine and chlorpromazine, but not by barbiturates.

These three animal tests showed that oxypertine was similar to chlorpromazine, and that it potentiated central depressant actions, blocked the actions of adrenaline and noradrenaline, but above all

it reduced or eliminated some of the basic conditioned reflexes such as the mouse head reflex.

The Rhesus Monkey

Another animal test involved the rhesus monkey. This animal is inherently aggressive, but this may be changed quite characteristically by psychoactive drugs.

If an intruder enters the monkey's cage, it will either run away by climbing -'flight', perhaps with an initial show of agressiveness, or fight. Reduction (taming) or any other change in behaviour can be assessed by a skilled observer and is very dependant on the amount of drug given.

Oxypertine (1mg/kg oral administration) reduced the flight or fight reaction in thirty minutes. The animal would sit at the open front of the cage and permit itself to be handled. The animal was still aware of its environment and would react to auditory stimulus. Increasing the intensity of the auditory stimulus would return the monkey to its hostile state. Therefore oxypertine produced a perceptible reduction in the avoidance behaviour of the monkey.

With a larger oral dose, 2mg/kg, the monkey would go through a similar period of less agressiveness, but sometimes froze in the middle of a movement, even in an awkward position, for 15 to 30

seconds. Further increasing of the dose to 8mg/kg produced a rapid increase in these cataleptic periods, with eventual complete central depression in which the animal remained immobile in bizarre positions. The animals appeared totally withdrawn from its environment and would not respond to pain. This crude overt behaviour study showed three phases of drug action, A) Taming B) Depression and C) Catatonia.

Overall the animal tests carried out by Wylie and Archer (1962) suggested that oxypertine could be an effective major tranquillizer. Cole and Edwards (1964) however showed that oxypertine at low doses (2mg/kg) potentiated the stimulatant effective of amphetamine on motor activity in rats, while larger doses depressed this effect. They suggested that with oxypertine it might be possible to activate the depressed but quieten the overactive, the degree of stimulation lessening with the increase in dose.

Biochemical Studies

The first biochemical, neuropharmacological studies were carried out by Spector et al, (1962). They showed that oxypertine lowered the noradrenaline content of the rat brain rapidly. A fall of 60% in three hours after a single intraperitoneal injection, 60mg kg, of oxypertine. There appeared to be no effect on the 5HT concentration of the brain at the dose and they also noted an obvious central nervous system depression.

Matsuoka (1964) and Fuxe et al (1967) showed that pretreatment with MAOI's prevented the decline in brain noradrenaline levels caused by oxypertine. From this they concluded that oxypertine does not inhibit the synthesis of the catecholamines.

Matsuoka et al (1965) carried out morphological studies in the rabbit hypothalamus and showed a reduction in the number of end terminal vesicles in the neurones, along with a reduction in noradrenaline levels. They suggested that the vesicles may be the storage sites for noradrenaline and the reduction in their number might account for the reduction in noradrenaline levels. Hassler and Bak (1966) repeated the experiment in the substantia nigra of the rat and found that oxypertine produced little effect on the vesicles, and suggested this was because they were dopamine storage sites rather than noradrenaline storage sites.

Work by other groups (Goldstein and Nakajima, 1966, Fuxe et al; 1967) using high doses of oxypertine (35mg/kg - 70 mg/kg, IP injection) showed a reduction in dopamine and 5-HT levels as well as noradrenaline levels. These results were conformed by Bak and Hassler (1968). Bak et al (1969) using a wider dose range of oxypertine than previously used (5mg/kg to 70mg/kg) showed differences in the time of onset of action, and duration of action of oxypertine on different amine containing neurones. At the highest dose used (70mg/kg) they suggested that actual disruption of the vesicles could occur, causing the escape of all the biogenic amines, rather than the release of the amines which occurs with the lower levels of oxypertine.

Penn (1972) suggested an additional action for oxypertine, as well as amine depletion from nerve endings. In animals pretreated with MAOI, hyperexcitability occurred if they had also been treated with reserpine, while in animals treated with oxypertine sedation still occurred. Penn attributed this observation to an adrenoceptor antagonism by oxypertine, which prevented excessive stimulation through the amines released by oxypertine. Miranda (1978) studying the effect of oxypertine on isolated rat vas deferens, showed that oxypertine antagonised completely the effects of noradrenaline, 5HT and dopamine on the tissue. Thus again suggesting that oxypertine could be an adrenoceptor antagonist.

Clinical Studies

Initial psychopharmacological studies were carried out by Flament et al (1962). They investigated the effects of oxypertine on a group of schizophrenic patients with as wide a range of symptomatology as possible. The effects of oxypertine included an activation of, or disinhibition of catatonic stupor, improved affective contact, slight reduction in the intensity and frequency of auditory hallucinations and a slight elevation of mood. Aside from some transient slowness and drowsiness, oxypertine did not control effectively overactivity and impulsivity. Delusions were not diminished and somatic reactions resembled in part those seen with neuroleptics. It was suggested from the slight elevation of mood and activation caused by the oxypertine treatment, that oxypertine might be useful in the treatment of patients manifesting psycho-motor retardation and stupor. A combination of the work by

Flament et al (1962) and other clinical studies of the time concluded that oxypertine would be useful in the treatment of withdrawn schizophrenic patients.

Various studies into the uses of oxypertine were carried out through the 1970's. Tyson (1970) showed that oxypertine had anxiolytic and tranquillizing effects, and Bonn et al (1971) compared it with the benzodiazepines for the treatment of anxiety. Remr et al (1974, 1975) showed that oxypertine was as effective as some phenothiazines in the control of chronic apathetic schizophrenics and could be used in cases where phenothiazines were contraindicated for somatic reasons.

Tardive Dyskinesia

With the emergence of tardive dyskenesia in schizophrenic patients on long term drug treatment, interest arose in the possible use of oxypertine in treating this condition. A preliminary report by Freeman and Soni (1980) indicated that oxypertine might be useful in the treatment of tardive dyskinesia. At the same time Kasamatsuri (1980) reported that 7 out of 10 patients, diagnosed as suffering from tardive dykinesia, had no involuntary movements when receiving oxypertine. Soni et al (1982) confirmed their previously reported observation of tardive dyskinesia sufferers after an 8 week controlled study, but warned, as the condition is an extremely complex one, great caution was needed in the evaluation of any possible methods of treatment.

Low Dose Oxypertine Treatment

Sterling (1979) suggested that, for the treatment of withdrawn psychotics by oxypertine a daily dose of 80 - 120 mg would be sufficient. This dose in an average size human of 70kg is approximately 1mg/kg - 2mg/kg. This dose differs considerably from the doses used in the earlier animal experiments when 35mg /kg - 70mg/kg was used.

In an attempt to update the dopamine theory of schizophrenia, Ashcroft et al (1981) proposed a model in which the limits within which dopamine activity is tolerated are reduced. So rather than there being an overactivity of the dopamine system, the thresholds within which certain types of activity are controlled are reduced. To test this theory, Palamo and Russell (1983), examined the effect of oxypertine on amphetamine-induced behaviours in rats. They concluded that the amphetamine model was a useful experimental tool to test Ashcroft et al's (1981) proposed hypothesis of the dopamine theory of schizophrenia and that oxypertine appeared to be a drug capable of expanding the tolerated limits for aminergic activity. It is interesting to note that the dose range of oxypertine used by Palomo and Russell (1983) was 1mg/kg to 16mg/kg, and that 1mg/kg or 4mg/kg, but not 16 mg/kg increased the exploratory behaviour in rats with amphetamine induced activity. Palomo and Reid (1983) argued that if this increase in exploratory behaviour after oxypertine treatment was due to oxypertine being an amine depleting agent then it should be prevented by pretreatment with MAOI. Using pargyline (a MAOI) at a dose to inhibit both types A and B MAO they

prevented the increased exploration caused by oxypertine during amphetamine induced activity in rats. Therefore, they suggested that oxypertine at a dose of 1mg/kg to 4mg/kg in amphetamine induced behaviour is an amine depleter, effecting the amphetamine releasable pool of amines. Scheel-Kruger (1971) reported that there are two different pools of neuronal amines, the reserpine-resistant pool (amphetamine releasable) and the reserpine-releasable pool (vesicular pool), which according to Arbilla et al (1984) accounts for over 90% of the neuronal dopamine. Palomo and Reid (1984) investigated whether oxypertine releases amines from the reserpine-releasable pool of amines as well as the reserpine-resistant pool of amines. They studied the effect of oxypertine and reserpine on amphetamine and methylphenidate induced behaviour (Methyphenidate causes the release of the amines from the reserpine releasable pool of amines). Their results are in line with their previous work (Palomo and Reid 1983), that oxypertine appeared to deplete the reserpine resistant pool of amines, at 4mg/kg and appears to have no effect on the reserpine-sensitive pool. They concluded that oxypertine might provide a means to interfere with the pool of newly synthesised amines but that biochemical data was essential to confirm the behavioural evidence.

METHODS

1. Animal Husbandry

In all the experiments, male rats of the RGIT/Sprague Dawley strain were used. The animals were housed in constant conditions, - 9 hours light, 09:00hrs to 18:00 hours, 15 hours dark, 18:00 hours to 09:00 hours (intensity 30 lumens), temperature of 21+/-2^{o}c, humidity 50% + RH. A diet of Oxoid pasteurized breeding diet for rats and mice and water were freely available.

2. Chromatographic Solvents and Equipment

All chromatographic solvents used HPLC grade, purified water. This is prepared from distilled water by filtering through a Millipore, Milli-Q filtration system.

Chemicals used in solvents.

1. Lauryl Sulphate (Sodium Salt), (purchased from Sigma Chemical Company Ltd., Poole).

2. Tetraethylammonium (purchased from Sigma Chemical Company Ltd., Poole).

3. Sodium Phosphate, dibasic (Na_2HPO_4) Anhydrous, (purchased from Sigma Chemical Company Ltd., Poole).

4. Ethylenediaminetetraacetic Acid, Free acid, purified grade, (purchased from Sigma Chemical Company Ltd., Poole).

5. Acetonitrile, ALN grade, (purchased from Rathburn Chemicals Ltd.)

All solvents used were degassed under vacuum by an ultrasonic bath. Equipment used in chromatographic analysis.

1. Waters Assoc. M6000 A pump.
2. Specra Physics SP.5400 Isocratic Pump.
3. Waters Assoc. M440 fixed wavelength (254 mm) detector
4. Bioanalytical Systems LC4 detector
5. Bioanolytical Systems LCA B/17 detector
6. Rheodyne 7125 valve

The electrochemical detectors were fitted with polished glassy carbon electrodes.

Columns used were 200 x 4.6mm, slurry packed with 5 m ODS Hypersil (purchased from Shandon Laboratories).

Sample Preparation

Rat brain samples were prepared using the following chemicals and equipment.

1. Tissues homogenised by 1ml Potterten Glass Rod Homogenisers, (purchased from Anachem Ltd., Luton).

2. Filtered with BAS microfilter, through 1 m regenerated cellulose filter, (purchased from Anachem Ltd., Luton).

3. Sample homogenised in Perchloric Acid ($HClO_4$)
(purchased from Sigma Chemical Company Ltd., Poole)

4. Samples centrifuged in Fisons MSE Chillspin Centrifuge.

Standard Compounds and Drugs Used

A. Standards Compounds

Chemical standards used in the preparation of standard curves for the estimation of tissue amine and metabolite levels.

1. Adrenaline:- Epinephrine Bitartrate, (purchased from Sigma Chemical Company Ltd., Poole).

2. Noradrenaline :- (±) - Arterenol Hydrochloride (purchased from Sigma Chemical Company Ltd., Poole).

3. Dopamine:- 3-Hydroxytryptamine Hydrochloride (purchased from Sigma Chemical Company Ltd., Poole).

4. 5-Hydroxytryptamine Creatinine Sulphate (purchased from Sigma Chemical Company Ltd., Poole).

5. Vanilmandelic acid:- DL-4-Hydroxy-3-Methoxy-Mandelic Acid (purchased from Sigma Chemical Company Ltd., Poole).

6. 3, 4-Dihydroxyphenylacetic Acid (purchased from Sigma Chemical Company Ltd., Poole).

7. MHPG,:- 4-Hydroxy-3-Methoxyphenylglycol, Hemipiperazine Salt. (purchased from Sigma Chemical Company Ltd., Poole).

8. 5-Hydroxyindole-3-Acetic Acid, Free Acid (purchased from Sigma Chemical Company Ltd., Poole).

9. Homovanillic Acid:- 4-Hydroxy-3-Methoxy Phenylacetic Acid. (purchased from Sigma Chemical Company Ltd.)

B. Drugs used in vivo

Unless otherwise stated, all pharmacological agents and vehicles controls were administered by intra-peritoneal injections. Vehicles of injection varied from drug to drug.

1. Oxypertine (a gift from Sterling Winthorp).
   24mg was dissolved in 2ml of 0.5m Ascorbic acid and made up to 6ml with water. Given as a 4mg/ml solution.

2. Reserpine (purchased from Sigma Chemical Company Ltd., Poole).
   125mg of reserpine + 375mg of citric acid was dissolved in 6ml of benzylalcohol (warmed). 15ml of Tween 80 was added and made up to a final volume of 100ml with distilled water and stored out of the light. Given as a final solution of 1.25mg/ml.

3. Haloperidol (purchased from Sigma Chemical Company Ltd., Poole).
   1mg of haloperidol was dissolved per 1ml of 0.9% saline.

4. ∝-Methyl-DL-p-tyrosine Methyl Ester (purchased from Sigma Chemical Company Ltd., Poole). Dissolved in distilled water.

5. DL-p-Chlorophenylalanine Methyl Ester (purchased from Sigma Chemical Company Ltd., Poole). Dissolved in distilled water.

CHAPTER 1

The Development of a HPLC-ECD system for the determination of the biogenic amines and their metabolites

Introduction - Development of ion exchange-desolvation theory of separation

The theoretical origins of the retention of ion-pair chromatography (IPC); that ion pairs are formed between the ionised solute and the added pairing ion, and this complex is adsorbed onto the hydrophobic stationary phase is discussed in the general introduction. Kissinger (1977) updated this theory when he showed that the pairing-ion is adsorbed by the stationery phase and proposed on alternative mechanism of separation based on the in situ formation of an ion-exchange matrix. However, Hung and Taylor (1980) pointed out that the ion-exchange mechanism fails to take into account the hydrophobicity of the solute, therefore implying that separation is based on differences in the ion-exchange constants of various solutes. This also omits the indication that desolvation of the ion-pair is more readily achieved than that of the ionised solute alone.

Several groups of workers (Knox and Laird, 1976, Horvath et al; 1977, Ghaemi and Wall; 1979, Knox and Jurand; 1978) have shown that, over a wide range of mobile phase pairing-ion concentrations, the relationship between capacity factor and pair-ion concentration is complex. It may reach a plateau or indeed pass through a maximum

suggesting that the processes occurring during such chromatographic separations are considerably more complex than the ion-pairing, or ion-exchange approaches.

Hung and Taylor's (1980, 1981) address of this more complex relationship, resulted in an ion-exchange-desolvation model for the chromatographic separations they observed. Examining the surface coverage of a C_{18} bonded phase, they showed that the hydrophobicity of the pairing ion is a relevant property in its level of adsorption. For the commonly used quaternary ammonium pairing-ions, they showed that their adsorption to C_{18} silica varies, and the extent of the absorption depends more on the nature of the pairing-ion than its aqueous-phase concentration. They also reported similar results for the adsorption of four anionic pairing-ions. As a consequence of this, the adsorbed ion-pairing species may act as an in situ ion-exchanger for other solutes, and therefore reduce the C_{18} surface area available for the desolvation of solutes. They also showed that the presence of an organic modifier, i.e. acetonitrile, reduced the extent of column loading by the pairing-ion and that the effect is concentration dependant.

On the basis of their results Hung and Taylor proposed a mathematical model for the retention of an ionised solute in the presence of a pairing-ion on a C_{18} reversed-phase system. They proposed that the retention may be considered by two separate mechanisms.

a) The desolvation of the solute on the C_{18} surface. This may be minimal in the case of a highly ionised solute resulting in short retention times, but may be appreciable if the solute is hydrophobic even when ionised.

The nett retention volume is given by.

$$Vr1 - Vm = K_1 A'_s \quad \ldots\ldots\ldots\ldots\ldots\ldots \text{(i)}$$

Where Vrl is the measured retention volume, Vm is the volume of the mobile phase in the column, A'_s the surface area of the stationary phase available for desolvation and K_1 the desolvation constant or partition coefficient.

B) An ion-exchange mechanism by an ion-exchange equilibrium of the form.

$$(P_n + C^{n-})\ org + nA^-aq \rightleftharpoons n(P^+A^-)\ org + C^{n-}aq$$

$(P_n{}^+ C^{n-})$ org refers to a monovalent pairing ion together with the possible multivalent (n) counter ion adsorbed to the C_{18} surface, and A^- a monovalent anion as solute.

An ion-exchange constant can be written as

$$K_{IE} = \frac{[P^+ \quad A^-]org\ [C^{n-}]\ aq}{[P^+_n\ C^{n-}]org\ [A^-]^n org} \quad \ldots\ldots\ldots\ldots \text{(ii)}$$

From which, if the distribution for A^- between organic and aqueous phases is written as

$$D_{A^-} = \frac{[P^+ A^-]org}{[A^-]aq} \quad \ldots\ldots\ldots\ldots \text{(iii)}$$

by substituting for $[P^+A^-]$ org, you have

$$D_{A^-} = \frac{K_{IE}\,[P^+_n\, C^{n-}]\ org}{[C^{n-}]\ aq} \quad \ldots\ldots\text{(iv)}$$

This relationship alone does not allow for any desolvation of the ion-pair formed at the C_{18} surface due to the hydrophobicity of the solute. It does not account for differences in distribution, and thus retention, amoung different solutes other than on purely electrostatic terms. An explanation of the variation in retention is that an ion-exchange reaction occurs between the ionised solute and the absorbed pairing-ion followed by desolvation of the neutralized solute onto the C_{18} surface. This desolvation will be proportional to the hydrophobicity of the solute as described by a desolvation constant K_2, which will act to increase the electrostatic effect of ion-exchange. K_2, although analogous to K_1, may be of a different magnitude, since it refers a solute with no nett ionic charge. The retention of the bonded species ion ion-exchange will also be proportional to the area of the stationary phase available for desolvation, so that the nett retention volume for a solute retained by an ion-exchange-desolvation mechanism is given by.

$$V_{r2} - V_m = K_2\, A_S' \left(\frac{KIE\,[P^+_n\, C^{n-}]\ org}{[C^{n-}]\ aq}\right)^{1/n} \quad \ldots\ldots\text{(v)}$$

The total nett retention volume (Vr2 - Vn) is given by

$$V_{r2}\ V_m = K_1 A_S' + K_2 A_S' \left(\frac{KIE\ [P^+_n\ C^{n-}]\ org}{[C^{n-}]\ aq}\right)^{1/n} \quad .....(vi)$$

and the column capacity factor k' by

$$k' = \frac{1}{Vm} \quad K_1 A_S^1 + K_2 A_S^1 \left(\frac{KIE\ [P^+_n\ C^{n-}]\ org}{[C^{n-}]\ aq}\right)^{1/n} \quad .....(vii)$$

A_s is related to the total area of the C_{18} stationary phase, as by the relationship

$$A_S' = A_S - [P_n^+\ C^{n-}]\ org\ Ap \quad ..... (viii)$$

where Ap is the area per mole occupied by a particular absorbed pairing-ion. A_s is difficult to define, but can be thought of as the effective area available for adsorption of any species in terms of C_{18} surface.

Substituting A'_s in the expression for k' we obtain

$$k' = \frac{1}{Vm} \left(A_S - [P_n^+\ C^{n-}]org\ A_p\right)\left(K_1 + K_2 \left(\frac{KIE\ [P^+_n\ C^{n-}]\ org}{[C^{n-}]\ aq}\right)^{1/n}\right) ..(ix)$$

This equation relates the capacity factor of a given solute to the absorbed pairing-ion concentration and mobile phase counter ion concentration, taking into account the effective area of C_{18} available for desolvation. It shows a much more complex dependance of k' on absorbed pairing-ion concentration than has hitherto been suggested.

The form of the dependance of k' on adsorbed pairing ion concentration can be seen more readily if the situation of monovalent counter-ions only is considered.

The equation can be rearranged after setting "n" to unity as

$$k' = \frac{1}{Vm}\left(A_s K_1 - K_1[P^+C^-]org\, A_p + K_2 K_{IE} A_s \frac{[P^+C^-]org}{[C^-]aq} - K_2 K_{IE}\frac{[P^+C^-]^2 org}{[C^-]aq}\right) \quad (x)$$

k' is thus expressed as a quadratic in $[P^+C^-]$ org which exhibits a maximum volume. A schematic representation of the two processes treated above is shown in figures 4.1.

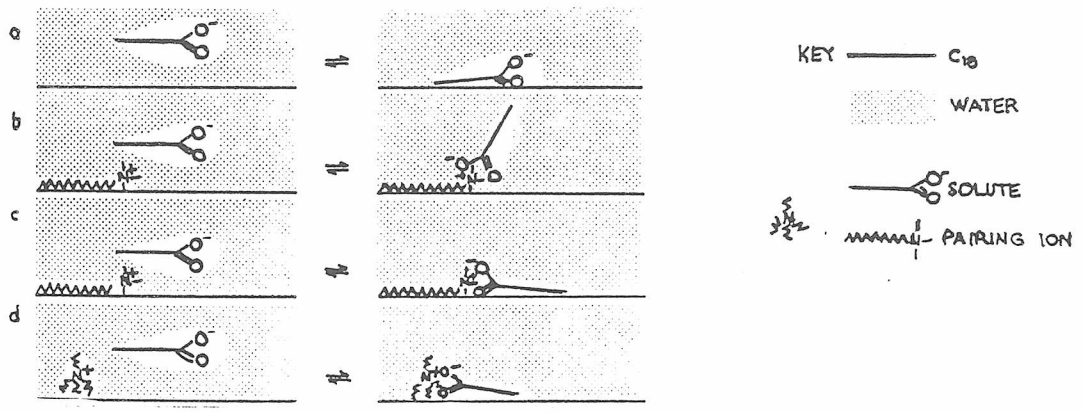


Fig.4.1 Schematic representation of the postulated retention mechanisms discussed in the text. (a) Desolvation of an ionised solute; (b) ion exchange involving an adsorbed pairing ion without subsequent desolvation of solute; (c) and (d) ion exchange reinforcing desolvation of solute for adsorbed surfactant (c) and non surfactant (d) pairing ions.

Following experimental testing of the mathematical model, Hung and Taylor (1980, 1981) concluded that the ion-exchange-desolvation model adequately represents the behaviour of both anionic and cationic solutes in the presence of pairing-ion, and that the electrostatic binding is approximately constant for all solutes and pairing-ions.

It also predicts the chromatographic behaviour of mixed element containing both water and organic modifier. One problem with the model though is with predicting the behaviour of neutral solutes. Hung and Taylor (1981) showed that the retention time of neutral solute is decreased with increasing the pairing-ion concentration. However, they could not adequately predict the behaviour of a neutral substance and explained this by the difficulty of quantifying the phase ratio in the presence of pairing ion.

It is on this theory that the following chromatographic separation described is based (see latter pages of chapter).

Why develop a new HPLC method?

There exist at present many difference HPLC/ECD assay procedures for the determination and quantification of the biogenic amines and their metabolites. There also exist, several procedures for the extraction of biogenic amines from difference biological matrices. For the needs of the following investigations, it is essential to have an extraction procedure that is both simple and fast, so that there is as little time lost as possible between brain tissue dissection and analysis. It is also essential that the full range of C.N.S. biogenic amines and their major metabolites (Bowman and Rand, 1980, Cooper et al, 1983) be recorded from a single sample.

Sample Preparation and chromatographic techniques

In the initial paper on the electrochemical detection, Refshauge et al (1974) extracted noradrenaline and dopamine from brain samples. These compounds were analysed after tissue homogenisation in a mixture of EDTA, $NaHSO_3$ and $HClO_4$ followed by extraction onto alumina oxide. The extraction of catecholamines by alumina adsorption was developed by Anton and Sayer (1962) and is still a useful technique (Causon and Carruthers, 1982, Salzman and Sellers, 1982). Alumina extractions are used to separate the catecholamines from the cell debris and endogenous material after homogenisation. This results in the biogenic amine metabolites being discarded along with the cell debris, and therefore alumina absorption is only useful if the amines are the compounds of interest.

Higa et al (1978) developed an extraction procedure using boric acid gel, instead of alumina oxide, and it has been used successfully in the extraction and amines from urine (Speeke, 1983) and brain tissue (Koike et al, 1982). Again, however this procedure does not allow for the extraction of the metabolites and therefore is of little use to the present problem.

Cross and Joseph (1981) used ethyl acetate extractions for the determination of the biogenic amines metabolites from brain tissue and cerebrospinal fluid. Both techniques involved the acidification of samples, in 0.1M citric acid and 0.2M sodium dihydrogenphosphate buffer for brain samples and 6M hydrochloric acid for cerebrospinal fluid. The metabolites are then extracted in 2 or 3 washes of ethyl acetate between which they are evaporated to dryness by dry nitrogen. For analysis they are reconstituted in water or buffer. This method allows for the analysis of VMP,MHPG,DOPAC,5HIAA and HVA from a single sample, but does not allow for the analysis of the biogenic amines.

Shibuya et al (1982) in an attempt to measure both the biogenic amines and their metabolites from one sample have used an organic solvent extraction. As can be seen from the flow chart in Figure 4.2, this extraction procedure involves several shaking and centrifugation steps with different solutions. This method produces two different samples, one containing the amines and the other the metabolites. This results in VMA, 5HIAA, HVA, DA, NA and 5HT being detected chromatographically, but no record of DOPAC, MHPG or adrenaline. Whether these compounds are not recorded through inadequacies

in the extraction process, the HPLC methodology or both is not explained, as one would expect DOPAC and adrenaline be recorded with this technique.

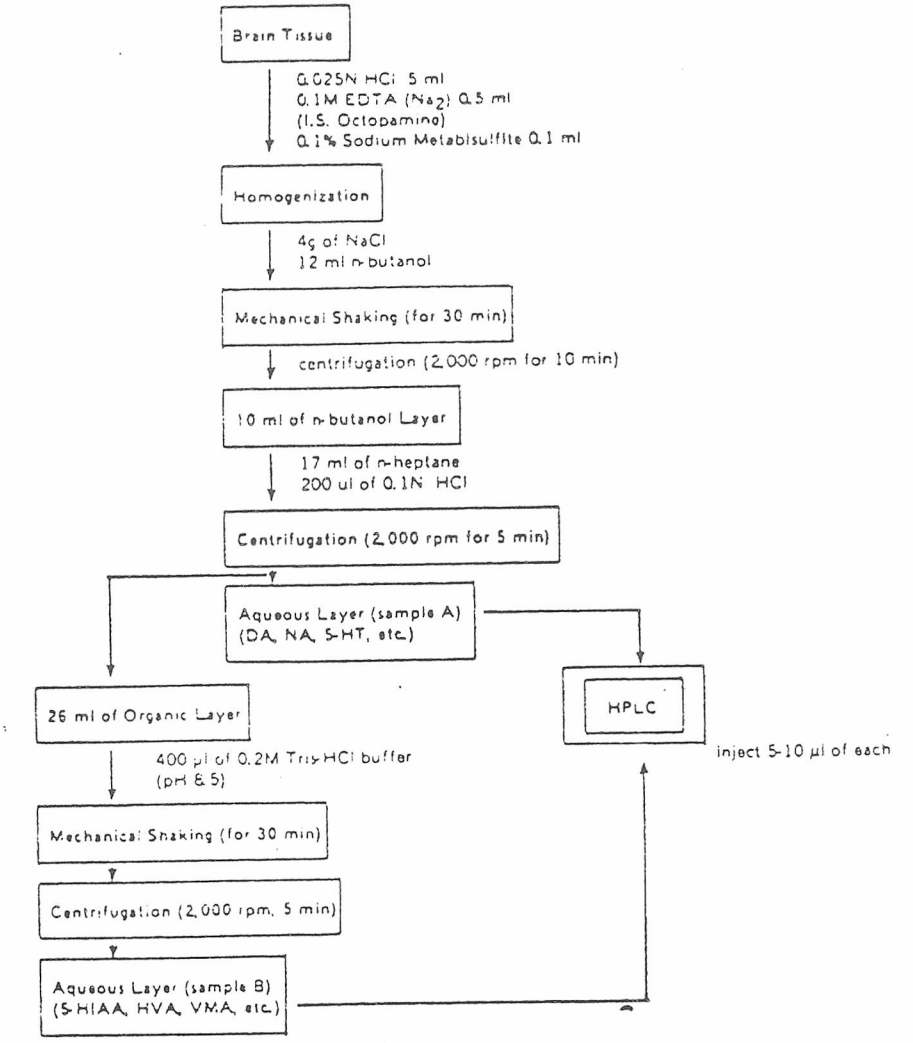


Fig.42 Tissue - sample cleanup method before HPLC biogenic amine and metabolite analysis.
From Shibuya et al (1982)

In preparing brain samples for extraction with alumina oxide, Refshauge et al (1974) initially homogenised the tissue in a mixture containing perchloric acid ($HClO_4$). $HClO_4$ is used to precipitate protein thus it will disrupt cell membranes and internal cellular

structures. It does not however degrade the catecholamines or their metabolites, therefore it provides a tool for the isolation of the biogenic amines and their metabolites. Various groups have homogenised brain tissue with different concentrations of $HClO_4$, Garnier et al, (1979) used 1lN $HClO_4$, Hefti (1979) used 0.1M $HClO_4$ followed by extraction in diethyl ether, (only interested in in DOPAC and HVA), Semerdian-Rouquier et al (1981), 0.1M $HClO_4$ and Lyness (1982), 0.2M $HClO_4$. All these groups used a mechanical homogeniser to disrupt the brain tissue and cells. The homogenisation was followed by centrifugation with variation in force and time from method to method. The supernatants were then injected directly onto the HPLC column for analysis. The advantage in using a system like this is its simplicity (ie homogenisation and centrifugation) and its quickness. The major draw back of the above systems is that they do not give an analysis of the full range of amines and metabolites mentioned earlier. Assuming that all the compounds are present in the supernatant after homogenisation and centrifugation, it appears that the incomplete analysis of the compounds is due to deficiencies in the different chromatographic conditions used. Saraswat et al (1981) and Hori et al (1982) replaced the mechanical homogenisation of the brain tissue in $HClO_4$ with homogenisation by sonication. This however did not increase the number of amines or metabolites recorded, confirming that the problem is a chromatographic rather than sample preparation one.

Ishikawa and McGaugh (1982) homogenised brain tissue in 0.1N hydrochloric acid followed by centrifugation. They then passed the supernatant through a series of extraction procedures, which resulted

in an aqueous medium containing the amines and an organic layer containing the metabolites. They then analysed the samples under different chromatographic conditions. Although they separated all the major amines and metabolites, with the exception of adrenaline, the overall resolution was inadequate for complete specificity of determination and the recovery of the catecholamines was less than that obtained by the alumina extraction technique.

Although many of the groups mentioned above use similar extraction procedures, the chromatographic analysis provided determinations of particular metabolites (Cross and Joseph, 1981, Hefti, 1979) or amines (Koike et al, 1982) or unique combinations of both (Shibuya et al, 1982, Garnier et al, 1979, Saraswate et al, 1981, Lyness, 1982, Neilson and Johnston, 1982), through the different chromotographic conditions used. The determination of the metabolites has been carried out in buffered solutions at pH values low enough to supress ionisation of the predominantly acid metabolites. This results in the retention of these compounds being based on the varying hydrophobicity of essentially neutral solutes. Under such conditions the amines, being fully protonated should elute rapidly. To over-come this problem Ishikawa and McGaugh (1982) have manipulated the pH so to achieve retention of certain amines as well as metabolites. The reciprocal methodology cannot be applied to the determination of the basic amines alone since at pH values high enough to deprotonate the bases completely, the catecholamines are chemically unstable (Krstulovic, 1982) and in addition, the bonded hydrocarbon stationary phase would be degraded (Ettre, 1980). To overcome this problem, hydrophobic pairing-ions such as octylsulphonate are included in a low

pH buffers, so that the bases are retained by ion-pairing and the acid and neutral metabolites are retained by ion suppression (Hegstrand and Eichelman, 1981). This procedure can result in crowded chromatograms with resolutions often inadequate for quantification.

The development of ion-exchange-desolvation theory of separation for ion-pairing chromatography by Hung and Taylor (1980, 1981) offers an explanation of the complex relationship between capacity factors and solvent pairing-ion concentrations. They have proposed that the retention of a solute on a C_{18} coated surface containing adsorbed pairing-ion of charge opposite to that of the solute is due to electrostatic binding coupled with desolvation of the neutralised solute on the available C_{18} surface, ie on surface unoccupied by adsorbed pairing ion. Thus if the adsorbed pairing-ion concentration is high due to either a high solvent concentration, or the use of a very strongly adsorbed pairing-ion, retention of both charged and neutral species will actually be reduced due to the unavailability of free C_{18} surface for desolvation. The mixture of basic amines and the predominantly acid metabolites constitutes an ideal system to which the above ion-exchange-desolvation ideas may be applied for the purpose of obtaining improved specificity in the assay of these compounds. At a low pH, the amines may be retained and resolved by a suitable choice of anionic pairing ion at an appropriate concentration while the retention of acidic and neutral metabolites may be minimal. Similarly at pH7, the acidic compounds are appreciably ionised and may be retained by use of a suitable cationic pairing ion while minimising the retention of the bases whether or not they are completely uncharged.

To achieve the optimum separation of the amines and metabolites from a single sample will require the use of two chromatographic systems. Hung and Taylor (1981) examined the relative properties of several anionic and cationic pairing-ions and sodium laurylsuphate (SLS) (anionic) and tetraethylammonium (TEA) (cationic) were chosen as the compounds with the best potential of producing good separation for the amines and metabolites. Using these compounds, two isocratic chromatographic systems were designed, from which it was hoped that one would separate the amines in the presence of the metabolites and the other vice versa. The preliminary chromatographic retention data was obtained using an ultraviolet dector and quantitation of the biological samples was by electrochemical detection.

Sample preparation

Mature male rats were killed by cervical dislocation. Their hypothalamus was removed, weighed and placed in a vessel in an ice bath within 2 minutes of death. Within one hour of removal from the animals the tissues were prepared for analysis. Approximately 0.1g pooled tissue samples were homogenised in 500 of 0.4M $HClO_4$ in glass rod homogenisers. The homogenates were then centrifuged at 3000g for 30min and the supernatant injected directly onto the column for analysis.

Chromatographic conditions and Solvent Systems

The equipment and columns used have been described in the general methods. The electrochemical detector was fitted with a polished glassy carbon electrode and operated at an applied potential of +0.7

volts relative to a silver/silver chloride reference electrode. The column to detector cell fittings were modified to reduce dead volume. The solvent for the separation of the amines consisted of the anionic pairing-ion SLS dissolved in 10mM disodium hydrogen phosphate, pH2 containing 25% acetonitrile and 0.5mM EDTA. The solvent for the separation of the metabolites consists of the pairing ion TEA disolved in 10mM disodium hydrogen phosphate, Ph7 containing 0.5mM EDTA.

Results Separation of the amines and metabolites

The effect of varying the pairing-ion concentration in the solvent on the capacity factor (k') for the biogenic amines and metabolites was studied.

Figure 4.3 shows the effect of SLS concentration in the solvent in the capacity factor for the amines and metabolites. The amines show the predicted maxima while the metabolites show very low retention which are further reduced by increasing the SLS concentration. Figure 4.3 shows that the pairing-ion concentration also dictates the degree of resolution obtained. While low concentrations (3-5mM) can provide adequate resolution of the amines, high concentrations (10-50mM) provide a less critical resolution with respect to pairing ion concentration and also avoid the problems associated with long equilibrium times (Knox and Jurand, 1978). These higher concentrations also further reduce the retention of the metabolites.

Figure 4.3

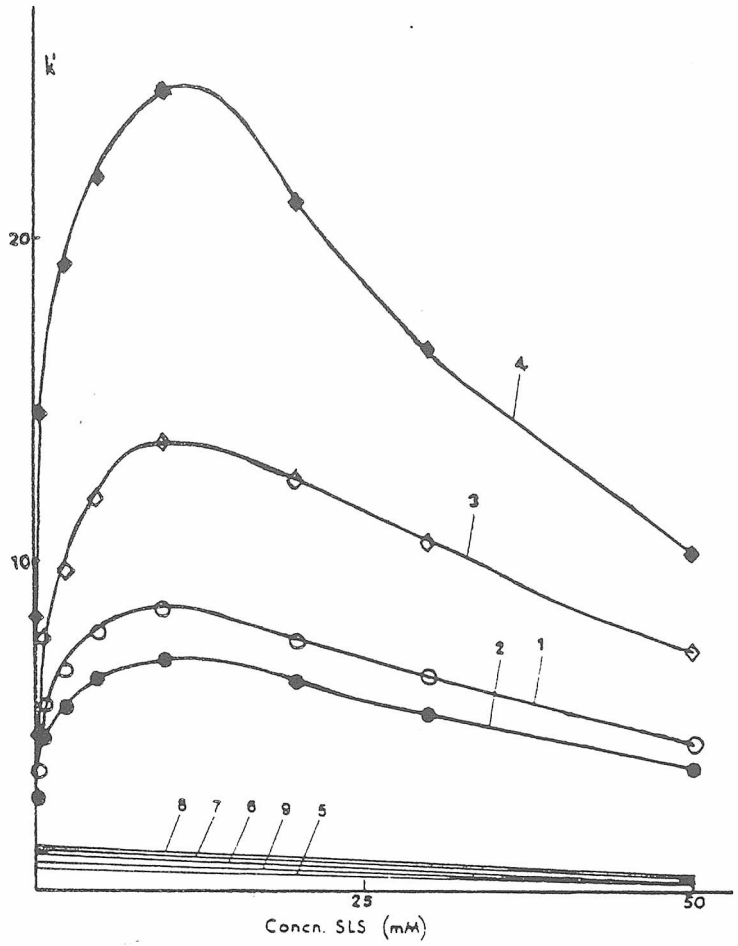


Fig. 4.3 Variation of capacity factor k' with SLS concentration for the nine amines and metabolites studied. Column 200 x 4.6 mm ODS Hypersil (5 μm); solvent 10 m*M* disodium hydrogen phosphate, pH 2 buffer containing 25% acetonitrile and 0.5 m*M* EDTA. Compound identification: 1 = A; 2 = NA; 3 = DA; 4 = 5HT; 5 = VMA; 6 = DOPAC; 7 = MHPG; 8 = 5HIAA; 9 = HVA.

Representative separations of the major amines in a protein precipitated homogenate are shown in Figure 4.4(a). Figure 4.4(b) shows the separation of a standard mixture of amines with their matabolites while Figure 4.4(c) shows the separation of a spiked homogenate extract, verifying the identities of the peaks shown. Comparison of Fig.4.4(a) and 4.4(b) indicates the absence of both endogenous compound and metabolite interference in the deterimination of amines using this system. The critical resolution was found to be the adrenaline/noradrenaline pair and in experiments comparing the

organic modifier used, acetonitrile gave better selectivity than methonol, which produced unreasonably long retention times for 5HT to achieve complete adrenaline/noradrenaline separation.

Figure 4.4

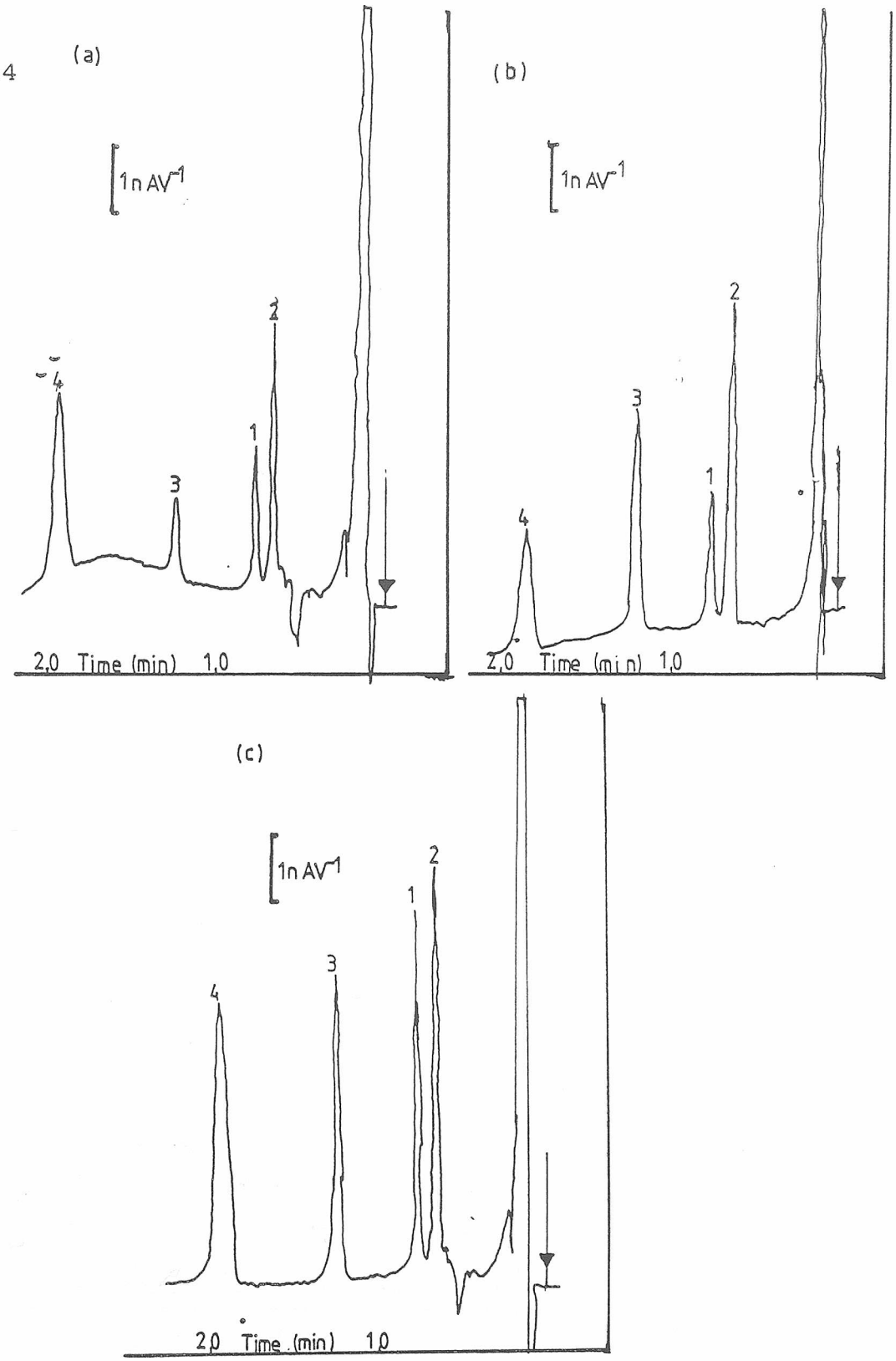


Fig 4.4 Representative chromatograms showing separation of the major amines. (a) Supernatant from protein-precipitated tissue homogenate; (b) all amines and metabolites in aqueous solution; (c) tissue homogenate spiked with added metabolites. Conditions and compound identification as in Fig 4.3 with SLS concentration 20 mM.

Figure 4.5 shows the effect of TEA concentration in the solvent on the capacity factor for the amines and metabolites. At low TEA concentrations, the neutral MHPG is highly retained as is 5HT, while retention of the acidic metabolites is inadequate for the reliable determination in biological matrices. Increasing the concentration of TEA only gradually reduces the retention of MHPG while all the other metabolites retentions are markedly affected. Increasing the TEA concentration produces an accumulation of charge on the C_{18} surface of the stationery phase, which results in a decrease in retention of 5HT and an increase in retention of the ionised acidic metabolites. In this system, the optimal concentration for TEA appears to be 30mM. At this concentration, noradrenaline, adrenaline and adrenaline are rapidly eluted, while 5HT in spite of its predominantly basic character is retained due to its hydrophobic nature but resolved from the metabolite species.

Figure 4.5

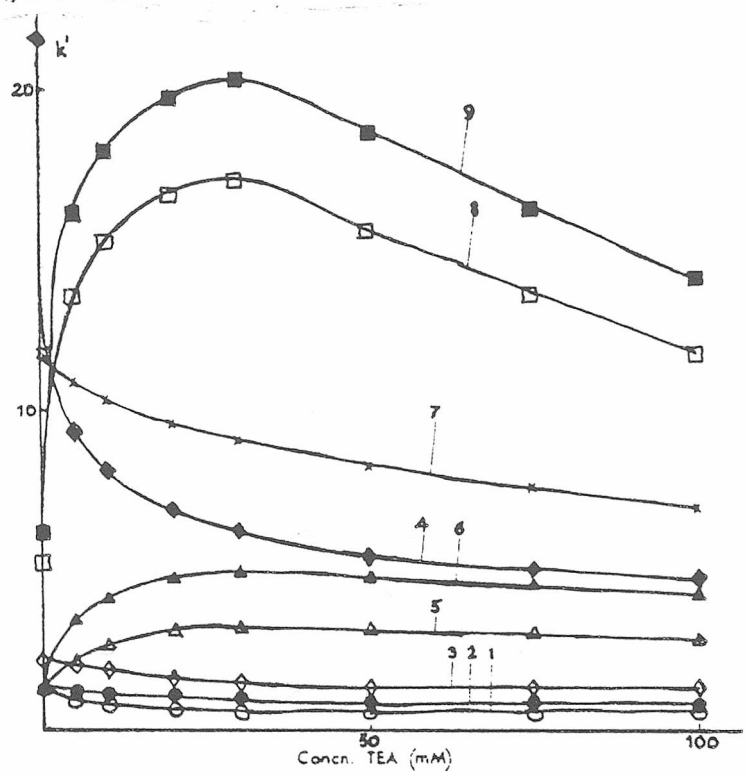


Fig.4.5 Variation of the capacity factor k' with TEA concentration for the nine solutes studied. Column 200 × 4.6 mm ODS Hypersil (5 μm); solvent 10 m*M* disodium hydrogen-phosphate, pH 7 buffer 0.5 m*M* in EDTA. Compound identification as in Fig. 4.3

Figure 4.6(a) shows a representative chromatogram of tissue extracts run on this solvent system. The signal due to endogenous material is much larger than in the case of the amine system but resolution is adequate. Figure 4.6(b) is a chromatogram of a standard mixture of amines and metabolites, verifying that the amines other than 5HT are eluted with the residual endogenous compounds. Figure 4.6(a) indicates that the absence of VMA in the sample and this was found to be the case for other hypothalamic samples measured. Figure 4.6(c), a chromatogram of tissue spiked with all the compounds, confirms the identities of the various peaks and the absence of VMA in the sample. In this solvent system a few unidentified peaks were evident at the sensitivity required for the major metabolites. In Figures 4.6(a) and (c) only one unidentified peaks is noted between 5HIAA while in other brain samples assayed an additional peak was occasion recorded after HVA. Resolution was adequate in all cases of quantitation of the specific metabolites.

Figure 4.6

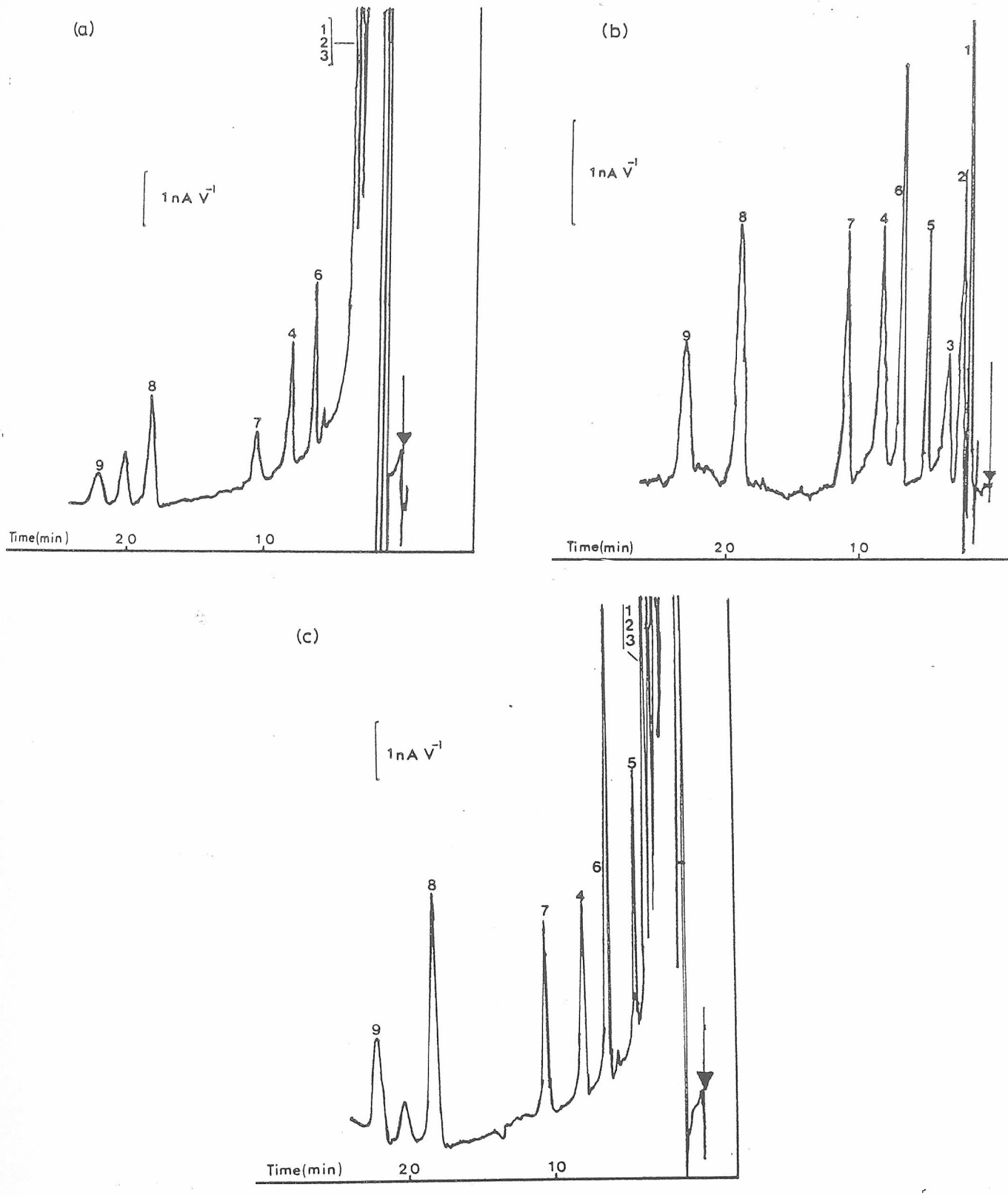


Fig 4.6 Representative chromatograms showing separation of the amine metabolites and 5HT. (a) Supernatant from protein-precipitated tissue homogenate; (b) all amines and metabolites in aqueous solution; (c) tissue supernatant spiked with added metabolites. Conditions as in Fig 4.5 with TEA concentration 30 m*M*. Compound identification as given in Fig. 4.3

Quantitation

For both the amines and their metabolites the standard addition method was applied to a pooled tissue sample to verify the linearity of the chromatographic response, i.e. known concentrations of standard compounds were added to samples before analysis, then the peak heights recorded for a series of concentrations. Figures 4.7(a) and (b) demonstrate the resultant plots, and confirm the linearity of the response and verify the specificity of the solute peaks. The constants of the regression lines are listed in Table 2.1. Concentrations of the endogenous amines and metabolites are not quoted since different pooled samples were employed, but the shapes of the regression lines indicate the relative sensitivities of the method for the different solutes. It is also seen from Table 2.1 that good agreement is obtained between experimental intercepts in Figures 4.7(a) and (b) and those obtained by regression analysis.

The limits of detection for the solutes by this method determined in aqueous solution are also included in Table 2.2. The chromatographic precision of the method (RSD=2.0%, n=10) was considerably below the biological variation shown in Table 2.2.

CONSTANTS OF THE REGRESSION LINES OBTAINED BY STANDARD ADDITION OF AMINES AND METABOLITES TO TISSUE SAMPLES

Compound	Constants of regression line					
	Slope	S.D. of slope	Intercept (regression)	Intercept (experimental)	S.D. of intercept	Correlation coefficient, r^2
A	0.125	$3.79 \cdot 10^{-3}$	37.3	38.4	1.04	0.998
NA	0.154	$3.32 \cdot 10^{-3}$	39.2	39.8	0.898	0.999
DA	0.205	$4.49 \cdot 10^{-3}$	5.02	6.3	1.16	0.999
VMA	0.282	$1.49 \cdot 10^{-3}$	13.7	14	1.99	0.994
DOPAC	0.433	$1.14 \cdot 10^{-3}$	11.4	12.4	1.54	0.998
MHPG	0.212	$8.19 \cdot 10^{-3}$	2.02	3.2	1.74	0.997
5HIAA	0.194	$1.13 \cdot 10^{-3}$	19.7	17.8	2.77	0.993
HVA	0.089	$5.43 \cdot 10^{-3}$	3.12	2.8	1.50	0.992
5HT bases	0.120	$6.11 \cdot 10^{-3}$	7.91	9.0	1.46	0.996
5HT acids	0.167	$9.58 \cdot 10^{-3}$	38.1	35.8	1.17	0.993

FIGURE 4.7

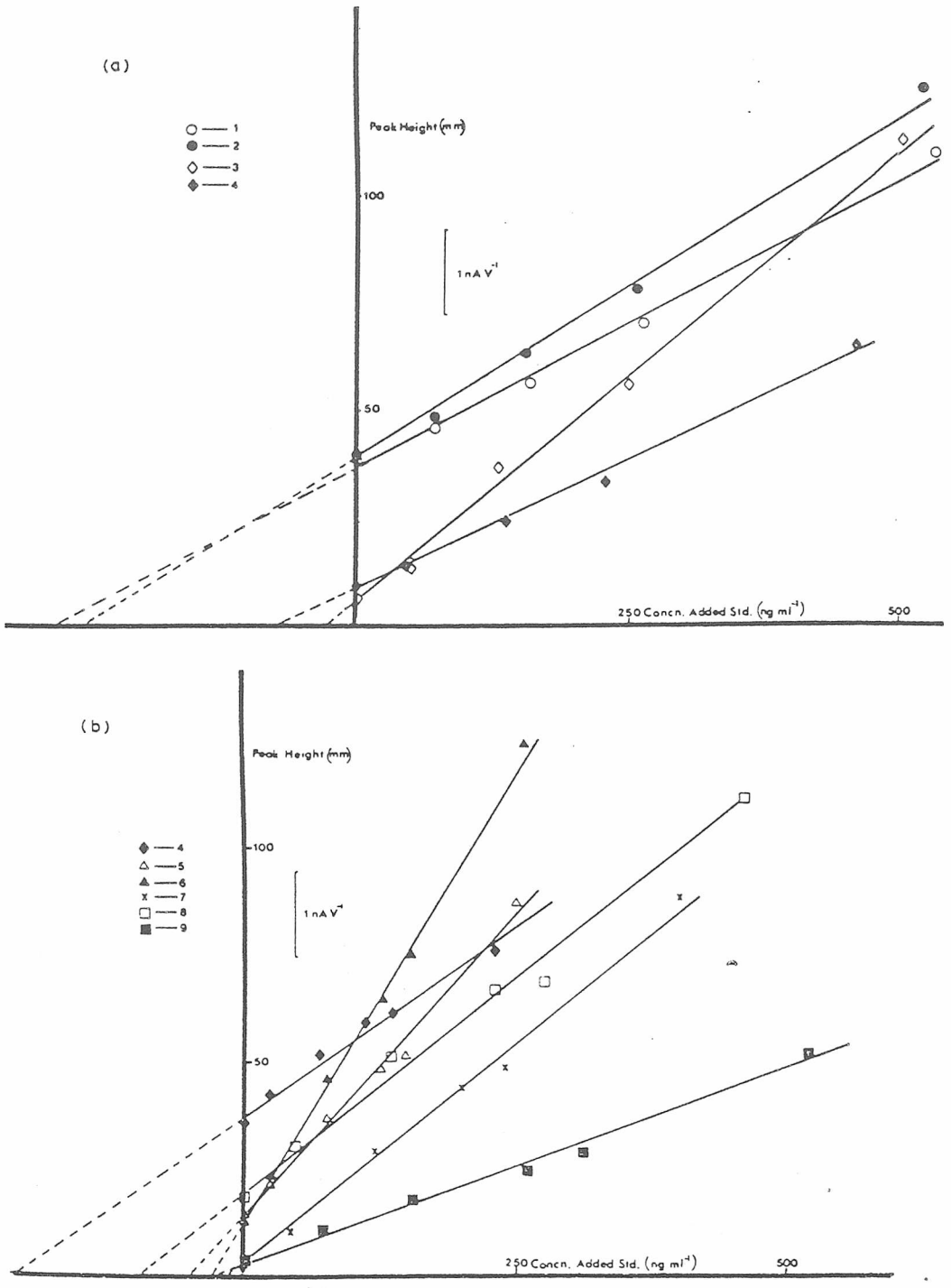


Fig4.7. Plots showing the variation of peak heights on addition of added standards to pooled protein-precipitated tissue homogenate. (a) Amines added, results from SLS system; (b) metabolites added, results from TEA system. Compound identification as in Fig.4.3

RESULTS OF AMINE AND METABOLITE DETERMINATIONS ON SIX TISSUE SAMPLES BY THE PRESENT METHOD TOGETHER WITH LITERATURE VALUES FOR CERTAIN COMPOUNDS IN THE MALE RAT HYPOTHALAMUS

Investigation method	Concentration ng/g wet tissue (RSD)								
	A	NA	DA	5HT	VMA	DOPAC	MHPG	5HIAA	HVA
This investigation	78.3	3066	502	1812	20	100	97	409	25
Rat hypothalamus (n=6)	(51)	(27)	(12)	(17)		(17)	(14)	(10)	
HPLC-ED Limit of detection (ng/ml)	33	26	21	26	6.6	9.4	8.5	11	25
HPLC-ED (Koike et al 1982)	-	808	150	-	-	-	-	-	-
HPLC-ED (Bennet et al 1981)	-	2780	596	1098	-	-	-	-	-
Gas chromatography -	-	2418	569	956	-	-	-	565	95
mass spectrometry	-	1740	488	1089	-	160	92	560	-
		1707	359	741	-	-	-	414	116
(Smythe et al 1982)	-	2327	465	931	-	-	-	483	111
HPLC-ED	64	2080	410	-	-	46	-	-	35
(Roth et al 1982)	58								
	66								

Table 2.2

Discussion

The resolution of the biogenic amines and their metabolites by the chromatographic method described shows that it is possible to extract these compound by a simple protein precipitation process. The homogenisation of the brain tissue in perchloric acid followed by centrifugation removes the need to use complex extraction procedures involving alumina or boric acid gel for the isolation of the biogenic amines and their metabolities. Combining this sample pretreatment method with the chromatographic system developed has produced the resolution of a larger range of compounds than has been previously described (see Table 2.2). The values obtained using this combination of methods are in general agreement with those reported in the literature (Table 2.2). However, the noradrenaline and 5HT levels are greater than the highest levels previously recorded. These differences are probably a reflection of the different techniques used, i.e. the minimal sample pretreatment, the speed of sample pretreatment with the consequent minimal opportunity for losses. Although HVA was reported in the literature it was not detected in the hypothalamic homogenates used for quantitation. It was, however present in the homogenate used to establish retention times as indicated in Figure 4.6(a) and (c). This difference may be due to inexperience in the dissection of the hypothalamus which might have resulted in minor variations in the size of tissue isolated. These minor variations may have reduced the levels of HVA to below the limits of detection of the system. It is also possible that the applied voltage (+0.7V) may not be fully oxidising all of the HVA present in the samples. Kissinger et al (1979) showed that the

half-wave potential of HVA in their system is +0.63V. Increasing the applied potential should increase the oxidation of HVA (also VMA, half-wave potential also +0.63), and thus increase the peak height of any HVA present. Figure 4.6(a) indicates an absence of VMA in the sample and this was found to be the case for other hypothalamic samples measured. The absence of VMA from the hypothalamic samples is in agreement with the proposed routes of noradrenaline metabolism within the CNS (see general introduction) which is through MHPG followed by conjugation.

It should be remembered that the two solvent systems described are not unique and have been chosen as optimum for the particular purpose of determining the specified compounds. The systems can be easily modified to alter retention times and order of elution by using different pairing ions and concentrations bearing in mind the dual retention characteristics of ion-exchange and hydrophobicity. It should also be possible to devise a system capable of isolating the precursors used in amine synthesis.

Overall the minimum pretreatment of tissue samples isolates all the compounds of interest with little opportunity for sample loss. The chromatographic systems represent an advance on current literature systems since they provide improved resolution over all but gradient elution methods (Tjaden et al, 1983) for all the accepted metabolites or amines and they also accommodate the presence of all these compounds. The disadvantage of the two-solvent column system is believed to be more than compensated by the completeness of metabolite detection, the high capacity factors and improved resolution.

CHAPTER 2

System Maintenance and Sample Preparation and Standardization Required for Routine Regional Brain Sample Analysis

Introduction

The theoretical and practical development of the chromatographical system have been discussed in the previous chapter. Initial experiments into the relative separation of compounds under different chromatographic conditions, used chemical standards prepared in aqueous solutions. Biological samples were used only in the latter experiments, concerned with sample preparation and quantitation of the technique. Daily use of the system for the analysis of biological samples has highlighted shortfalls in the system maintenance, sample preparation and handling and also the preparation of standard curves for the calculation of tissue amine levels.

System Maintenance

Under ideal operating conditions, the chromatographic techniques described in chapter one requires two separate HPLC-ECD systems on the bench. However, it is possible to undertake the analyses of the amines and metabolites with only one HPLC pump and electrochemical detector, by changing columns and solvents. The main obstacle associated with changing the columns and solvents between the analysis of the amines and metabolites is time.

Ideally, the amine and metabolite content of a sample should be analysed at the same time, which is only possible with two separate chromatographic systems. This ensures against any sample degradation through storage.

With a single chromatographic system, changing of the columns and solvents for analysis, takes approximately 40-45min. After disconnecting one column, the solvent for that column has to be flushed out of all the connecting tubing before the second column can be plumbed in. Water is first washed through the tubing, followed by methanol. This prevents the precipitation of the pairing ions within the tubing and the blocking of the system. The second column is then plumbed into the system and the appropriate solvent passed down the column. It then takes approximately 30 minutes for a solvent to equilibrate within the column (as seen be a steady base line on the pen recorder) after which samples may be analysed by the system.

If there is a large number of samples to be analysed in a day, it is most practical to first analyse the amine content of the sample then the metabolite content of the samples, to reduce to a minimum the amount of time lost through column changing. With a large number of samples being analysed in this way, the storage conditions of the samples is important, and it will be discussed under the heading sample preparation.

The problem of changing columns does not occur if two HPLC-ECD systems are in operation. However it is still advisable to flush the system overnight with water when in daily use, to prevent the pairing-ion precipitating in the tubing or column packing. This helps in limiting flow problems with the solvent and increases the working life of the column (especially the TEA column). It also aids in the maintenance of the electrochemical detector by helping to clean the surface of the glassy-carbon electrode and moisten the reference electrode.

The sensitivity of the system depends on the condition of the surface of the glassy-carbon working electrode. Over a period of time the sensitivity of the electrode decreases. To keep the electrode in top working order it is recommended that it be polished with alumina powder. When working with aqueous samples, the recommended time interval is 6 months. There is however no recommended interval between polishing when biological samples are being analysed. With the constant daily use the systems received it was found necessary to polish the electrode weekly.

The working life of the column also has to be closely monitored. A well maintained column with SLS as its pairing ion should give at least 12 months good service before having to be renewed. Columns with TEA as their pairing ion have a working life of approximately 2-3 months. Break down of the column can be seen in an alteration in the order in which the metabolites are eluted coupled to a shortening in retention times. This short working life is in part due to the action of TEA on the packing

material and the pH (pH7) of the solvent (the packing material starts to break down between pH 8-9).

Sample Preparation

In the analysis of hypothalamic samples during the methodological development, pooled hypothalamic samples (weighing 0.1g approx) were homogenised in 500μl of 0.4m $HClO_4$ and centrifuged. The supernatants were decanted off and kept in an ice bucket until analysed. Converting this initial sample preparation and storage technique to an everyday methodology capable of examining drug effects on amine levels in individual animals required several small changes in design. Usually, two hypothalamic samples pooled together weighed approximately 0.1g. This was homogenised in 500μl of $HClO_4$ so that several injections of the same sample could be analysed in the methodological development. Under normal experimental conditions only 2 samples of 50μl are required for analysis (1 sample for each column), with a further 2 in reserve in case of problems (i.e., air bubble in the system, electrical interference). It therefore seemed logical to halve the volume of $HClO_4$ used for the homogenisation of a single hypothalamus, (average weight, 50mg). This gives a homogenisation volume of 250μl, 0.4M, $HClO_4$ from which four 50μl sample can be easily taken. Increasing the volume of $HClO_4$ used for the homogenisation diluted the amine and metabolite levels per 50μl injection, thus reducing the peak heights recorded. It is important to record the maximum peak height possible from a sample to ensure the most accurate estimate of tissue levels. In

an attempt to decrease the amount of cell debris present in the sample after homogenisation, a centrifugal micro-filtration step was introduced. This step has increased the life of the column top, which provides indirect evidence that the samples are "cleaner". (Removing the dirty packing material from the top of the column and repacking it with new packing material increases the sharpness of the peaks, and reduces peak broadening).

Therefore the overall methodology for sample preparation is as follows.

1. Male Sprague Dawley rats, 250g were killed by cervical dislocation. The brains removed and the hypothalamus and striatum dissected out (maximum time, 2 min from cervical dislocation to dissection).

2. The dissected brain areas were homogenised in 250μl 0.4M $HClO_4$ using 1ml glass rod homogenisers.

3. The homogenisation tube (containing the homogenate) was then centrifuged for 30 min at 3000g, 4^{o}c.

4. The supernatant was decanted off into the centrifugal microfiltration tube. This was centrifuged for 15min at 3000g. 4^{o}c. during which time the sample is filtered though a 1 micron regenerated cellulose filter. The homogenate pellet was resuspended in 500μl 0.1M NaOH and stored at -20^{o}c until analysed for protein content.

5. * 50µl of the filtered supernatant was then analysed for its amine and metabolite content.

If a single HPLC-ECD system is being used for the measurement of amine and metabolite levels the storage of samples between samples analysis on both columns is of prime importance. The biogenic amines are notoriously unstable and storage in an ice bucket on the bench provides insufficient protection against decay.

Attempts to use the antioxidant agents ascorbic acid and meta-bisulphate to prevent decay proved unsuccessful as they increased the solvent front of the chromatograms, interfering with the resolution of some compounds. The best storage for the samples between preparation and analysis proved to be frozen between $-5^{o}c$ and $-10^{o}c$, as little loss seen over the short storage time required when using two HPLC-ECD systems for the analysis.

Preparation of Standards

In chapter one, Figure 1.7 shows that the addition of known aqueous standards to pooled protein-precipitated tissue homogenate produces a linear increase in peak height. From this, it was decided to prepare aqueous standards fresh, each day, for quantifying the amine and metabolite tissue levels.

The two column method employed in measuring the amines and their metabolites, records 5HT on both systems.

The levels of 5HT in a specific sample was calculated by measuring the results obtained from both columns, as in chapter one.

Table 2.3

	Amine Column	Metabolite Column
5HT Aqueous Standards	960 +/- 40 (n=20)	104.9 +/- 5.2
5HT Added to frontal cortex samples	1937 +/- 165 (n=5)	1678 +/- 94

All values in ng/g wet tissue +/- SD.

Looking at the 5HT levels recorded on the individual columns (Table 2.3), it can be seen that the 5HT level from the amine column is much larger than the 5HT level from the metabolite column. On reflection it is not surprising that the calculated results from the SLS column differ from TEA column for several reasons. There are many differencies between biological matrices and aqueous solutions. In brain tissue, 5HT is stored within specialised compartments within the cell, where it is protected from metabolic enzymes, other subcellular activity, and variations in pH. In aqueous solution, (pH,7 approximately) 5HT is in equilibrium

between its ionised/unionised state particular to that pH. After homogenisation of the brain tissue, the amines and their metabolites are present in a strongly acidic, low pH solution. This will alter the equilibruim of the ionised/unionised 5HT molecules greatly. Therefore, there is a great difference between the aqueously prepared standards, and the biological prepared samples. The solvents in which the analysis takes place are also different. The SLS solvent is weakly acidic at pH2 and the TEA is neutral at pH7. Injection of the aqueous standards into the TEA solvent will not alter the equilibrium of the ionised/unionised 5HT, while injection of the standards into the SLS solvent will alter the equilibruim ration. The reverse will happen with the biological samples as they are already in a strongly acid, low pH medium. Injection into the SLS solvent will not alter the equilibrium ratio, while injection into the TEA will alter the equilibrium ratio. The extent to which this will be altered though is hard to predict as the samples are highly acidic, and the volume of solvent the samples are injected into are small. The ratio of ionised/ unionised 5HT molecules is important as retention of compounds by the column is primarily through the formation of ion-pairs.

In an effort to improve the estimation of 5HT levels, a method simlar to the technique used in chapter one to show the linear relationship between amount of standard added and peak hieght measured was devised. Pooled samples of frontal cortex were homogenised and centrifuged and the supernatant drawn off. The

supernatant was then devided into four parts, to three of which known quantities of aqueous standards were added(in 10µl of water). These three samples along with the frontal cortex sample were analysed. The frontal cortex sample acts as a blank for the known standard samples. So any amine or metabolite peak recorded from this sample is substracted from the peak hieghts recorded by standard samples, thus giving the absalute peak hieghts of the standards.

Using this method, the 5HT levels recorded by the SLS column were compared to the 5-HT levels recorded by the TEA column. (See Table 2.3). It can be seen that there is little difference between the 5HT levels recorded in both columns. It is therefore assumed that this method of preparing standard mixtures overcomes the problems associated with the aqueous standards.

Many groups of workers incorporate internal standard to the chromatographic analysis (Koike et al, 1982, Smythe et al 1982, Roth et al, 1982) from which the calculate percentage recovery of the amines and metabolites. The standard addition technique used above does not include an internal standard. The theory behind including an internal standard is that the percentage recovery of compounds of interest can be calculated, ie. the amount lost though the extraction prosess. It was realised after the experiment that an internal standard calculation of some sort should have taken place.

The incorperation of an internal standard prosess into the homogenisation and centrifugation the standards are subjected to above mentioned frontal cortex procedure proved rather simple. By adding the standards to the frontal cortex samples before the same conditions as the endogenous compounds, and it is assumed that any losses experience by the compounds will be mirrored by the standards

Therefore the overall protocol for the preparation of the standard curves is as follows.

1. Several frontal cortex samples were pooled (0.2g approx) and blended together. This frontal cortex "paste" is divided into 4 and each part placed in a glass homogenisation tube.

2. Standard mixtures were prepared in such a way that a 50 injection of supernatant will place, 2ng, 8ng and 16ng of amines and metabolites on column. This is achieved by preparing the standards so that 10µl of water contained, 10ng 40ng or 80ng of each compound. Then 10µl of the standard mixtures were added to 240µl of 0.4M $HClO_4$ giving 3 standard mixtures at 10ng, 40ng and 80ng in 0.4m $HClO_4$. Each of these perchloric acid mixtures was homogenised with a portion of the "frontal cortex past". The homogenate was then centrifuged and filtered as described previously and 50µl of the supernatant of each mixture is analysed. Therefore the final amount of the standards on column are 2ng, 8ng and 16ng. This only uses 3 of the 4 frontal cortex sample

prepared. The 4th sample is homogenised in 250µl of 0.4M $HClO_4$ and this acts as a blank from which the peak heights of the standard mixtures are calculated ie. The size of any peaks recorded in the blank are substracted from the peak heights of the known standards.

3. The graphs of peak height against amount of standard or column were constructed and these were used to calculate the amount of amines and metabolites per sample. Final results were given as ng/mg of protein. The protein content of each sample was estimated using the Lowry method. Details of which can be found in,

Data for Biochemical Research, Dawson, RMC et al, eds. 2nd edition Oxfor Press, 1969

Discussion

Converting the original chemical assay for catecholamine standards into a reliable technique for the everyday measurement of biological samples required time and patience in solving the many problems that arose. Solving problems meant initiating a sequence of system checks on isolated pieces of equipment which aids fault finding. The development of these techniques and the identifying of problems has only been made possible through the constant use of the system. To ensure the smooth operating of the above detailed chromotographic system it is essential that it is regularly serviced to ensure it is free from mechanical failure. Use of the above system on a regular basis is not as difficult as it may first appear. Everyday use of the system will quickly familiarize an operator with the limitation of the system. When operational and well maintained the system is easy to use and is reliable, it is only when the system is misused that serious problems will arise. The greater the experience of the operator of the system has the easier it is to use. The only difference between the above system and other HPLC systems is the electrochemical detector. This is probably the most sensitive part of the system and therefore requires the greatest amount of attention. Again though, once the operation of the detector is understood, its maintenance is simple.

Overall the system is simple to operate and maintain to the experienced user, although ones knowledge of HPLC use is never complete.

CHAPTER 3

Effect of Oxypertine on Biogenic Amines Levels in the Hypothalamus and Striatum and Motor Behaviour

Introduction

The initial biochemical studies into the action of oxypertine, after its synthesis in the late, 1950's by Spector et al (1962) showed it to have a noradrenaline depleting action in rat brain at a dose of 60mg /kg (I.P. injection). Work on oxypertine continued throughout the 1960's and early 1970s which increased the number of pharmacological actions attributed to the compound. Goldstein and Nakajina (1966) and Fuxe et al (1967) showed that oxypertine reduced dopamine and 5-HT levels as well as noradrenaline levels (following IP injections of 35mg/kg to 70mg/kg). Bak et al (1969) suggested that the depleting action of oxypertine might be dose dependent. Using a dose range of 5mg/kg to 70mg/kg (I.P. injection) they suggested that at the lower doses, oxypertine cause the release of the biogenic amines from storage vesicles, while at higher doses, it causes the disruption of the biogenic amine storage vesicles.

Penn (1972) suggested that as well as being an amine depletor, oxypertine is an adrenoceptor antagonist. Miranda (1978) demonstrated that oxypertine could inhibit the actions of noradrenaline, dopamine and 5-HT on the isolated rat vas deferens preparation, lending further evidence to the suggestion of receptor

antagonism. Receptor binding studies carried out by Nakahara et al (1980) concluded that oxypertine is a weak dopaminergic antagonist and a potent 5-HT receptor antagonist.

In a reappraisal of the treatment of withdrawn psychotics with oxypertine, Sterling (1979) suggested that a daily dose of 80-120mg should be sufficient for symptom control. This dose range equates to a dose range of 1mg/kg to 2mg/kg in rats, compared to the 35mg/kg to 70mg/kg used in previous animal experiements. Palomo and Russell (1983) examined the behavioural effects of oxypertine (1mg/kg to 16mg/kg) on amphetamine-induced behaviour in rats and noted that the doses of 1mg/kg and 4mg/kg increased the rats exploratory behaviour. From this and studies with MAOI (Palomo and Reid, 1983) it was suggested that oxypertine is an amine depletor, acting on the amphetamine releasable pool of amines as opposed to the reserpine releasable (vesicular) pool of amines and Arbilla et al (1984) showed that the amphetamine releasable pool of dopamine accounts less than 10% of the total neural dopamine content. Palomo and Reid (1984) concluded from their behavioural studies that oxypertine might provide a means to interfere with the pool of newly synthesised amines (amphetamine releasable) but that biochemical data was essential to confirm their behavioural evidence.

Using the same dose range as Palomo and Reid (1983), the experiment was designed to examine the biochemical action of oxypertine on hypothalamic and striatal amine and metabolite levels. The action of oxypertine was compared with the action

of reserpine (an amine depletor acting on the vesicular pool of amines) and haloperidol (a dopamine receptor antagonist). The behavioural studies of Palomo and Reid (1984) were also repeated so that the biochemical action of oxypertine could be compared with its behavioural effects.

Methodology

Male Sprague Dawley rats (250g) were used in the experiment. They received one I.P. injection of drug solvent (see general methods) or a dose of oxypertine (4mg/kg, 8mg/kg and 16mg/kg, I.P injection). 90 minutes after the drug administration the rats motor behaviour was recorded for 4 minutes. The motor behaviour was examined using the hole-board apparatus designed by Palomo and Reid (1983). The board is divided by lines into 16 squares with 25 holes evenly distributed in the board at 1 hole per 100 cm^2. the main behaviour parameters recorded were:

A) Locomotion: one unit each time that the rat travelled from one square to the next in any direction.

B) Exploration: one unit each time that the rat reared or stopped to examine a corner etc. of the board or dipped its head into a hole.

C) Stereotypy: one unit each time that the rat repeated subsequently and in the same place any motor activity.

Immediately after the behavioural examination on the hole board the rats were killed by cervical dislocation, the brains removed and hypothalamus and striatum dissected out and stored at -80°C until analysed. The amine and metabolite levels were estimated by the HPLC/ECD method described previously (see general methods) with the final results given in ng/mg protein.

Results

The effect of the three drugs (Oxypertine, Reserpine and Haloperidol) on the rats motor behaviour is shown in Table 3.1. Grouping locomotion and exploration together it can be seen that all 3 drugs reduced locomotor activity. The 3 different doses of oxypertine all reduced locomotor activity slightly but as the recorded locomotor activity for all 3 doses is small and of a similar magnitude, no overall difference can be seen between doses. Reserpine completely blocked any exploratory behaviour, while a small amount of locomotor activity was recorded in one rat. The rats treated with reserprine exhibited the classical signs of treatment with the drug. The rats remained motionless, curled up in a ball when placed in the hole board apparatus, their fur showed signs of pilo-erection and the animals often shivered. Haloperidol reduced locomotor and exploratory behaviour but not to the extent of reserpine. The effect of drugs on stereotypy behaviour produced few results. Oxypertine treatment produced the same levels of stereotypy as the control. No activity at all was recorded in the reserpine or haloperidol treated animals.

The effects of the three drugs on the biogenic amines and metabolites can be seen in Tables 3.2 (a), (b) and Tables 3.3 (a), (b). Table 3.2 (a) shows the effect of the durgs on the levels of the amines in the hypothalamus. The table shows that oxypertine at all three doses (4 mg/kg, 8 mg/kg and 16 mg/kg) had no significant effect on noradrenaline, dopamine or 5-HT levels, using a one tailed, Multiple Range t-Test. While both reserpine and haloperidol significantly reduced noradrenaline, dopamine and 5-HT levles. Reserpine reduced the levels of noradrenaline significantly (P 0.05). Haloperidol reduced all the amines significantly (P 0.05). Table 3.2(b) shows the effect of the drugs on the metabolite levels in the hypothalamus. The table shows that the DOPAC levels in the control animals were below the sensitivity of the method. Treatment with oxypertine, and haloperidol increased the levels of DOPAC to just above the level of sensitivity of the method. As can be seen the levels recorded are all small with little difference between either the dose or drug administered. The reverse of the effect on DOPAC levels is exhibited by the HVA levels. The initial control level of HVA is high while treatment with oxypertine at all 3 doses reduces the HVA level to below the sensitivity of the method. Reserpine and haloperidol both reduce the levels of HVA, but not significantly from control values. This in part may be due to the high standard error of mean exhibited by the control values. There is no change in the 5HIAA levels recorded irrespective of the drug or dose of drug used. Table 3.3(a) shows the effect of the drug on the biogenic amine levels in the rat striatum. The noradrenaline level under normal conditions is small in the striatum as can be seen

from the recorded control values. The 3 doses of oxypertine have all slightly increased the noradrenaline levels and with them all having small SEM this has resulted in all the oxypertine levels being significantly different (P 0.05) from the control level. Reserpine reduced the noradrenaline level to below the sensitivity of the method and haloperidol reduced the noradrenaline levels significantly (p 0.01). The striatum contains a large number of dopamine containing fibres which results in the high control values recorded for dopamine. All 3 oxypertine doses again caused a slight increase in the levels of dopamine recorded, but no significant difference in levels was noted. Reserpine produced a significant decline in the dopamine content of the striatum (p 0.01). While haloperidol produced a different effect to both oxypertine and reserpine. It caused a slight reduction in the dopamine levels, but showed no significant change from control values. The drugs produced a range of effects on the 5-HT levels depending on dose and compound administered. Oxypertine, at 4mg/kg produced a significant increase in 5-HT levels (p 0.05) but 8mg/kg and 16mg/kg oxypertine had no effect on 5-HT levels. Both reserpine and haloperidol significantly reduced the 5-HT levels (p 0.01).

Table 3.3(b) shows the effect of the drugs on the metabolite levels in the striatum. All the drugs at all doses used increased the recorded DOPAC levels. As the dose of oxypertine is increased from 4mg/kg to 16mg/kg the recorded change in DOPAC increases until it is significantly different at 16mg/kg (p 0.01). The increases recorded by reserpine and haloperidol are of a similar magnitude to those produced by 4mg/kg and 8mg/kg oxypertine so therefore are not

significantly different from control values. The effect of oxypertine on HVA levels is similar to those observed in the hypothalamus. Oxypertine reduces the HVA levels at all the doses used but only 8mg/kg and 16mg/kg significantly reduce (p 0.01) the levels of HVA. Reserpine slightly increases the HVA levels, but to not significantly, while haloperidol significantly increases (p 0.01) the levels of HVA. The 3 doses of oxypertine, reserpine and haloperidol treatment had no effect on the 5HIAA levels recorded in the striatum as with the hypothalamus.

Overall the results show that oxypertine has little effect on the biogenic amines levels in the hypothalamus and striatum apart from an increase in noradrenaline in the striatum. Reserpine reduced the levels of all the biogenic amines found in the hypothalamus and striatum, while haloperidol also reduced the levels of the amines but not to the same extent as resperpine.

On the metabolite levels, oxypertine at all 3 doses increased the levels of DOPAC while reducing the levels of HVA. Reserpine and haloperidol both increased the levels of DOPAC in the hypothalamus and striatum but had differing effects on HVA levels. In the striatum they both increased HVA levels while in the hypothalamus they reduced HVA levels. No drug had any effect on 5HIAA levels in the hypothalamus or striatum.

Discussion

The oxypertine doses were chosen from the results of Palomo and Reid (1983) who studied the effect of oxypertine and haloperidol on amphetamine induced behaviour in rats. Using a dose range of 0.25mg/kg to 16mg/kg body weight for oxypertine and 0.025mg/kg to 1.6mg/kg for haloperidol and recording stereotyped and exploratory behaviour before and after 8mg/kg amphetamine they showed that oxypertine alone had no effect on stereotyped or exploratory behaviour. After amphetamine treatment (8mg/kg), 1mg/kg, 4mg/kg and 16mg/kg oxypertine reduced stereotyped activity, 1mg/kg and 4mg/kg oxypertine increased exploratory behaviour while 16mg/kg reduced exploratory behaviour. From this they concluded that both 1mg/kg and 4mg/kg oxypertine were doses of interest as they increased exploration while reducing sterotyped activity. They did not report any results for haloperidol given alone but reported on its effect in behaviour after 8mg/kg amphetamine. Haloperidol blocked stereotyped activity at a dose of 0.025mg/kg or more and exploratory behaviour was also reduced, and although it seemed somewhat boosted at 0.025mg/kg dose, the deviation from linearity was not significant. From these results it was decided to use 4mg/kg, 8mg/kg and 16mg/kg for oxypertine and 1mg/kg for haloperidol. Palomo and Reid (1983) used 4mg/kg oxypertine and 1mg/kg haloperidol in further studies into the action of oxypertine and these doses were chosen from the results produced by Palomo and Russell (1983). The dose of 5mg/kg for reserpine was chosen as Palomo and Reid (1983, 1984) used this dose when comparing the

behaviour effect of reserpine against oxypertine (4mg/kg) in amphetamine and methylphenidate induced activity in rats.

The behaviour studies confirmed the previous results of Palomo and Russell (1983) and Palomo and Reid (1983, 1984). The results for the 3 doses of oxypertine show no significant difference from control for both locomotor and exploratory behaviour. The small levels of stereotyped activity in the control group and oxypertine treated animals resulted from one or two rats repeatedly investigating one of the holes in the apparatus. The rats treated with reserpine showed the classical signs of reserpine treatment, pilo-erection, shivering, and a disregard for their surroundings. It is therefore not surprising that the rats showed no exploratory, locomotor or stereotyped behaviour. The rats treated with haloperidol showed similar behaviour paterns to those in the experiments of Palamo and Reid (1983). The locomotor and exploratory behaviour was confined to one or two animals while no stereotyped activity was recorded. In general the rats treated with haloperidol sat motionless when placed in the hole-board apperatus uninterested in their surroundings. From the behavioural evidence alone it can be suggested that oxypertine has a different mode of action from reserpine and haloperidol. Combining these results with the behavioural results of Palomo and Russell (1983) and Palomo and Reid's (1983, 1984) studies into amphetamine and methylphenidate included behaviour, there appears little doubt that oxypertine has a different mode of action to reserpine and haloperidol.

Palomo and Reid (1983, 1984) suggestion that oxypertine is an amine depletor acting on the reserpine-resistant (amphetamine releasable) pool of amines appears to be supported by the biochemical data presented in tables 3.2(a) (b) and 3.3 (a), (b). Arbilla et al (1984) showed that the reserpine resistant pool of dopamine in the striatum accounts for less than 10% of the amine in that area. Therefore, the reserpine releasable pool of dopamine in the striatum should be approximately 90% of the total striatum dopamine content. As the reserpine releasable pool of amines is vesicular in nature, and the action of reserpine is non specific, any amine stored in a vesicle will be released by reserpine. Therefore in the hypothalamus, with noradrenaline being the principal amine stored, it may be predicted that noradrenaline levels will be reduced by approximately 90%, similar to dopamine in the striatum.

The results in Table 3.2(a) (b) and 3.3(a), (b) have been discussed in detail earlier, but in general oxypertine had no significant effect on biogenic amine content in the hypothalamus or striatum apart from an increase in striatum noradrenaline levels. Reserpine on the other hand significantly reduces (p 0.05) all the biogenic amines from the hypothalamus and striatum. Therefore, a straight comparison between the action of oxypertine (all 3 doses) and reserpine on amine levels shows an immediate difference in action. This supports the original behaviour data of Palomo and Reid (1983, 1984) and their conclusion that oxypertine acts on a different pool of amines from reserpine. The increase in noradrenaline levels produced by oxypertine in the striatum may

give a clue to the actual mechanism of oxypertine. The behavioural and biochemical evidence above suggests that oxypertine acts on the non-vesicular (amphetamine releasable) pool of amines. Scheel-Kruger (1971) described this pool as the newly synthesised, fast turnover pool of amines. Korf (1981) concluded that the importance of this pool on dopamine level control would depend on the size of this. If it is small alterations in its activity will not effect overall levels, and if it is large, alterations in its activity will effect overall levels. It is therefore suggested that in the striatum, the noradrenaline fast turnover pool accounts for more than 10% of the total noradrenaline content and any disruption of this pool by oxypertine will alter the control mechanism of the synthesising enzymes. This alteration in enzyme control may allow for an increase in synthesis, resulting in an overall increase in noradrenaline levels. (The action of oxypertine on the non-vesicular pool of amines is discussed in greater detail in the later chapters).

Haloperidol, a neuroleptic type drug is commonly used in the treatment of schizophrenic and psychotic symptoms of excitement and agitation. Haloperidol, reduced significantly ($p < 0.05$) the levels of all 3 amines in the hypothalamus and reduced significantly ($p < 0.01$) the levels of noradrenaline and 5HT in the striatum. Although, the dopamine levels in the striatum are reduced, the reduction is not significant. This, however might in part be due to the high level, with the resultant high SEMs, recorded. Haloperidol's therapeutic mode of action is thought to be through its dopamine receptor antagonism, but Jenner and Marsden (1983)

remind us that most neuroleptic drugs do not act specifically on cerebral dopamine receptors but may also interact with noradrenaline, 5HT, histamine and acetylcholine receptors. The neuronal control of dopamine function is complex, with many factors able to influence synthesis and release. As well as end product control over the synthesis of the enzymes, there are various feedback mechanisms (positive and negative) controlling the release of dopamine. Jenner and Marsden (1983) point out that the various actions of haloperidol at non-dopaminergic receptors may themselves serve, to modulate cerebral dopamine function, and that changes in dopamine turnover are a most indirect index of postsynaptic receptor function. The reduction in amine levels produced by haloperidol is discussed in a review by Bunney (1984). Comparing the action of several neuroleptic drugs Bunney (1984) reports that haloperidol (acute treatment) increases the firing rate of the A9 and A10 dopamine neuron tracts (see general introduction for anatomy of dopamine neurons). This increased firing of the dopamine neurons, combined with antipsychotic drugs ability to block autoreceptors, would lead to a significant increase in dopamine release. This increased release of dopamine will reduce the dopamine in the neurons and increase the metabolism of dopamine (effect on metabolite levels discussed later). Bunney (1984) as well as discussing the effect of neuroleptics and neuron firing rates, discussed the mechanics of several dopamine neuron feed back systems, which confirms the complexity of the neuronal control mechanisms as outlined by Jenner and Marsden (1983).

Tables 3.2(b) and 3.3(b) shows the effect of the drugs on the metabolite levels in the hypothalamus and striatum. Both tables show similar patterns of drug effect on the metabolite levels, and the change in the metabolite levels may also help explain some of the changes in amine levels recorded. DOPAC and HVA are the major metabolites of dopamine, DOPAC being synthesised by MAO (an intraneuronal enzyme) and HVA being synthesis from DOPAC by COMT (an extraneuronal enzyme). In both tables, oxypertine (all 3 doses) increases the DOPAC levels, while the HVA levels are decreased. By comparing the sites of synthesis of these two metabolites, it can be seen that there is an increase in DOPAC, the intraneuronal metabolite and a decrease in HVA, the extraneuronal metabolite. This suggests that oxypertine site of action is intraneuronal, increasing the amount of free dopamine in the cytoplasm, and making it available for degredation by MAO. If it were possible to measure MHPG sulphate, by this method, the main metabolite of noradrenaline in the rat, it would have been interesting to see if it also increases after oxypertine treatment, further suggesting that oxypertine causes the release of amines from the non-vesicular amine pool.

Haloperidol, produces a different effect on metabolite levels from oxypertine, with both HVA and DOPAC levels increased. As has been discussed earlier, haloperidol increases the release of dopamine, therefore increasing the amount of dopamine available for metabolism, both intraneuronolly after reptake and extraneuronally. A similar increase in DOPAC and HVA levels was reported by Shore (1976) using the same dose of haloperidol

(1mg/kg). The different profile of action for haloperidol against oxypertine my tell us something about the dopamine receptor activity of oxypertine. Several groups of workers (Penn, 1972, Miranda 1978, Nakahara et al, 1980) have suggested a dopamine receptor antagonist activity for oxypertine (see into introduction). At the doses used (4mg/kg, 8mg/kg, 16 mg/kg) it appears that oxypertine is free from any dopamine receptor antagonism, if haloperidols action is used as a model for a dopamine receptor antagonist drug.

The effect of reserpine on metabolite levels does not give us as much information as the effects of oxypertine and haloperidol. Reserpine produces a slight increase in DOPAC levels without effecting HVA levels. It may be that the effect of reserpine in the vesicles is so dramatic, that initally there may be a large increase in both metabolite levels but after a short period of time the levels might return to normal as the non vesicular pool of amines assumes total amine synethesis for the neurons.

Overall the biochemical data appears confirm the behaviour conclusions of Palomo and Reid (1983, 1984) in that oxypertine acts on the readily synthesized pool of amines as opposed to the vesicular pool of amines.

TABLE 3.1

Rat Motor Behaviour after Single IP Injection of Oxypertine,

	Control (Solvent)	Oxypertine (4 mg/kg)	Oxypertine (8 mg/kg)
LOCOMOTION	6.6	4.6	2.8
	(± 1.2)	(± 1.6)	(± 0.06)
EXPLORATION	7.2	4.2	2.8
	(± 2)	(± 0.7)	(± 0.6)
STEREOTYPED	1.6	2.6	1.2
	(± 1)	(± 2.6)	(± 0.8)

$\bar{x}$ ± SEM, n = 5

, Reserpine or Haloperidol

Oxypertine (16 mg/kg)	Reserpine (5 mg/kg)	Haloperidol (1 mg/kg)
1.8 (± 0.4)	0.2 (± 0.2)	1.4 (± 1)
2.4 (± 0.5)	-	0.6 (± 0.6)
1.4 (± 1.4)	-	-

TABLE 3.2 (a)

Effect of Oxypertine, Reserpine and Haloperidol on Hypothalamic Biogenic Amine levels

	Control (Solvent)	Oxypertine (4 mg/kg)	Oxypertine (8 mg/kg)	Oxypertine (16 mg/kg)	Reserpine (5 mg/kg)	Haloperidol (1 mg/kg)
NORADRENALINE	78 (± 31.5)	43.7 (± 5.4)	45 (± 6.1)	34.2 (± 7.2)	7.1** (± 4.1)	17.4* (± 1.1)
DOPAMINE	26.1 (± 10.7)	30.5 (± 3.2)	21.5 (± 2.1)	17.9 (± 1.9)	0.3* (± 0.1)	0.5* (± 0.2)
5 - HT	42.2 (± 16.7)	37.2 (± 7.7)	29.4 (± 3.8)	32.1 (± 9.3)	3.3* (± 2.2)	3.6* (± 1.6)

All levels in ng/mg protein. n = 5, (± SEM)

* Signficant difference from control at 5% using one tailed multiple range t - Test

** Significant difference from control at 1% using one tailed multiple range t - Test

TABLE 3.2 (b)

Effect of Oxypertine, Reserpine and Haloperidol

All levels in ng/mg protein (± SEM) n = 5

	Control (Solvent)	Oxypertine (4 mg/kg)
DOPAC	Below Sensitvity	1.9 (± 1.9)
HVA	30.1 (± 15.8)	Below Sensitvity
5 HIAA	23.7 (± 9.9)	18.1 (± 3.6)

* Significantly different from control at 5%,

on Rat Hypothalamic Biogenic Amine Metabolite Levels

Oxypertine (8 mg/kg)	Oxypertine (16 mg/kg)	Reserpine (5 mg/kg)	Haloperidol (1 mg/kg)
9.9 (± 4.1)	3.1 (± 3.1)	4.2 (± 1.8)	7.4 (± 0.5)
Below Sensitvity	Below Sensitvity	3.3 (± 3.3)	6.7 (± 4.2)
20.6 (± 4)	15.8 (± 0.8)	20 (± 5.7)	18.8 (± 6.2)

using one tailed multiple range t - Test

TABLE 3.3 (a)

Effect of Oxypertine, Reserpine and Haloperidol on Biogenic Amine Levels from Rat Striatum

All values in ng/mg protein (± SEM) n = 5

	Control (Solvent)	Oxyperine (4 mg/kg)	Oxypertine (8 mg/kg)	Oxypertine (16 mg/kg)	Reserpine (5 mg/kg)	Haloperidol (1 mg/kg)
NORADRENALINE	11.1 (± 1.6)	19** (± 2.7)	16.6* (± 0.8)	18.2** (± 0.9)	Below Sensi ity	0.2** (± 0.1)
DOPAMINE	333.3 (± 23.9)	397.2 (± 98.2)	381.9 (± 69.8)	448.9 (± 54.6)	12 ** (± 2.5)	264 (±44.1)
5 - HT	27.6 (± 1.7)	41.7* (± 4)	24.6 (± 4.4)	25.5 (± 2.9)	5.5** (± 3.4)	8.6** (± 3.6)

* Significant difference from control at 5%, using one tailed multiple range t - Test

** Significant difference from control at 1%, using one tailed multiple range t - Test

TABLE 3.3 (b)

Effect of Oxypertine, Reserpine and Haloperidol in Rat Striatum Biogenic Amine Metabolite Levels

All values in ng/mg protein (± SEM) n = 5

	Control (Solvent)	Oxypertine (4 mg/kg)	Oxypertine (8 mg/kg)	Oxypertine (16 mg/kg)	Reserpine (5 mg/kg)	Haloperidol (1 mg/kg)
DOPAC	65.4 (± 4.1)	160.2 (± 50.7)	171.7 (± 4.1)	271.5** (± 20.6)	102.6 (± 12.3)	168.7 (± 9.9)
HVA	51.1 (± 2.3)	23.6 (± 4.2)	17* (± 7.5)	22.3* (± 4.7)	70.1 (± 3.4)	115.7** (± 14.8)
5 HIAA	21.9 (± 1.6)	15.5 (± 4.4)	18.2 (± 2.2)	26.4 (± 1.8)	25.6 (± 2.8)	17.4 (± 4.1)

* Significant difference from control at 5%, using one tailed multiple range t - Test

** Significant difference from control at 1%, using one tailed multiple range t - Test

CHAPTER 4

Kinetics of Catecholamine Turnover

The term "turnover" was first described by Zilversmit et al (1943) who said that, turnover, is the amount of a metabolite pool that is transported or metabolised. The term "turnover rate", is often used instead of turnover, although both expressions have the same dimensions of mass. time^{-1}. In turnover studies that assume the transmitter is in a steady state (Brodie et al, 1966); the rate of synthesis equals the rate of removal or metabolism, or, that the synthesis or breakdown has been completely blocked by an appropriate enzyme inhibitor, in which case either the rate or depletion or accumulation of transmitter can be determined as an estimate of turnover (Korf, 1981) Brodie et al (1966) were the first group to present a mathematical model that (assuming steady state kinetics) could be used to calculate the turnover rate and the turnover time for a specific catecholamine. Tozer et al (1966) had shown that the rate of 5HT synthesis in brain could be measured non-isotopically by taking advantage of steady state kinetics, in which rate of synthesis and loss were equal. Brodie et al (1966) used this in their argument, that is if the tissue stores of noradrenaline were also maintained at a constant level, a balanced process of synthesis and removal existed, and that the catecholamine levels would decline exponentially after blockade

of synthesis. In support of this, they reported on some unpublished preliminary studies of Dlabac which showed that brain catecholamine levels did disappear at an exponential rate following catecholamine synthesis blockage by -methyl-tyrosine. In their own experiments, Brodie et al (1966) used -methyl-paratyrosine to inhibit noradrenaline synthesis and monitored its effect on noradrenaline levels in rat brain and heart and showed that noradrenaline levels declined as single exponentially curves. In the case of heart noradrenaline, the slope of decline was similar to the decline in specific activity after H^3-noradrenaline labelling. They concluded that the efflux of noradrenaline in the normal steady state is exponential and advantage of this could be taken, since rates of synthesis and removal are equal.

Mathematical Model

The rate of catecholamine synthesis (K) may be expressed as

$$K = k[CA]_o \qquad (1)$$

where k is the rate constant of CA efflux and $[CA]_o$ the amine concentration at time zero (ie normal amine concentration).

After inhibition of synthesis, the concentration of CA declines at a rate that is proportional to the concentration, ie.

$$\frac{-d[CA]}{dt} = k[CA]$$

Integrating this expression gives,

$$[CA] = [CA]_o e^{-kt}$$

and converting to $\log_{10}$,

$\log [CA] = \log [CA]_o - 0.434\ kt$ (2)

where $[CA]_o$ is the initial catecholamine level, [CA] is the catecholamine level after time .t,

A plot of the graph, log [CA] vs, time yields a straight line, the slope of which is 0.434 times k., the rate constant of CA efflux. Substituting in equation (1), the product of k, (from equation (2)) and $[CA]_o$ will give K, the rate of CA efflux, which is equal to the rate of [CA] synthesis, and therefore the turnover rate of catecholamines.

It should be remembered that at steady state, as well as the rate of synthesis equalling the rate of efflux, the rate of efflux is proportional to the initial concentration of catecholamines. Expression of this relationship is possible through equation (1). $K = k\ [CA]_o$). On rearrangement, this becomes $k = K/[CA]_o$. Expressed in this form, it is evident that k, is the fraction of the total CA that is formed and lost per unit of time (fractional turnover rate). The reciprocal of k or the turnover time equals, $[CA]_o/K$ and is the time interval required for the biosynthesis of the amount of CA equal to that stored in the tissue.

In practical terms, from knowing the initial concentration of catecholamine, $[CA]_o$, the concentration of catcholamine after time t, [CA], and the time of synthesis inhibition, t, it is possible to calculate.

A) the rate constant of amine loss k, by rearranging equation (2).

$$k = \frac{\log [CA]_o - \log [CA]}{0.434t.}$$

After calculating k, the turnover rate (K) can be calculated.

ie. $K = k[CA]_o$

and the turnover time (TT), calculated by.

$$TT = [CA]_o/K$$

When reporting their results, Brodie et al (1966) present the initial amine level ($\pm$ S.E), the rate constant of amine loss ($\pm$ S.E.), the turnover rate and turnover time. For the calculation of turnover rates, the values for tissue levels of catecholamines were logarithmically transformed for calculation of linearity of regression, and the standard error of the regression coefficients. What they failed to provide was any statistical analysis of the turnover rate or turnover time.

Hohn and Wuttke (1978), in an attempt to provide some statistical analysis of the turnover rate and turnover time calculated the standard error of mean (SEM) for both of these figures. They proposed that, since the mean turnover rate is a function of two independant variables (ie initial catecholamine level $\pm$ S.D. and end concentration after α-MPT), means and standard deviations of these rates can be estimated. The mean is the product of initial catecholamine (A) level and the rate constant (k) (R=Ak). The standard deviation of this mean can be estimated by the law of the propagation of errors, ie, if $y= f(x_1, x_2 \ldots . x_n)$ where $x_1 \ldots . x_n$ are means of random variables and $s_1 \ldots . s_n$ are their standard deviation, then the standard deviation of y is

$$Sy = \sqrt{\frac{(Y_1)^2}{(X_1)} S_1^2 \pm \ldots\ldots\ldots + \frac{(Y_n^2) S_n^2}{(yx_n)^2 S_n^2}}$$

What the law of the propagation of errors does is, it incorporates the standard deviation of S, into the calculation of the standard deviation of Sn.

Without further explanation Hohn and Wuttke (1978) presented tables of results including the SEM of turnover rate and turnover time. They did not explain how they substituted the measureable variables, ie. $[CA]_o$, [CA], or t into the basic mathematical formula, this making it difficult to repeat their work. In the mathematical model proposed by Hohn and Wuttke (1978), they argue that the turnover rate is a function of two independant variable. That is, the concentration of catecholamine after time t. of MPT treatment is independent of the initial concentration of

catecholamine. This assumption is in direct contrast to the steady state assumptions of Brodie et al (1966), who it should be remembered said, "The rate of efflux is proportional to the initial concentration of catecholamines", implying that the concentration of catecholamines after time t, is dependant in the initial concentration.

It was not until 1982, that the equations used for calculating the SEM's of turnover rate and time were published by Hiemke and Ghraf. These being derived from the law of the propagation of errors first proposed by Hohn and Wuttke (1978). The equations are,

$$\text{SEM of TR} = \sqrt{\frac{1}{t^2}\ln^2\frac{Coe}{Ct}\,SEM_{Co}^2 + \frac{Co}{t^2}^2\frac{SEM\ Ct}{Ct}^2}$$

$$\text{SEM of TT} = \sqrt{\frac{t^2\ SEM^2\ Co^2}{[(\ln Co-\ln Ct)^2\ Co]^2} + \frac{t^2\ SEM^2\ Ct^2}{[Ct\ (\ln Co-\ln Ct)^2]^2}}$$

where C_o = catecholamine concentration at time zero

Ct = catecholamine concentration after time t of MPT treatment

t = time of MPT treatment.

These equations, being based in the orginal paper of Hohn and Wuttke (1978) assue that the variables are independant of each other. Hiemke and Ghraf (1982), however did not give the derivation of these equations from first principles therefore making it impossible to check their mathematics. (See appendix 1).

In between the papers of Hohn and Wuttke (1978) and Hiemke and Ghraf (1982), Wuttke published a paper in association with another worker (Honma and Wuttke (1982)). This paper again calculated SEM of TR and TT based on the method proposed by Hohn and Wuttke (1978), but their explanation of the assumptions used, differed form the orginal paper. Honma and Wuttke (1980) proposed that, "The turnover rates are a function of two or more variables (ie. catecholamines concentrations $\pm$ SD at time zero and at the various times after MPT). Hence, the different mean catecholamine concentrations after MPT and their SD are dependent upon the mean catecholamine concentrations at time zero and on the SD of this mean." Therefore rather than the variables being independent of each other, they are dependant upon each other. Honma and Wuttke (1980) did not explain if this difference in variable dependence affected the mathematical calculation. Rance et al (1981) also in attempting to improve the analysis of turnover rates, produced a equations for the calculation of the standard deviation of turnover rate.

$$SD_{TR} = \sqrt{(k)^2[SD(Co)]^2 + 2(k)(Co)(Covarience) + (SDk)^2Co^2}$$

In calculating their turnover rates and rate constants, they used the method of Brodie et al (1966), therefore assuming that Ct is dependent upon Co. They derived the SD_{TR} from the law of the propagation of errors as suggested by Hohn and Wuttke (1978)(See appendix 2), but rather than assuming that the variables were

independent, they followed through the assumptions of Brodie et al, (1966) in that the variable were dependent upon each other.

In assuming that the variables are dependent upon each other, it introduces a convariance term into the equation. Unfortunately, Rance et al, (1981) do not explain how to calculate this term, making it difficult to check their mathematics or use the equation. Appendix 3 gives details of how to calculate the covariance factor and the mathmatical proof of the covariance.

Rance et al (1981) and Hiemke and Ghraf (1982) have both calculated terms of statistical inference for the turnover rate. Both groups used the law of the propagation of errors for the derivation of their equations with the only difference being Rance et al (1981) assumed Ct was dependant upon Co while Hiemke and Ghraf (1982) assumed Ct independent from Co. If the same assumptions were applied to both equations, it should be possible to show that they are the same.

ie $$SD_{TR} = \sqrt{(k)^2 (SDCo)^2 + 2(k)(Co)(Covarience) + (SDk)^2 CO^2}$$

Rance et al (1981)

$$SEM_{TR} = \sqrt{\frac{1}{2} \ln^2 : \frac{Coe}{Ct} : SEM\,Co^2 + \frac{CO^2}{t^2} \frac{SEM\,Ct^2}{Ct^2}}$$

Hiemke and Ghraf (1982)

See appendix 4

In the original assumptions of Brodie et al (1966), Ct was said to be dependant upon Co, and as Hiemke and Ghraf (1982) and Hohn and Wuttke (1978) both used the methods of Brodie et al (1966) for the calculations of turnover rates and turnover times it is difficult to know why they said it was independant of Co. Rance et al (1981) have shown that it is possible to calculate the SD of turnover rate using the law of the propagation of errors assuming that Ct is dependant upon Co. As Hiemke and Ghraf (1982) calculated the SEM of turnover time as well as turnover rate it would be interesting to see what difference there would be in their equations for these calculations assuming that Ct is dependant upon Co. (See appendix 5).

$$SEM_{TR} = \sqrt{\frac{1}{t^2}\ln^2\frac{Coe}{Ct}SEM^2 Co + \frac{Co^2}{t^2}\frac{SEM^2}{Ct^2}Ct - \frac{4Co}{t^2 Ct}\ln\frac{Coe}{Ct}\frac{cov\,[co,\ Ct]}{n}}$$

$$SEM_{TT} = \sqrt{\frac{t^2}{Co^2(\ln Co - \ln Ct)^2}SEM^2 Co \frac{t^2}{Ct^2(\ln to - \ln Ct)^2} - \frac{2t^2}{Co\ Ct(\ln Co - \ln Ct)^2}\frac{cov[co,\ Ct]}{n}}$$

With the assumption that Ct^1 is dependant upon Co it can be seen the extra term included in the equations will alter the calculated value of SEM_{TR} and SEM_{TT}.

The extent to which the inclusion of the convariance term will alter the SEM_{TR} or SEM_{TT} has still to be calculated. It may be

that the overall change in the SEM_{TR} or SEM_{TT} is small enough to ignore so that the orginal equations proposed by Hiemke and Ghraf (1982) are sufficient for the calculations.

Drugs Used For Amine Synthesis Inhibition

Since Brodie et al (1966) introduced their method for the calculation of amine turnover rates, many groups of workers have repeated their experiments. Variations in the methodology used do occur, with one of the main differences being in the dose of -methyl-p-tyrosine used to inhibit the synthesis of the enzyme tyrosine hydroxylase. Brodie et al (1966) used 200mg/kg of α methyl tyrosine dissolved in 4N NaOH, adjusted to pH9 with 4N $HClO_4$ and diluted with water to form a 2% solution. An additional dose of 75 mg/kg or 100mg/kg was sometimes given after 3 hrs to ensure synthesis blockage for up to 10hrs. The development of the methylester of α-methyl -paratyrosine (MPT) with different solubility characteristics from α-methyltyrosine removed the drug solubility problem encountered by Brodie et al (1966), as the methylester is soluble in water. Spector et al (1975), Kizer et al (1975), Versteeg et al (1975) and Hohn and Wuttke (1978) showed that 250mg/kg body weight, of the methylester of α MPT blocked catecholamine synthesis for at least 3 hrs. Rance et al (1981) used a dose of 400mg/kg body weight of the amino acid αMPT to produce an exponential decline in noradrenaline and dopamine levels for 24 hrs. 5HT synthesis has been shown to be blocked by a dichlorophenylalanine (pCPA) through an inhibitory action on tryptophan hydroxylase.

Jequie et al (1968) and Lovenberg et al (1968) showed that a single dose of pCPA (300mg/kg body weight) produces a 80% depletion of brain 5HT which persists for 4-5 days, however the onset of action is slow compared to the action of αMPT.

A preliminary study was carried out to find the dose of αMPT and pCPA that produced the best inhibition of the synthesis enzymes. 500mg/kg αMPT (methyl ester) and 300mg/kg pCPA (methyl ester) were found to be the doses that best inhibited synthesis. The effect of the drugs was studied over 16 hrs and it was found that noradrenaline, and dopamine levels fluctuated about the 0hr value for the first 2 hrs, before declining monophasically till 8 hrs after administration. The levels of the amines stopped declining after 8 hrs and in some cases (dopamine in hypothalamus) increased between 8 and 16 hrs. It was found that under the chromatographic conditions used MPT is eluted at the same time as DOPAC. This makes it impossible to measure the DOPAC levels in the samples and attempts to separate the two peaks proved unsuccessful. HVA declined in a pattern similar to dopamine and noradrenaline. 5HT and 5HIAA levels declined slowly but steadily over the 16 hrs of the experiment, similar to the effect of pCPA observed by the other groups (see discussion above).

Materials and Methods

Male Sprague Dawley rats (200-250g) were used in all experiments. Each animal received a single IP injection of 500mg/kg αMPT methyl ester, and 300mg/kg pCPA methyl ester. The animals were killed 0 hrs, 2 hrs or 8hrs after drug administration and the amine and metabolite content the hypothalamus and striatum analysed using the HPLC/ECD method described previously. Results are given in ug/g wet tissue. Catecholamine turnover rates and turnover times are calculated using the method of Brodie et al (1966) SEMs of turnover rate and turnover time are calculated assuming variable independence (Hiemke and Ghraf 1982) and variable dependence (see chapter introduction) and the difference between the two methods compared.

Results

Tables 4.1 and 4.2 show the effects of 500mg/kg αMPT and 300mg/kg pCPA on hypothalamic and striatal amine and metabolite levels for the given times after administration. The 0 hrs times for each group may be used as control values as the animals were killed immediately the drugs were injected. It can be seen that the 2 hr values are slightly higher or lower than the 0hr value giving an indication of the fluctuation in values that was seen in preliminarly investigation into drug dosage. A sharp drop in levels between 2 hrs and 8 hrs is shown by noradrenaline, dopamine, and HVA (which is assumed to be monophasic) while a slow decline in 5HT and 5HIAA levels occurs from the time of administration.

Table 4.3 compars the SEMs of turnover rate and time as calculated by both methods for noradrenaline from the hypothalamus and dopamine from the striatum. This shows that if independence of variables is assumed, the SEM's of turnover rate and turnover time are both smaller than if dependence of variables is assumed ie. for noradrenaline from the hypothalamus, TR, $\pm$25.46 against $\pm$53.4 and TT, $\pm$0.48 as against $\pm$0.71.

Appendix 6 shows the calculations required in calculating the SEM of TR and TT when variable independence is assumed while appendix 7 shows the computer programme used for the calculation of the SEM of TR and TT assuming variable dependence.

Discussion

There is much confusion in the literature about the methods available for the calculation of turnover rates and turnover times and the relative accuracy of calculations. As all workers use the paper of Brodie et al (1966) as the starting point from which the calculations are based, it is difficult to see why Hohn and Wuttke (1978) assumed that Ct is independant of Co, rather than Ct being dependant upon Co. Hohn and Wuttke (1978) failed to show any of the mathematical formula they used to calculate their results and also failed to explain fully the origins of the mathematical derivations. It was not until 4 years later that Hemke and Ghraf (1982) published equations for the calculations of SEM fo TR and TT based on the work of Hohn and Wuttke (1978) i.e. using the laws of

the propation of errors. Rance et al (1981) also used the laws of the propagation of errors to derive their calculation for the standard deviation of turnover rate, but used the orginal assumption that Ct is dependant on Co. This necessitates the inclusion of a covariance factor into the calculation, but similar to the in the other workers they failed to explain fully their derivation from first principles or fully explain the use of their calculation. When the two equations proposed for the calculation of the SD and SEM of the turnover rates are compared (see Appendix 4) it can be shown that if the covariance factor is assumed to be zero (ie. it independant of Co) then the equations are similar. Since the equations are the same, it is possible to alter the equations of Hiemke and Ghraf (1982) to include a covariance factor by assuming the Ct is dependant upon Co. Appendix 5 shows the additional terms required for this transition and these are the equations used in the computer programme (Appendix 7).

The comparasions in the different methods for the calculations of the SEM of TR and TT (Table 4.3) has produced an interesting result. The SEM calculated when variable independence was assumed are lower than when variable dependence was assumed. The SEM"s for the TR are more the 50% less for both amines chosen while the SEM of TT of noradrenaline is aproximately 30% less and the SEM of TR is again 50% less. If the difference between the calculated results had been small, it may have been possible to ignore the covariance factor in the calculation and use the equation as first proposed by Hohn and Wuttke (1978). With such a large difference between the calculated values though, any calculation undertaken

using Hohn and Wuttke's equations will have a false degree of accuracy about them. This could lead to the misintrepretation of results and misinterpretation of a drugs properties.

The more accurate estimation of SEM of turnover rates and times achieved when Ct is assumed dependant on Co, is useful when the significant difference between turnover rates is being calculated. Rance et al (1981) showed that it is possible to undertake this calculation by calculating the Z values between two ratios. Where

$$Z = \frac{K_1 - K_2}{\sqrt{(SEK_1)^2 + (SEK_2)^2}}$$

Z is then entered on a table of standard normal distribution. Use of this calculation should make it possible to examine the effect of different drugs on amine turnover and compare drugs of different action.

TABLE 4.1

Effect of 500 mg / kg αMPT and 300 mg / kg pCPA on Biogenic Amine and Metabolite Levels in Rat Hypothalamus

Time after injection	Noradrenaline	Dopamine	5 -HT	5 HIAA	HVA
0 hrs	2048 (± 69.7)	1999 (± 112.8)	5409 (± 256.2)	1854 (± 224)	3924 (± 555.6)
2 hrs	1552 (± 100.6)	2584 (± 666)	5373 (± 83.4)	839.8 (± 160.3)	2998 (± 978.7)
8 hrs	612 (± 39.5)	668 (± 121.4)	3789 (± 342.7)	618 (± 43.9)	1841 (± 223)

All values in ng/g wet tissue (± SEM, n = 10)

TABLE 4.2

Effect of 500 mg / kg αMpT and 300 mg / kg pCPA on Biogenic Amine and Metabolite Levels in Rat Striatum

Time after injection	Noradrenaline	Dopamine	5-HT	5HIAA	HVA
0 hrs	297 (± 34.2)	34540 (± 868.3)	2672 (± 99.6)	1614 (± 144.9)	5628 (± 1040.7)
2 hrs	330 (± 34.7)	23316 (± 1795.7)	2069 (± 309.1)	674 (± 86.4)	1832 (± 312.9)
8 hrs	142 (± 17.1)	7118 (± 619.8)	1777 (± 147.6)	590 (± 59.3)	152 (± 84.7)

All values in ng/g wet tissue (± SEM, n = 10)

TABLE 4.3

Comparison between the two methods for the calculation of SEMs of turnover rates and turnover times

Amine	Initial Conc	Concentration After Time	Rate Constant of Amine Loss	Turnover Rate	Turnover Time
	ng/g ± SEM	ng/g ± SEM	k (hr -1)	ng/g / hr ± SEM	hr ± SEM
NORADRENALINE	2048	612	0.151	309.5 (± 25.36)*	6.62 (± 0.48)
(HYPOTHALAMUS)	(± 69.7)	(± 39.5)		(± 53.4)**	(± 0.71)**
DOPAMINE	34540	7118	0.198	6839 (± 468)*	5.05 (± 0.46)*
(STRIATUM)	(± 868.3)	(± 619.8)		(± 1216)**	(± 1.15)**

* SEM TR and SEM TT assuming independance of variables

** SEM TR and SEM TT assuming dependance of variables

Appendix 1

Derivation of Equations Proposed by Hiemke &Ghraf (1982)

The law of the proagation of errors.

If $Y = f(x_1, x_2, \text{-------}, x_n)$

$$Sy = \sqrt{(\frac{dY^2}{dX_1})S_1^2 + \text{------} + (\frac{dY^2}{dX_n})S_n^2}$$

assuming $Cov(x_i, x_j) = 0$ for $i \neq j$

i.e. (x_i, x_j) mutually independant.

From Brodie et al. (1966)

$$-\frac{dC_t}{dt} = kC_t$$

$$C_t = C_o e^{-kt}$$

$$\Leftrightarrow \ln C_t = \ln C_o - kt$$

$$\Leftrightarrow k = \frac{\ln C_o - \ln C_t}{t}$$

$$TR = K = kC_o$$

$$\hat{K} = C_o\hat{k} = \frac{C_o(\ln C_o - \ln C_t)}{t} \quad \text{---------- (1)}$$

By the law of the propagation of errors.

$$SEM\ \hat{K} = \sqrt{(\frac{d\hat{K}}{dC_o})^2\ SEM^2 + (\frac{d\hat{K}}{dC_t})^2\ SEM\ C_t^2}$$

$$\frac{d\hat{K}}{dC_o} = \frac{\ln C_o - \ln C_t}{t} + \frac{C_o}{tC_o} \qquad \text{using equation (1)}$$

$$= \frac{\ln C_0 - \ln C_t}{t} + \frac{1}{t}$$

$$= \frac{\ln C_o - \ln C_t + \ln e}{t}$$

$$= \frac{1}{t}\ln\left|\frac{C_o e}{Ct}\right| \qquad e = 2.7183$$

$$\frac{dK}{dC_t} = -\frac{C_o}{tC_t}$$ using equation (1)

$$\therefore SEM_{TR} = \sqrt{\frac{1}{t^2}\ln^2\left|\frac{C_o e}{C_t}\right| SEM^2_{C_o} + \frac{C_o^2}{t^2 C_t^2} SEM^2_{C_t}}$$ Hiemke & Ghraf (1982)

$$\text{Turnover time} = \frac{1}{K} = \frac{t}{\ln C_o - \ln C_t}$$

By the law of the propagation of errors.

$$SEM_{TT} = \sqrt{(\frac{dTT}{dC_o})^2 SEM^2_{C_o} + (\frac{dTT}{dC_t})^2 SEM^2_{C_t}}$$

$$\frac{dTT}{dC_o} = \frac{t}{C_o(\ln C_o - \ln C_t)}$$

$$\frac{dTT}{dC_t} = -\frac{t}{C_t(\ln C_o - \ln C_t)}$$

$$\therefore SEM_{TT} = \sqrt{\frac{t^2}{C_o^2(\ln C_o - \ln C_t)^2} SEM^2_{C_o} + \frac{t^2}{C_t^2(\ln C_o - \ln C_t)^2} SEM^2_{C_t}}$$

Hiemke & Graf (1982)

Appendix 2

Proof of Rance et al (1981) mathematic derivation

To prove

$$SD[XY] = \sqrt{\mu x^2 [SD(Y)]^2 = 2\,\mu x \mu y\, cov\,[X,Y] + \mu y^2\,[SD(X)]^2}$$

μx = mean of X = E [X]

μy = mean of Y = E [Y]

Proof: $XY = \mu x\,\mu y + (x-\mu x)\mu y + (y-\mu y)\mu x + (x-\mu x)(y-\mu y)$ ------(1)

$\therefore \Sigma[XY] = \mu x\ \mu y + cov\,[X,Y]$ $\quad \Sigma[x-\mu x] = 0$

$\Sigma[y-uy] = 0$

$$(1) \Leftrightarrow [XY]^2 = \mu x^2\ \mu y^2 + (x-\mu x)^2\ \mu y^2 + (y-\mu y)^2\ \mu x^2 + (x-\mu x)^2\,(y-\mu y)^2$$

$$+ 2\mu x\,\mu y^2\ (x-\mu x) + 2\mu x^2\ \mu y\ (y-\mu y) + 2\mu x\,\mu y\ (x-\mu x)(y-\mu y)$$

$$+ 2\mu x\ (x-\mu x)(y-\mu y)^2 + 2\mu y(x-\mu x)^2\ (y-\mu y)$$

$$+ 2\mu x\ \mu y\ (x-\mu x)(y-\mu y)$$

$$\therefore \Sigma[(XY)^2] = \mu x^2\ \mu y^2 + \mu y^2\ var\,[X] + \mu x^2\ var\,[Y]$$

$$+ 4\,\mu x\,\mu y\ cov\,[X,Y] \qquad \text{APPROXIMATELY}$$

ignoring terms of 3rd and higher order.

$(\Sigma[(X-\mu x)^2] = var[X],\ \Sigma[(X-\mu x)(Y-\mu y)] = cov[X,Y])$

But varience of XY = $var\,[X,Y] = \Sigma[(XY)^2] - (\Sigma[XY])^2$

=P.T.O.

$$= \mu x^2 \mu y^2 + \mu y^2 \, var[X] + \mu x^2 \, var[Y] + 4\mu x \, \mu y \, cov[X,Y]$$
$$= \mu x^2 \mu y^2 - 2\mu x \, \mu y \, cov[X,Y] - cov^2[X,Y]^*$$

* can ignore this term.

$$\therefore \; var[X,Y] = \mu x^2 \, var[Y] + 2\mu x \, \mu y \, cov[X,Y] + \mu y^2 \, var[X]$$

$$\text{ie.} \quad (SD[X,Y])^2 = \mu x^2 (SD[Y])^2 + 2\mu x \, \mu y \, cov[X,Y] + \mu y^2 (SD[X])^2$$

In our problem, $\mu x = k$

and, $\mu y = C_o$

$$\therefore \; SD_{TR} = \sqrt{(k)^2 (SD(C_o))^2 + 2(k)(C_o)(\text{covariance}) + C_o^2 (SDk)^2}$$

Appendix 3 Calculation of Covariance Factor

Theory: $\text{cov}\,[\bar{Y}o;k] = -X[S.D.(k)]^2$

Proof: Consider data as follows:-

Xo	X1
Yo1	Y11
Yo2	Y12
"	"
"	"
Yon1	Y1n2

Regression line is $Y = a + kX$

$$\bar{Y}_o = \frac{1}{n1} Yo1 + \frac{1}{n1} Yo2 + \text{----------} + \frac{1}{n1} Yon1$$

$$k = \frac{(Xo - \bar{X})Yo1}{\sum(xi - \bar{X})^2} + \frac{(Xo - \bar{X})}{\sum(xi - \bar{X})^2} + \text{-------} + \frac{(Xo - \bar{X})Yon1}{\sum(Xi - \bar{X})^2} + \frac{(X1-\bar{X})Y11}{\sum(Xi-\bar{X})^2} + \text{---}$$

$$\therefore \text{cov}\,[Yo,k] = \left\{ \frac{(Xo-\bar{X})}{n1\sum(Xi-\bar{X})^2} + \underbrace{\frac{(Xo-\bar{X})}{n1\sum(xi-\bar{X})^2} + \text{----} + \frac{(Xo-\bar{X})}{n1\sum(xi-\bar{X})^2}}_{n1} \right\}^2 S$$

since Yi is independant

$$= \frac{(Xo-\bar{x})^2 S}{\sum(xi-\bar{x})^2}$$

$$= (Xo-\bar{x})^2 [SD\ k)]$$

In our problem Xo = 0

$$\therefore \quad \text{cov}\,[\bar{Y}_o,\ k] = -\bar{X}\left\{SD\,[k]\right\}^2$$

The SDk is calculated by the method of least squares after log transformation of catecholamine concentrations.

$$\text{ie.} \quad SDk = \sqrt{\frac{1}{n-2}\left(\Sigma(y-\bar{y})^2 - \frac{[\Sigma(x-\bar{x})(y-\bar{y})]^2}{\Sigma(x-\bar{x})^2}\right)}$$

where x = Co and Y=Ct.

Therefore the calculation of the covariance.

covariance = $-x\ (SD[k])^2$

$$= -\bar{Co}\left[\frac{1}{n-2}\left(\Sigma\,(Ct - \bar{Ct})^2 - \frac{[\Sigma(Co-\bar{Co})(Ct-\bar{Ct})]}{\Sigma(Co-\bar{Co})^2}\right)\right]$$

Appendix 4

In calculating the SEM of turnover rate and time, Hiemke and Ghraf (1982) assumed that C_o and C_t were independant of each other, Rance et al (1981) in calculating the SD of turnover rate assumed that C_t was dependent upon C_o. Since they both used the law of propagation of errors in the derivation of their equations it should be possible to show that the equation are the same using the same initial assumptions.

ie. $$SD_{TR} = \sqrt{(k)^2\,[SD\,(C_o)\,]^2 + 2\,(k)\,(Co)\,(\text{covariance}) + (SDk)^2 Co^2}$$

Rance et al (1981)

$$SEM_{TR} = \sqrt{\frac{1}{t^2}\,\ln^2\left|\frac{Co^e}{Ct}\right| SEM^2 Co \quad + \quad \frac{Co^2}{t^2}\cdot\frac{SEM^2 Ct}{Ct^2}}$$

Hiemke and Ghraf (1982)

Since SEM = SD/$\sqrt{n}$., multiplying SEM by $\sqrt{n}$ converts SEM to SD.

$$\therefore \quad SD_{TR} = \sqrt{\frac{1}{t^2}\ln^2\left|\frac{Co^e}{Ct}\right| SEM^2 Co \; + \; \frac{Co^2}{t^2}\;\frac{SD^2 Ct}{Ct^2}} \quad \ldots\ldots\ldots(1)$$

Using the equation of Rance et al (1981), if it were assumed to C_t was independent from C_o, this would remove the covariance term from the equation, since when variable are independent of each other, Covariance = 0

Rance et al, becomes

$$SD_{TR} = \sqrt{(k^2)\;[SD(Co)]^2 \; + \; (SD_k)^2\; Co^2}$$

From Brodie et al, (1966)

$$k = \frac{\ln Co - \ln Ct}{t}$$

$$= \frac{\ln \left|\frac{Co}{Ct}\right|}{t} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (2)$$

By the of the propagation of errors,

$$SD_k^2 = \left(\frac{dk}{dCo}\right)^2 SD^2Co + \left(\frac{dk}{dCt}\right)^2 SD^2Ct$$

$$\Leftrightarrow \left(\frac{1}{tCo}\right)^2 SD^2Co + \left(\frac{1}{tCt}\right)^2 SD^2Ct$$

$$\Leftrightarrow \frac{SD^2Co}{t^2Co^2} + \frac{SD^2Ct}{t^2Ct^2} \qquad \ldots\ldots\ldots\ldots\ldots\ldots\ldots\ldots (3)$$

From Rance et al,

$$SD^2TR = (k)^2[SD(Co)]^2 + (SDk)^2Co^2$$

Substituting k^2 by equation (2) and SDk by equation (3)

$$SD^2TR = \left(\frac{\ln \left|\frac{Co}{Ct}\right|}{t}\right)^2 SD^2Co + \left(\frac{SD^2Co}{t^2Co^2}\,\frac{SD^2Ct}{t^2Ct^2}\right)Co^2$$

$$= \frac{\ln^2 \left|\frac{Co}{Ct}\right|}{t^2} SD^2Co + \frac{SD^2Co}{t^2} + \frac{Co^2SD^2Ct}{t^2Ct^2}$$

$$= \left(\frac{\ln^2 \left|\frac{Co}{Ct}\right| + 1}{t^2}\right) SD^2Co + \frac{Co^2SD^2Ct}{t^2Ct^2}$$

$$= \frac{1}{t^2}\ln^2 \left|\frac{Co^e}{Ct}\right| SD^2Co + \frac{Co^2SD^2Ct}{t^2Ct^2}$$

$$SDTR = \sqrt{\frac{1}{t^2}\ln^2 \left|\frac{Co^e}{Ct}\right| SD^2Co + \frac{Co^2}{t^2}\,\frac{SD^2Ct}{Ct^2}}$$

This the same as equation (1). It is therefore possible to derive the of Hiemke and Ghraf (1982) from the equation of Rance et al (1981) assuming independance of variables.

Appendix 5

Hiemke and Ghraf (1982) used the law of the propagation of errors to calculate SEM of turnover rate and turnover time.

ie. If $Y = f(x_1, x_2, \text{----------}, x_n)$

$$SD_Y = \sqrt{\{\frac{dY}{dX_1}\}SD_1^2 + \text{---------} + \{\frac{dY}{dX_n}\}^2 SD_n^2}$$

Assuming $Cov[xi,xj] = \emptyset$ for $i \neq j$, ie. (xi,xj) mutually independent . For the more general situation where (xi,xj) are dependant upon each other, $Cov[xi,xj] \neq \emptyset$ for $i \neq j$.

$$\therefore \quad SD_Y = \sqrt{\{\frac{dY}{dX_1}\}^2 SD_1^2 + \text{---} + \{\frac{dY}{dX_n}\}^2 SD_n^2 + 2\frac{dY}{dX_1}.\frac{dY}{dX_2} cov[x_1, x_2]} \text{ ---}$$

In our problem, wher Co and Ct are not independent, $Cov[Co,Ct] \neq \emptyset$, and since calculating SEM, instead of SD

$$SEM_Y = \frac{SD_Y}{n}$$

$$= \sqrt{\{\frac{dY}{dX_1}\}^2 \frac{SD_1^2}{n} + \text{---} + \{\frac{dY}{dX_n}\}^2 \frac{SD_n^2}{n} + 2\frac{dX}{dX_1}.\frac{dY}{dX_2} \frac{cov[X_1, X_2]}{n}}$$

$\therefore$ The extra term to be included into the equations are

$$SEM_{TR}', \quad \{2\frac{d\hat{K}}{dCo}.\frac{d\hat{K}}{dCt}.\frac{cov[Co,Ct]}{n}\}$$

$$SEM_{TT}', \quad \{2\frac{dTT}{dCo}.\frac{dTT}{dCt}.\frac{cov[Co,Ct]}{n}\}$$

ie. $2\frac{d\hat{K}}{dCo}.\frac{d\hat{K}}{dCt}.\frac{cov[Co,Ct,]}{n}$

$$= 2\{\frac{1}{t}\ln\left|\frac{Co^e}{Ct}\right|\}\{-\frac{Co}{Ct}\}\frac{cov[Co,Ct]}{n} \qquad \text{from appendix 1.}$$

$$= -\frac{4Co}{t^2 Ct}\ln\left|\frac{Co^e}{Ct}\right|\frac{cov[Co,Ct]}{n}$$

and $2\frac{dTT}{dCo}.\frac{dTT}{dCt}.\frac{cov[Co,Ct]}{n}$

$$= 2\frac{t}{Co(\ln Co - \ln Ct)}.\ (-)\frac{t}{Ct(\ln Co - \ln Ct)}.\frac{cov[Co,Ct]}{n}$$

from appendix 1

$$= (-)\frac{2t^2}{CoCt(\ln Co - \ln Ct)^2}.\frac{cov[Co,CT]}{n}$$

Covariance of [Co,Ct] calculated by

$$cov[Co,Ct] = \sqrt{\frac{\Sigma(Coi - Co)(Cti - Ct)}{n - 1}}$$

$$= \sqrt{\frac{\Sigma CoiCti - 1/n \Sigma Coi \Sigma Cti}{n - 1}}$$

Overall, the equations for the calculation of the SEM of turnover rate and turnover time, when Ct is dependant upon Co are,

$$SEM_{TR} = \sqrt{\frac{1}{t^2} \ln^2 \left| \frac{Co}{Ct}^e \right| SEM^2 Co + \frac{Co^2}{t^2} \cdot \frac{SEM^2 Ct}{Ct^2} - \frac{4Co}{t^2 Ct} \ln \left| \frac{Co}{Ct}^e \right| \frac{cov[Co,Ct]}{n}}$$

$$SEM_{TT} = \sqrt{\frac{t^2}{Co^2 (\ln Co - \ln Ct)^2} SEM^2 Co + \frac{t^2}{Ct^2 (\ln Co - \ln Ct)^2} SEM^2 Ct - \frac{2t^2}{CoCt(\ln Co - \ln Ct)^2} \cdot \frac{cov[Co,Ct]}{n}}$$

Appendix 6

(1) Calculation of Turnover Rate and Turnover Time assuming variable independence.

Compound - Noradrenaline

Brain Region - Hypothalamus

Time 0hrs, NA_0 concentration = 2048 ng /g wet tissue

Time 8hrs, NA_t concentration = 612 ng /g wet tissue n = 10

From Brodie et al (1966)

Rate of Synthesis = Turnover rate, = K

$K = k [NA]_o$ ---------- (1), k = rate constant of efflux.

After blockage of synthesis,[NA] declines at a rate proportional to concentration.

ie $-d[NA]/dt = k [NA]$

Integrated, $[NA]t = [NA]oe^{-kt}$

$= \log[NA]t = \log[NA]o - 0.434kt$ ---------- (2)

Substituting, [NA]o, [NA]t and t into equation (2).

$\log [612] = \log [2048] - .0434 k \times 8$

$2.787 = 3.311 - 3.472 k$

$k = \frac{3.311 - 2.787}{3.472}$

$k = 0.151, \quad k (hr^{-1})$

Turnover rate = K = [NA]ok

= 2048 x 0.151 ng/g/hr

Turnover rate = 309.25 ng/g/hr

Turnover time = 1/k = 1/0.151

Turnover time = 6.62hrs

Assuming [NA]o and [NA]t independant

$$SEM_{TR} = \frac{1}{t^2}\ln^2\frac{Co^e}{Ct}\,SEM^2Co + \frac{Co^2}{t^2Ct^2}SEM^2Ct$$

Co = 2048 ng/g wet tissue, SEMCo = 69.7 t = 8

Ct = 612 ng/g wet tissue, SEMCt = 39.5 e = 2.7183

$$SEM_{TR} = \sqrt{\frac{1}{8^2}\ln^2\left|\frac{2048x2.7183}{612}\right| 69.7^2 \quad \frac{2048^2}{8^2 \times 612^2} \times 39.5^2}$$

$$= \sqrt{\frac{1}{64} \times \ln^2\left|9.096\right| \times 69.7^2 + \frac{4194304}{64 \times 374544} \times 39.5^2}$$

$$= \sqrt{\frac{1}{64} \times 4.875 \times 4858.09 + 0.175 \times 1560.25}$$

$$= \sqrt{370.05 + 273.04}$$

$SEM_{TR} = 25.36$

$$SEM_{TT} = \sqrt{\frac{t^2}{Co^2\,(\ln Co-\ln Ct)^2}\,SEM^2Co + \frac{t^2}{Ct^2\,(\ln Co-\ln Ct)^2}\,SEM^2Ct}$$

$$= \sqrt{\frac{8^2}{2048^2\,(\ln2048-\ln612)^2} \times 69.7^2 + \frac{8^2}{612^2\,(\ln2048-\ln612)^2} \times 39.5^2}$$

$$= \sqrt{\frac{64}{6120596.8} \times 4858.09 + \frac{64}{546558.6} \times 1560.25}$$

$$= \sqrt{0.051 + 0.183}$$

$SEM_{TT} = 0.48$

For noradrenaline from the hypothalamus

Initil NA concentration = 2048ng/g wet tissue ± 69.7

After time t, NA concentration = 612 ng/g wet tissue ± 39.5

k, rate constant of efflux = 0.151, $k(hr)^{-1}$

Turnover rate = 309.25 ng lg 1hr ± 25.36

Turnover time = 6.62 hrs ± 0.48

(ii) Compound Dopamine

Brain Region Striatum

Time, 0hrs, DAo concentration = 34540 ng/g wet tissue ± 868.3

Time, 8 hrs, DAt concentration = 7118 ng/g wet tissue ± 619.8

From Brodie et al (1966)

K = k [DA]o, ---------- (1)

log [DA]t = log [DA]o - 0.434 kt ------- (2)

Substituting, [DA]o, [DA]t and t into equation (2)

log [7.12] = log[34.54] - 0 .434 x 8 x k

0.852 = 1.538 - 0.434 x 8 x k

$$k = \frac{1.538 - 0.852}{3.472}$$

$$k = 0.198 \qquad k(hr^{-1})$$

Turnover Rate = K = [DA]ok

$$= 34.54 \times 0.198$$

Turnover Rate = 6.839 ug/g/hr

Turnover Time = $^{1}/k = {}^{1}/0.198$

Turnover Time = 5.05 hrs

Assuming [DA]o and [DA]t independent

$$SEM_{TR} = \sqrt{\frac{1}{2}\ln^{2}\left|\frac{Co}{Ct}\right|^{e} \; {}^{2}SEM\,Co^{2} + \frac{{}^{2}Co^{2}}{{}^{2}t\;{}^{2}Ct}\,SEM\,Ct^{2}}$$

$$SEM_{TR} = \sqrt{\frac{1}{2}\ln^{2}\left|\frac{34.54 \times 2.7183}{7.12}\right| 0.8683^{2} + \frac{34.54^{2}}{{}^{2}8\;{}^{2}7.12} \times 0.6198^{2}}$$

$$= \sqrt{\frac{1}{64}\ln^{2}\left|\frac{93.89}{7.12}\right| 0.754 + \frac{1193}{3244.4} \times 0.384}$$

$$= \sqrt{0.0784 + 0.1412}$$

$$SEM_{TR} = 0.468 \text{ ug/g/hr}$$

$$SEM_{TT} = \sqrt{\frac{{}^{2}t^{2}}{{}^{2}Co^{2}\,(\ln Co - \ln Ct)^{2}}\,SEM\,Co^{2} + \frac{{}^{2}t^{2}}{{}^{2}Ct^{2}\,(\ln Co - \ln Ct)^{2}}\,SEM\,Ct^{2}}$$

$$SEM_{TT} = \sqrt{\frac{8^2}{34.54^2\,(\ln 34.54 - \ln 7.12)^2} \times 0.8683^2 + \frac{8^2}{7.12^2\,(\ln 4.54 - \ln 7.12)^2} 0.619^2}$$

$$= \sqrt{\frac{6A}{2978.2} \times 0.754 + \frac{64}{126.57} \times 0.384}$$

$SEM_{TT} = 0.458$ hrs

For dopamine from the Striatum.

k, rate constant of efflux = 0.198

Turnover Rate = 6.839 u/g/hr $\pm$ 0.468

Turnover Time = 5.05 hrs $\pm$ 0.458 hrs

Appendix 7

Computer programmefor the calculation of SEM of turnover rate and time.

```
00050 PRINT "NAME OF COMPOUND"
00053 INPUT Q$
00055 PRINT "COMPOUND =";Q$
00056 PRINT
00060 PRINT "BRAIN REGION"
00062 INPUT R$
00064 PRINT "BRAIN REGION ="R$
00065 PRINT
00066 PRINT
00100 REM MEAN SEM SD
00110 PRINT "ENTER NO OF OBSERVATIONS"
00120 INPUT N
00130 PRINT "ENTER DATA"
00140 FOR I = N
00145 INPUT CO(I),CT(I)
00150 NEXT I
00152 PRINT "DATA USED ARE"
00154 FOR I = 1 TO N
00155 PRINT, CO(I),CT(I)

00156 NEXT I
00157 PRINT,"CO VALUES",,"CT VALUES"
```

```
00320 E1 = S1/SQR(N)
00330 E2 = S2/SQR(N)
00340 PRINT "MEAN OF CO =";CO1/N
00345 PRINT
00350 PRINT "SEM OF CO =";E1
00355 PRINT
00360 PRINT "SD OF CO =";S1
00363 PRINT
00365 PRINT
00370 PRINT "MEAN OF CT =";CT1/N
00375 PRINT
00380 PRINT "SEM OF CT =";E2
00385 PRINT
00390 PRINT "SD OF CT =";S2
00395 PRINT
00500 REM TO CALCULATE TURNOVER RATE
00510 PRINT "ENTER TIME T OF STUDY"
00520 INPUT T
00530 KE = (LOG10(CO1/N)-LOG10(CT1/N))/(0.434*T)
00550 REM TO CALCULATE "K" RATE OF SYNTHESIS
00560 K = (CO1/N)*KE
00580 REM TO CALCULATE TURNOVER TIME
00590 TT = 1/KE
00610 REM TO CALCULATE TURNOVER RATE
00620 TR = (CO1/N)*(LOG(CO1/N)-LOG(CT1/N))/T
00700 REM TO CALCULATE COVARIENCE COMPONENT OF EQUATION
```

```
00710 FOR I = 1 TO N
00720 COV = COV + CO(I)*CT(I)
00730 NEXT I
00740 COV1 = COV - ((1/N)*CO1*CT1)
00749 COV1 = ABS(COV1)
00750 COV2 = SQR(COV1/(N-1))
00800 REM TO CALCULATE SEM OF TURNOVER RATE
00810 REM CALCULATION ASSUMING DEPENDANCE OF VARIABLES
00820 P1 = ((CO1/N)*2.7183)/(CT1/N)
00830 P2 = ((CO1/N)*(CO1/N))/((T*T)*(CT1/N)*(CT1/N))
00840 P3 = (4*(CO1/N))/((T*T)*(CT1/N))
00850 H1 = (1/(T*T))*(LOG(P1)*LOG(P1))*(S1*S1)
00860 H2 = P2*(S2*S2)
00870 H3 = P3*P1*(COV2/N)
00900 SEMTR = SQR(H1+H2-H3)
00950 REM TO CALCULATE SEMTT
00960 J1 = (LOG(CO1/N)-LOG(CT1/N))*(LOG(CO1/N)-LOG(CT1/N))
00970 J2 = ((T*T)/((CO1/N)*(CO1/N))*(J1))*(E1*E1)
00980 J3 = ((T*T)/((CT1/N)*(CT1/N))*(J1))*(E2*E2)
00990 J4 = ((2*(T*T))/((CO1/N)*(CT1/N)*(J1)))*(COV2/N)
01040 SEMTT = SQR(J2 + J3 -J4)
01090 PRINT
01095 PRINT
01100 PRINT "RATE OF EFFLUX ="; KE
01110 PRINT
01120 PRINT
```

```
00320 E1 = S1/SQR(N)
00330 E2 = S2/SQR(N)
00340 PRINT "MEAN OF CO =";CO1/N
00345 PRINT
00350 PRINT "SEM OF CO =";E1
00355 PRINT
00360 PRINT "SD OF CO =";S1
00363 PRINT
00365 PRINT
00370 PRINT "MEAN OF CT =";CT1/N
00375 PRINT
00380 PRINT "SEM OF CT =";E2
00385 PRINT
00390 PRINT "SD OF CT =";S2
00395 PRINT
00500 REM TO CALCULATE TURNOVER RATE
00510 PRINT "ENTER TIME T OF STUDY"
00520 INPUT T
00530 KE = (LOG10(CO1/N)-LOG10(CT1/N))/(0.434*T)
00550 REM TO CALCULATE "K" RATE OF SYNTHESIS
00560 K = (CO1/N)*KE
00580 REM TO CALCULATE TURNOVER TIME
00590 TT = 1/KE
00610 REM TO CALCULATE TURNOVER RATE
00620 TR = (CO1/N)*(LOG(CO1/N)-LOG(CT1/N))/T
00700 REM TO CALCULATE COVARIENCE COMPONENT OF EQUATION
```

```
01130 PRINT "RATE OF SYNTHESIS ="; K
01140 PRINT
01150 PRINT
01160 PRINT "TURNOVER TIME ="; TT
01170 PRINT
01180 PRINT
01190 PRINT "SEM OF TURNOVER TIME ="; SEMTT
01200 PRINT
01210 PRINT
01220 PRINT "TIME OF STUDY ="; T
01230 PRINT
01240 PRINT
01250 PRINT "TURNOVER RATE ="; TR
01260 PRINT
01270 PRINT
01280 PRINT "SEM OF TURNOVER RATE ="; SEMTR
```

CHAPTER FIVE

The Action of Oxypertine on ∝MPT and pCPA Induced Synthesis Inhibition

Introduction

In chapters 3 and 4 the effects of oxypertine on biogenic amine levels and the kinetic analysis of biogenic amine turnover has been studied. The results from chapter 3 appear to confirm the suggestions of Palamo and Reid (1983, 1984) that oxypertine acts on the newly synthesised (amphetamine releasable) pool of amines rather than the vesicular (reserpine releasable) pool amines. The results from chapter 4 show that ∝MPT produces a monophasic decline in catecholamine levels after an initial "lag phase" in which the amine levels fluctuate about the zero time value. The existence of a reserpine resistant noradrenaline fraction in the peripheral nervous system was first shown by Sedvall and Thorson (1963), who showed that the reserpine resistant fraction could be rapidly depleted by electrical stimulation. Sedvall (1964) suggested that this result implies the existance of two storage forms of noradrenaline is sympathetic nerves; one large pool containing about 85% of the total catecholamine which can be rapidly depleted by reserpine, the remaining 15% stored in a reserpine-resistant pool which can be released by nerve stimulation. Groppetta et al (1977) observed that the metabolites of dopamine had a higher specific activity following injection of labelled tyrosine than dopamine itself, indicating the existance of a dopamine compartment

with a high turnover. Korf, (1981) stated that this observation contradicted the idea of single compartment kinetics (ie monophasic decline of amines after αMpT), unless the compartment with a high turnover is very small compared to other dopamine compartments. Arbilla et al (1981) showed this high turnover compartment accounts for less than 10% of the total striatal dopamine content, thus it should not contradict the idea of single compartment kinetics. The "lag phase" seen in the onset of action of αMPT may also suggest the existence of several amine containing compartments with different rates of turnover. It should be remembered though that Costa et al (1975) and Moleman and Bruinvels (1978) showed that it takes at least 30 minutes post-administration before a substantial inhibition of synthesis is obtained. This time for the onset of action will be further complicated by various compensatory feedback mechanism which regulate the synthesis of the amines (Sharman, 1981). Therefore this "lag phase" in amine decline is probably due to a combination of these effects.

Since oxypertine probably acts on the newly synthesised pool of amines, it is of interest to see if the combining of oxypertine with αMPT does alter the monophasic decline in amines previously observed.

Materials and Methods

Male Sprague Dawley rats (200g-250g) were used in this experiment. The control group of animals received an I.P. injection of 500mg/kg αMPTand 300mg/Kg pCPA and an IP injection of ascorbic acid (vehicle of oxypertine injection). The test group of

animals received an IP injection of 500mg/kg ∝MPT and 300mg/kg pCPA and of 4mg/kg oxypertine. The animals were killed either, 0hrs, 2hrs or 8hrs after the administration of the drugs and the hypothalamic and striatal amines and metabolite levels measured using the methods described previously. The final results are given in ng/mg of protein.

Results

The effect of ∝MPT(500mg/kg) and pCPA(300mg/kg) on the biogenic amine levels of the hypothalamus and striatum are given in Tables 5.1 and 5.3 respectively. The effect of combining oxypertine (4mg/kg) with MPT and pCPA treatment on biogenic amine levels is given is Tables 5.2 and 5.4.

The results in Tables 5.1 and 5.3 for the action of ∝MPT and pCPA, show a similar pattern of amine level decline as in Chapter 4. The noradrenaline and dopamine levels show little alteration in levels between 0hrs and 2hrs which is followed by a large decline in levels between 2hrs and 8hrs. The effect in HVA is not as clear with little difference overall in hypothalamic levels and a large decline in striatal levels between 0hrs and 2hrs with no decline between 2hrs and 8hrs. There is no overall change in 5HT levels through the 8hrs of the experiment while 5HIAA levels fall initial between 0hrs and 2hrs then remain static between 2hrs and 8hrs.

The results presented in Tables 5.2 and 5.4 show a different pattern of effect from the results presented in Tables 5.1 and 5.3. The addition of oxypertine to the drug treatment has reduced the noradrenaline and dopamine, in the hypothalamus within the first 2 hrs. after administration, with a further reduction in levels between 2 hrs and 8 hrs. In the striatum the noredrenaline levels appears to be unaffected in the first 2 hours while being reduced in the following 6 hrs. Dopamine levels have declined within the first 2 hrs and continue to decline between 2 hrs and 6 hrs. Again the effects on HVA levels is seen within the first 2 hrs after administration, but this is followed by a decline in levels in the next 6 hrs. In the striatum there is an immediate decline in levels within the first 2 hrs after administration, followed by a small decrease over the next 6 hours. 5HT levels in both the hypothalamus and striatum show no decline over the first 2 hrs after administration, but show a small fall in levels over the next 6 hours.

In a attempt to examine whether the additional injection of oxypertine had any effect on the amine synthesis inhibition produced by ∝MPT and pCPA, the turnover rates and times for various lengths of treatment were calculated. These are presented in Tables 5.5,5.6 and 5.7 Table 5.5 shows the effect of oxypertine on the turnover rates for hypothalamic noradrenaline, Table 5.6 shows oxypertines effect on turnover times and rates for hypothalamic dopamine and Table 5.7 shows oxypertines effect on turnover times and rates for striatal dopamine. Three different time intervals (ie Ct values) were chosen to see if the decline in amine levels

follows a single exponential curve as described by Brodie et al (1966). 0hrs to 8hrs was chosen, as used in the previous chapter, 0hrs and 2hrs to see if any change occurs over the first 2 hours after administration and 2hrs to 8hrs to see if this altered from the 0hrs to 8hrs values.

The results of ∝MPT an pCPA treatment show a similar pattern for all 3 amines examined. No calculation was possible for the 0hrs/2hrs time interval for noradrenaline and dopamine from the hypothalamus. This was because no difference in noradrenaline levels is seen over the 2 hrs, while the dopamine levels appear slightly increased after 2 hours (this has a high SEM though). It is possible to calculate a turnover rate and time for dopamine from the striatum over this time interval, but the turnover rate calculated is low and the time high when compared to the other time intervals for this amine. Suggesting that the decline in levels is due to biological variation within the samples and not through the action of ∝MPT. For all the amines investigated the turnover rates are faster and turnover times shorter for the 2hrs/8hrs time interval when compared to the 0hrs/8hrs time interval. For noradrenaline from the hypothalamus and dopamine from the striatum the difference is small but for dopamine from the hypothalamus these differences are large. This may be due to the fact that the 2hr value is much higher than the 0hr values, thus giving an inflated Co value upon which the calculation are based.

TABLE 5.1

Effect of ∝MpT + pCPA on biogenic amine levels -
All values in ng/mg of protein ± SEM (n = 10)

Time after Injection	NA	DA
0 hours	57.3 ± 5.1	55.9 ± 5.1
2 hours	51.3 ± 4	89.9 ± 28.5
8 hours	28.7 ± 2.1	32.7 ± 6.6

- Control, Area - Hypothalamus.

5HT	5HIAA	HVA
161.2 ± 13.4	52.6 ± 9	110.9 ± 21.4
175 ± 12.5	29.2 ± 7.4	101 ± 44.9
187 ± 15.9	27.9 ± 2.1	87 ± 13.4

TABLE 5.2

Effect of Oxypertine, αMpT and pCPA on biogenic amine levels
Area - Hypothalamus. All values in ng/mg of protein ± SEM (n = 10)

Time after Injection	NA	DA	5HT	5HIAA	HVA
0 hours	61.5 ± 5.1	92.9 ± 16.3	162.1 ± 14.8	80.7 ± 11.4	92.9 ± 12.7
2 hours	37.2 ± 4.7	43.6 ± 6.6	150 ± 17.5	27.5 ± 6.2	83 ± 13.3
8 hours	22.6 ± 3.2	20.7 ± 5.7	121.9 ± 5.7	21.5 ± 2.2	49 ± 5.7

TABLE 5.3

Effect of αMpT and pCPA on biogenic amine levels - All values in ng/mg protein ± SEM (n = 10)

Time after Injection	NA	DA
0 hours	8.7 ± 1	1020.3 ± 99
2 hours	10.7 ± 1.1	829 ± 105.3
8 hours	5.9 ± 1.1	264.5 ± 24.2

Control, Area Striatum.

5HT	5HIAA	HVA
74.6 ± 6.6	48.3 ± 7	128.9 ± 14.4
77.8 ± 16.1	23.5 ± 3.6	63 ± 11.1
65.2 ± 5.3	21.5 ± 2.3	57.5 ± 5.9

TABLE 5.4

Effect of Oxypertine, αMpT and pCPA on biogenic amine levels, Area-Striatum.
All values in ng/mg protein ± SEM (n = 10)

Time after Injection	NA	DA	5HT	5HIAA	HVA
0 hours	6.8 ± 0.4	780 ± 46.9	66.9 ± 5.8	36.6 ± 4.3	118.5 ± 19.9
2 hours	6.9 ± 1	490 ± 47.4	64.1 ± 4.3	19 ± 2.7	42.4 ± 6.5
8 hours	2.9 ± 0.4	198 ± 40.6	40.6 ± 7.4	13.7 ± 1.7	30:5 ± 3.4

TABLE 5.5

Calculated turnover rate and times for A) ∝MpT and pCPA and B) ∝MpT pCPA, and oxypertine on Hypothalamic Noradrenaline

	A Brain Area - HYPOTHALAMUS Compound - NORADRENALINE		B Brain Area - HYPOTHALAMUS Compound - NORADRENALINE
TIME		TIME	
0 hrs to 8 hrs	Rate of Efflux, k = 0.0864 Turnover Rate = 4.954 ± 1.588 Turnover Rate = 11.563 ± 0.528	0 hrs to 8 hrs	k = 0.1254 TR = 7.705 ± 3.528 TT = 7.972 ± 1.319
0 hrs to 2 hrs	No calculation possible as no change in amine levels	0 hrs to 2 hrs	k = 0.2509 TR = 15.412 ± 12.47 TT = 3.985 ± 0.0705
2 hrs to 8 hrs	k = 0.0969 TR = 4.966 ± 1.935 TT = 10.321 ± 0.121	2 hrs to 8 hrs	k = 0.0836 TR = 3.111 ± 2.962 TT = 11.959 ± 0.468

Units rate of efflux, k (hr -1) Turnover rate, ng /g /hr ± SEM, Turnover time, hrs ± SEM

TABLE 5.6

Calculated Turnover rates and times for A) ∝MpT and pCPA, and B) ∝MpT, pCPA and oxypertine on Hypothalamic Dopamine

TIME	A Brain Area - HYPOTHALAMUS Compound - DOPAMINE	B Brain Area - HYPOTHALAMUS Compound - DOPAMINE
0 hrs to 8 hrs	k = 0.0671 TR = 3.750 ± 4.518 TT = 14.9 ± 0.847	k = 0.188 TR = 17.474 ± 10.894 TT = 5.317 ± 3.899
0 hrs to 2 hrs	Calculation produces negative value as 2 hr reading larger than 0 hr reading	k = 0.3747 TR = 34.810 ± 22.26 TT = 2.668 ± 0.342
2 hrs to 8 hrs	k = 0.1687 TR = 15.153 ± 9.918 TT = 5.92 ± 2.27	k = 0.1257 TR = 5.522 ± 6.337 TT = 7.953 ± 1.386

Units rate of efflux, k (hr -1) Turnover rate, ng / g / hr ± SEM, Turnover time, hrs ± SEM

TABLE 5.7

Calculated turnover rate and times for A) ∝MpT and pCPA, and B) ∝MpT, pCPA and oxypertine on striatal dopamine

	A Brain Area - STRIATUM Compound - DOPAMINE	B Brain Area - STRIATUM Compound - DOPAMINE
TIME		
0 hrs to 8 hrs	k = 0.1689 TR = 172.17 ± 42.1 TT = 5.92 ± 1.45	k = 0.1715 TR = 133.86 ± 92.57 TT = 5.828 ± 3.338
0 hrs to 2 hrs	k = 0.1039 TR = 105.98 ± 227.825 TT = 9.62 ± 0.034	k = 0.233 TR = 182.35 ± 122.38 TT = 4.288 ± 0.102
2 hrs to 8 hrs	k = 0.190 TR = 157.79 ± 43.4 TT = 5.25 ± 1.07	k = 0.1514 TR = 74.307 ± 77.202 TT = 6.604 ± 1.706

Units, rate of efflux, k (hr -1) Turnover rate,ng / g / hr ± SEM, Turnover time,hrs ± SEM

The results for ∝MPT, pCPA plus oxypertine treatment show a different pattern of results for ∝MPT and pCPA treatment alone. In all three amines investigated it is possible to calculate turnover rates and times for the 0hrs/2hrs time interval. Comparing the turnover rates and times for this time interval (0hr/2hr) against the other time intervals for particular amines, shows that the turnover rate is much faster and turnover time much shorter than either the 0hrs/8hrs or 2hrs/8hr time interval. Comparing the 2hrs/8hrs time interval against the 0hr/8hr time interval shows that the turnover rates are slower and turnover times longer for the 2hrs/8hrs tine to interval. This differs from the results obtained when only ∝MPT and pCPA were administered in that the 0hr/8hr time interval have the slower turnover rates and longer turnover times.

Discussion

The results presented in this chapter confirm results in previous chapters and help explain the actions of ∝MPT and oxypertine. In chapter 4 ∝MPT was used as an catecholamine synthesis inhibitor so that the turnover rates and times of noradrenaline and dopamine could be calculated. It was noted however that unlike some previous reports (Brodie et al, 1966 Spector et al, 1975, Hohn and Wuttke, 1978), the catecholamine levels appeared to fluctuate about the zero time value for up to two hours before a significant decline takes place. These fluctuations are explained through the time dependent onset of ∝MPT's action, a minimum of 30 minutes (Costa et al;1975, and Molemen and Bruivels, 1976) and the various

feedback mechanism that control the rate of synthesis. (Sharman, 1981). After the turnover rates and times were calculated in chapter 4 using 0hrs as the initial time point it was suggested that the 2hr point might provide a more accurate point to start the calculation from. The result presented in Tables 5.1,5.3,5.5,5.6,and 5.7 appear to support this suggestion after close examination. In Tables 5.1 and 5.3 it can be seen that the noradrenaline and dopomine levels after 2 hours show no consistance of effect, ie levels have increased, decreased or remained constant. This confirms the results in chapter 4 in which the amine levels also fluctutated within the first 2 hours after administration. These fluctuation in amine levels were also noted by Hallman and Jonsson (1984) who recorded slight increase in amine levels between 30 min and 60 minutes following αMPT. Hallmann and Jonsson (1984) did not observe these fluctuation in all areas they examine and attempted to showed a multiphasic decrease in the amines, as opposed to a monophasic decrease. The differences seen in the profile of amine decrease by Hallman and Jonsson may be due to their more detailed dissections and selection of brain areas examined compared to the general dissections of the hypothalamus and striatum carried out in this experiment. Their explanation of the increase in amine levels, observed between 30min and 60 minutes, was that a temporary reactivation of tyrosine hydroxylase occurs, through an allosteric reactivation of the enzyme (as first proposed by Paden, 1979). The attempts to calculate turnover rates and times for 0hrs to 2hrs further confirms that no overall change is taking place.

With no apparent reduction in the amine levels occurring within the first 2 hrs, it appears that, as suggested in Chapter 4, that turnover rates and times calculated using the 2 hrs levels as the initial time point will give a more accurate results. A comparison between the 2/8hr rates and times against the 0/8hr times seems to confirm this suggestion. The 2/8hr turnover times are slightly faster than the 0/8hr times, as would be expected since time is the only factor altered in the calculation. The large difference seen in the hypothalamus dopamine turnover rate and time is due to the large difference in dopamine levels in the initial time (Co) chosen in the calculation. The decline in amine levels appears to be monophasic in nature, which is in agreement with Anderson et al (1985), who showed that catecholamine stores in various types of hypothalamic, pre-optic and telencephalic catecholamine nerve terminals disappear monophasically. Anderson et al (1985) also showed that in peri-and paraventricular dopamine nerve terminals, a multiphasic disappearance of dopamine takes place. (Similar to Hallman and Jonsson (1984)). In both these studies through, detailed disection of discrete catecholamine nerve terminal systems take place as apposed to the general disection carried out in this experiment. It is therefore possible that the pieces of tissue used in this experiment contain both neurons with amines that will decline monophasically and multiphasically, with the monophasic neurons accounting for the higher catecholamine content. It is though that the multiphasic disappearance of catecholamines (as seen by Hallman ans Jonsson, (1984), Paden, (1979)) supports the existance of a functional pool of catecholamines with a higher turnover of newly synthezised catecholamines. If this was the

case, then according to the observation of Korf (1981) this rapid turnover pool will be large when compared to other compartments. It therefore appears that the sizes of amine compartments within separate neurons may vary, in that highly active neurons may have large readily synthesised pool and that neurons with a lower activity have a small readily synthesised pool.

It is difficult to compare turnover rates and time for noradrenaline and dopamine from this experiment with those calculated by Hallman and Jonsson, (1984) and Anderson et al(1985) for a combination of factors. Hallman and Jonsson (1984) used a HPLC/ECD for measuring the amine levels, and presented their final results in ng/g wet tissue weight (own results given in ng/mg protein) and the turnover times were given as a series of t 1/2 -values over a varying time scale. (t 1/2 denotes the time when noradrenaline and dopamine concentrations reached 50% of control value). Anderson et al (1985) used a combination of HPLC techniques and quantitative histofluroimetric anlaysis which produced a 20-30 time difference in sensitivity. They also presented their results in nmol/g wet tissue, both groups of workers did find that the dopamine turnover time was always shorter than the noradrenaline turnover time, which corresponds to the results presented here.

The incorporation of oxypertine into the experimental design produced a interesting effect on all the turnover rates and times calculated. Comparison of the results presented in Tables 5.2 and 5.4 shows that the addition of oxypertine to αMPT and pCPA

treatment reduces the amine levels after 2 hours. From these results it is possible to calculate turnover rates and times for the amines. A comparison of the turnover rates and times for the 0/2hr interval against both the 0/8hr and 2/8hr intervals shows that the 0/2 hr rates and times are both faster and shorter respectively, for any amine from any region. It therefore appears that the addition of oxypertine has converted a monophasic decline of amines into at least a biphasic decline, if not a multiphasic decline in amines. How may oxypertine accomplish this? If oxypertine has the ability, to disrupt the fast synthes pool without inhibiting the synthesis enzymes, the synthesis enzymes of the fast synthesis pool will be released in the neuronal cytoplasm. Under normal conditions, free in the cytoplasm they will be able to synthesize amines unchecked, but in the presence of αMPT the enzymes will become quickly inhibited. This almost instant inhibition of this small, but significant pool of synthesising enzymes may be reflected in the sharp fall in amine levels observed within the first 2 hours, and the fast turnover rate and short turnover time may seem reflected the different rates of synthesis between the enzymes of the fast synthesis pool and vesicular pool of amines. Further evidence of this biphasic decrease in amine levels may be seen in a comparison of the 0/8hr interval with the, 0/2hr and 2/8 intervals. In choosing the 0/8 hr interval it is assumed that the decline in amines is monophasic and the results produced are similar to the results produced in animal treated wtih only αMPT and pCPA. However the result for the 0/2hr and 2/8hr intervals suggest a biphasic decline in the amines, with an initial fast turnover followed by a slower turnover. Further

comparison of the results shows that the turnover times calculated for 0/8hr interval are almost the means of the two other intervals. Therefore the effect of the 0/8hr interval is to average out the results of the other two time intervals, and if this were only interval chosen important results would be missed.

The existance of at least two separate pools of amines in neurones has been known since the 1960's (Sedvall and Thorson, 1963), and they are generally referred to as the vesicular and non-vesicular pools. The vesicular pool of amines, as its name suggests, is present in vesicles in the nerve fibre ending and are clearly seen with electron microscopy. An actual site for the catecholamine non-vesicular pool has still to be identified, but there is now evidence from other chemically based transmitter systems about a possible site of the non-vesicular pool. Dunant and Israel (1985) have suggested that acetylcholine released by nerve terminals does not originate from the vesicles, but is derived directly from the cytoplasm. The release of this cytoplasmic acetylcholine appear to be through a protein embedded in the nerve cell membrane and the protein may act as a valve enabling actylcholine to pass through the membrane. A similar type of system is thought to occur in 5-HT neurones, in which a membrane bound protein synthesis site and release point for newly-synthesized 5HT exists (Tamir and Huang, 1975, Tamir and Kuhar, 1975, Tamir et al 1976). It does not seem unreasonable to suggest that a similar system may be present in catecholamine containing neurones, ie that the fast-synthesis pools integrity is through a membrane bound protein, and this protein provides the

correct environment for amine synthesis and also a release mechanism from the neuron. Sharman (1981) suggested that the concentration of dopamine will depend on the relative contribution of various feedback systems that control the synthesis of dopamine. Included in these feedback systems is receptor mediated control. It is therefore possible that this membrane bound protein that may hold together the newly-synthesised pool may cross both layers of the lipid-membrane. Pre-synaptic receptor feedback mechanisms may be part of this protein complex. Therefore, the suggestion is that the membrane bound protein provides the correct environment in which the fast amine synthesing enzymes exist, a transport mechamism out of the neuron for the amines and part of the control mechanisms that regulate synthesis. With this in mind it is suggested that oxypertine disrupts the integrity of this protein causing the release of the fast synthesising amines into the cytoplasm. Oxypertine itself does not affect the synthesising enzymes, so they are free in the cytoplasm to function as normal releasing amines into the cytoplasm that may be released or metabolised.

CHAPTER SIX

The Reserpine Resistant Pool of Amines

Introduction

In the previous chapter, oxypertine was shown to alter the onset of action of αMPT. It's suggested mode of action, being through oxypertine disrupting the integrity of the reserpine resistant pool, and leaving the amine synthesising enzymes open to the action of MPT. The idea of single or multi compartment kinetics was also discussed which deals with the relative size of the non-vesicular pool of amines. Since Arbilla et al (1984) showed that this pool accounts for less than 10% of total striatal dopamine content it is of interest to see if this pool can be studied in isolation, what effect oxypertine may have on the isolated pool and if kinetic analysis of turnover can be applied to the pool. In an attempt to completely remove the reserpine releasable pool of amine, rats were pretreated with a double dose of reserpine (Palomo and Reid, 1984). The effect of oxypertine and the combined effect of MPT and oxypertine was then studied in the reserpinized rats.

Materials and Methods

Male Sprague-Dawley rats (200g-250g) were used in the experiments.

Experiment 1

1st Group; Control animals received 2 injections of the reserpine vehicle (see general methods). The first injection at -24hrs, and the second injection at -1.5hrs. The animals were killed at 0hrs.

2nd Group; Reserpine treated rats received 2 injections of reserpine (5mg/kg)(see general methods). The first injection at -24hrs, the second injection at -1.5hrs and the animals were killed at 0hrs.

3rd Group; Oxypertine treated animals received 2 doses of reserpine (5mg/kg) and a single dose of oxypertine (4mg/kg). The first reserpine injection at -24hrs, the second injection of reserpine and single injection oxypertine at -1.5hrs, and the animals were killed at 0hrs. Once killed the amine and metabolite levels of the hypothalamus and striatum were analysed (using methods described previously) and the final results given in ng/mg of protein.

Experiment 2

4th Group; Each animal received 2 doses of reserpine (5mg/kg), the first injection at -24hrs and the second injection at -0.5 hours. A single dose of αMPT (500mg/kg) was injected at 0hrs, and the animals were killed at 0hrs or + 4hrs.

5th Group; Each animal received 2 doses of reserpine, 5mg/kg), the first injection at -24hrs and the second injection at -0.5hrs. The animals also received a single injection of oxypertine (4mg/kg) at -0.5hrs. The animals were then killed at 0hrs or +4hrs.

6th Group; Each animal received, 2 doses of reserpine (5mg/kg), the first injection at -24hrs and the second injection at -0.5hrs. A single dose of oxypertine (4mg/kg) was injected at -0.5hrs, and a single dose of αMPT (500mg/kg) was given at 0hrs. The animals were killed at 0hrs and +4hrs.

The amine and metabolite levels of the striatum were analysed (using the methods described previously) and the final results given in ng/mg of protein.

Results

The results of Experiment 1 are shown in Tables 6.1 and 6.2. Table 6.1 shows the effect of the drug combination on the hypothalamus amine levels and Table 6.2 shows the effect on the striatal amine levels. From Table 6.1 it can be seen that the reserpine treatment (Group 2, two doses reserpine, 5mg/kg at -24 hrs and -1.5hrs) reduces the levels of all the biogenic amines, ie noradrenaline, dopamine and 5-HT, but has no effect on the HVA and 5HIAA levels. The oxypertine treatment (Group 3, two doses of reserpine, (5mg/kg) at -24 hrs and -1.5 hrs, and one dose of oxypertine (4mg/kg) at -1.5hrs) has the same effect as the reserpine treatment. It reduces the biogenic amine levels to a similar extent and again appears to have no effect on metabolite levels (5HIAA and HVA). A similar pattern can be

seen for the striatal amines (Table 6.2) Reserpine treatment (Group 2) reduces the initially small noredrenaline levels, greatly reduces and dopamine level and the 5HT levels, but again it appears to have no effect on the metabolite levels. Oxypertine treatment (Group 3) reduces the amines to the same extent as reserpine treatment (Group 2) and again has no effect on the metabolite levels.

The results of Experiment 2 are shown in Tables 6.3 and 6.4. In this experiment only amine levels from the striatum were measured as the results from experiment 1 indicated that reserpine treatment reduced hypothalamic amine levels to just above the detection limits of the method and with such small amine levels, any changes occurring would be difficult to detect. Table 6.3 gives the results of the different drug treatments at 0hrs after αMPT treatment and Table 6.4 after 4hrs of MPT treatment. In Table 6.3 Group 4 shows the effect of 2 doses of reserpine (5mg/kg) at -24hrs and -0.5 min, and 1 dose of αMPT (500mg/kg) at 0hrs. Group 5 shows the effect of 2 doses of reserpine (5 mg/kg) at 0hrs. Group 5 shows the effect of 2 doses of reserpine (5mg/kg) at -24 hrs and -0.5 min and a single dose of oxypertine at -30 min. Group 6 shows the effect of a double dose of reserpine (5mg/kg) given at -24hrs and -0.5min, a single dose of oxypertine (4mg/kg) given at -0.5min, and a single dose of αMPT given at 0hrs. The Group 4 results show that the amine levels have been reduced by reserpine treatment, (ie the amine levels are very small when compared to control amine levels recorded in previous experiments) and again with no apparent effect on the metabolite levels. Groups 5 and 6 the groups of animals that received a single dose of oxypertine at -0.5hrs show a different effect on amine levels

than group 4 (non-oxypertine treatment). The noradrenaline levels in Groups 5 and 6 are similar to that in Group 4 through this is probably due to the fact that under normal conditions noradrenaline levels are very small. The dopamine levels in Groups 5 and 6 are 5 to 6 times greater than the levels in Group 1 but have large SEMs associated with them. The 5HT levels in groups 5 and 6 show an increase in levels when compared in group 4, but again have large SEMs. The 5HIAA levels show a slight reduction in levels for groups 5 and 6 with little or no effect seen in the levels of HVA.

Table 6.4 shows the effect the drug combinations have on amine levels following a 4 hour delay in killing after the final injection. The levels of noradrenaline in all 3 groups are on or below the limits of detection of the method which is similar to the levels in Table 6.3. The dopamine levels of Group 4 have been reduced by the 4 hour treatment with αMPT, while there is no change in 5HT. For the metabolites 5HIAA shows a slight reduciton in levels while HVA shows a large reduction in levels. In Group 5 dopamine levels show a large reduction when compared to the levels at 0hrs (Table 6.3) and 5HT also shows a reduction when compared to the 0hr level. Of the metabolites, 5HIAA shows a slight increase in levels while no effect is observed in the HVA levels. For Group 6, the dopamine levels show a large reduction between 0hrs and 4hrs while the 5HT levels also show a reduction. Of the metabolites, 5HIAA shows an increase in levels while no effect is seen in the HVA levels.

TABLE 6.1

Experiment 1 Hypothalamus, all values in ng/mg protein ± SEM (n = 10)

Treatment	NA	DA	5HT	5HIAA	HVA
Group 1	50.1 ± 2.8	41.3 ± 5.4	133.6 ± 10.8	61.1 ± 9.6	144.5 ± 23.9
Group 2	0.97 ± 0.23	3.6 ± 1.4	5.2 ± 1.6	70.8 ± 12.5	120 ± 14.6
Group 3	3.1 ± 1.4	2.9 ± 0.4	6.6 ± 1.8	50 ± 10	97.7 ± 26.6

TABLE 6.2

Experiment 1 Striatum, all values in ng/mg of protein ± SEM (n = 10)

Treatment	NA	DA	5HT	5HIAA	HVA
Group 1	5.1 ± 0.6	749.2 ± 77	71.8 ± 8.1	42.5 ± 7.5	162.4 ± 38.7
Group 2	1.4 ± 0.7	10.9 ± 2.1	5.8 ± 2.8	47.5 ± 8.1	132.5 ± 17.4
Group 3	0.7 ± 0.2	16.9 ± 2.2	5.1 ± 1.3	49.2 ± 9.7	128.3 ± 17.9

TABLE 6.3

Experiment 2 - Striatum - Zero time. All values in ng/mg protein ± SEM (n = 5)

Treatment	NA	DA	5HT	5HIAA	HVA
4th Group	1.3 ± 0.3	28.5 ± 5.1	8.3 ± 1.5	60.7 ± 13.1	150.5 ± 23.9
5th Group	1.7 ± 0.6	155.4 ± 111.8	27.6 ± 13.8	45.7 ± 6.2	143.7 ± 30
6th Group	2.6 ± 1	199.1 ± 105.5	23.8 ± 10.4	33.6 ± 3.8	120.3 ± 16.6

TABLE 6.4

Experiment 2 - Striatum - + 4 hours. All values in ng/mg of protein ± SEM (n = 5)

	NA	DA	5HT	5HIAA	HVA
4th Group	0.2 ± 0.2	4.8 ± 0.7	5.24 ± 1.5	39.5 ± 2.3	48 ± 3.1
5th Group	0.3 ± 0.1	19.2 ± 2.6	3.9 ± 2.1	68.7 ± 9	154.1 ± 37.2
6th Group	-	8.2 ± 3.9	8.9 ± 2.2	74.5 ± 7.8	143.9 ± 26.2

Discussion

Experiment 1 was designed to see if it is possible to carry out studies on the reserpine resistant pool of amines after the reserpine releasable pool of amines had been totally depleted by reserpine. Reserpine however does not have an all or nothing effect on amine storage. The mechanism by which reserpine causes depletion of catecholamine stores from neurons depends on the dose (Bowman and Rand, 1980), Depletion of amine stores is secondary to inhibition of amine uptake into vesicles. Doses which are sufficient to cause transmission failure produce a reversible impairiment of the uptake and binding of amine in storage vesicles. A large dose of reserpine causes irreversible damage to the storage vesicles after which amine storage does not return until new storage vesicles have been manufactured in the cell and transported to the axon terminals (Bowman and Rand, 1980). The rate of amine turnover also affects the amine depletion by reserpine. The increase in turnover rate of amine stores produced by neurone stimulation facilitates the depleting action of reserpine. A double supra-maximal dose of 5mg/kg body weight of reserpine (Palomo and Reid, 1984) was used so to produce a long lasting irreversible depletion of amine stores. (Hong et al (1985) used 0.3mg/kg/day reserpine in rats as an equivalent to daily clinical dose in man). The first dose of reserpine was given 24 hours before killing and the second dose was given 1.5 hrs before killing to ensure that maximum amine depletion had occurred. The amine and metabolite levels recorded for the control treatment (vechicle of reserpine injection) are in agreement with normal levels recorded in previous chapters. Treatment with reserpine produced the expected reduction

in the amine levels in both the hypothalamus and striatum (at least 90%). Reserpine failed to have any effect on the metabolite levels (HVA and HIAA same as control values). This is in agreement with the results presented in chapter 3, when a single dose of reserpine (5mg/kg) also failed to reduce metabolite levels. The addition of oxypertine (4mg) to the reserpine treatment (Group 2) produced the same results as the reserpine treatment apart from a slight increase in dopamine levels in the striatum (see later part of discussion). In this experiment the amine levels recorded in the hypothalamus are just above the levels of sensitivity of the method. Any small changes in hypothalamic amine levels may be lost because of this so it was decided to use only striatal samples when investigating the properties of the reserpine resistant pool.

Oxypertine's suggested mode of action is a disruption of the non-vesicular pool, which causes the release of the synthesising enzymes into the cytoplasm. The injection of oxypertine at -1.5hrs was chosen as Palomo and Reid (1983) used this time when studying the behavioural actions of oxypertine. Previous to this, Palomo and Russell (1983) had shown that oxypertine produced behavioural effects within 40 minutes of administration. In experiment 1 it might be that analysis of amine levels 1.5hrs after oxypertine administration is too long, as the high cytoplasmic reserpine concentration may inhibit the amine synthesising enzymes when released into the cytoplasm. With this in mind, experiment 2 was designed with a shorter time between oxypertine administration and amine level analysis and αMPT was included in the design to see if kinetic analysis of turnover rates and time is possible.

The results from experiment 2 may be analysed is several ways Kinetic analysis of turnover rates and times may be possible using the results in Table 6.3 as the Co values and the Table 6.4 result as the Ct values. Comparisions between the length of oxypertine treatment are possible using Tables 6.2 and 6.3. This is possible, because the animals that received MPT in experiment 2 were killed immediately after being injected, preventing MPT from having any effect. To make this analysis, Group 4 (Table 6.3) should be compared against reserpine treatment (Group 2) (Table 6.1) and treatment Group 5 and 6 (Table 6.3) compared against oxypertine treatment (Group 2 , Table 6.2). This shows that both reserpine treatment (Group 2 and 4) reduced the amine levels without reducing the metabolite levels. The oxypertine treatments, Groups 5 and 6 produce an interesting result. Both of these groups, with short injection times for oxypertine (0.5 hrs) show an increase in dopamine and 5HT levels. Disruption of the non-vesicular pool of amines with oxypertine will release the synthesising enzymes into the cytoplasm. On release into the cytoplasm, the enzymes will be free to operate at their maximum rate. The uninhibited activity of the emzymes appears to be short as the amine levels recorded for 1.5 hrs oxypertine treatment (Group 3) are no different from reserpine treatment (Group 2) (i.e. after 1.5 hrs the enzymes released into the cytoplasm have been inhibited by cytoplasmic material and/or the reserpine).

The effect of αMPT treatment is seem through comparing the results in Tables 6.3 and 6.4 MPT reduces the HVA levels in reserpine treatment (Group 4) and slightly reduces the dopamine levels. The

only other differences between the groups is the increased dopamine and 5HT levels in Tables 6.3, for which an explanation is given above. The reduction in HVA levels is similar to the results of MPT treatment seen in chapter 4. A similar reduction in HVA levels may been have expected in the groups 5 and 6 levels, but no reduction is seen. If the HVA synthesising enzymes are opperating at their maximum, the increase in dopamine synthesis produced by oxypertine (Table 6.3) may form a sizable pool of amines that takes some time to clear. This overloading of the catalytic enyzmes may not occur under normal conditions as any excess dopamine synthesised may be taken into storage vesicles.

Overall the change in dopamine levels produced by MPT appear too small to attempt any kinetic analysis upon. Oxypertine appears to produce a short term increase in dopamine and 5HT synthesis in the reserpinized animals. A fuller investigation into this action of oxypertine is required to achieve a better understanding of its action.

GENERAL DISCUSSION

The combination of the behavioural data (Palomo and Reid, 1983, 1984) and biochemical results (Chapters 3,5,6) suggest that oxypertine disrupts the non-vesicular pool of amines. It has been speculated that this pool of amines may be formed about a membrane bound protein that provides the correct environment for the synthesis, storage and release of the amines.

The dopamine hypothesis of schizophrenia has been discussed in detail in the introduction, but roughly it proposes that an increased dopamine neuron activity results in schizophrenia. The question that therefore arises is; "Why and how does oxypertine have an action in schizophrenia and tardive dyskinesia?" Flament et al (1962) report on the action of oxypertine in a wide range of schizophrenic patients (see introduction) and concluded that oxypertine would be useful in the treatment of withdrawn schizophrenics. Remr et al (1974,1975) further verified the use of oxypertine in the treatment of schizophrenia by showing that it is as effective in the control of chronic apathetic schizophrenics as some of the phenothiazines. In both these groups oxypertine treatment produced an elevation in mood and activity of the patient. These workers showed that oxypertine is not "the magic bullet" of schizophrenia treatment as it failed to produce symptom remission in all the patients treated. It did however show again the complexity of the condition and reaffirm that the dopamine hypothesis is too simple an explanation for schizophrenia.

Palomo and Russell (1983) showed that oxypertine's, action in increasing exploratory behaviour in rats with amphetamine induced activity is dose dependent. It is with these doses that oxypertine's depletor action in the non-vesicular pool is suggested. This is the action of oxypertine that could be producing the above positive clinical effects. Sedvall (1964) showed that in the peripheral nervous system, the non-vesicular pool of amines could be released by electrical stimulation after complete depletion of the vesicular pool. Thus suggesting that the non-vesicular, fast turnover pool plays an important part in the chemical transfer of nervous impulses. Depletion, or disruption of this pool may reduce the flow of nervous impluses in aminergic neuronal pathways. Remembering, that in its simplest form, the dopamine hypothesis suggests that schizophrenia results from an increase dopamine neuronal activity. Any decrease in dopamine activity may be useful in the treatment of schizophrenia. Since oxypertine is seen to be effective, depletion of the fast turnover pool may be sufficient to cause symptom relief, in some schizophrenics, but not in other schizophrenics.

The same explanation may be put forward for oxypertine use in the treatment of tardive dyskinesia. Tardive dyskinesia is thought to occur through a hypersensitivity of the dopaminergic system, ie. an increase in the number and/or sensitivity of dopamine receptors. Decreasing the amount of dopaminergic neuronal transmission with oxypertine will reduce dopamine receptor stimulation and thus decrease the number of tardive dyskinetic symptoms.

To date all the behaviour and biochemical studies undertaken with oxypertine have been acute experiments. It may be that with chronic experiments, oxypertine's profile of action may change completely. In chronic experiments, the accummulative effect of oxypertine treatment would have to be examined closely. This may result in an adrenoceptor and 5-HT receptor antagonism as reported by Nakahara et al (1980) and /or a post-synaptic dopamine receptor effect as suggested by Hong et al (1984). Experiments using a wider dose range of oxypertine may also prove useful. This would re-examine the early biochemical work undertaken, and shown if high doses (70mg/kg) do infact release the amines from the reserpine resistant pool of amines. Tardive dyskinesia is mainly reported in schizophrenics who have been receiving neuroleptics for 10-20 years, and it has been suggested that it is in fact an acceleration of a naturally occuring degenerative condition in the brain. Oxypertine may provide only short term relief form this condition and it may be that it itself might cause the onset of this condition if give to patients on a long term basis. Overall, a fuller profile of oxypertine's actions is still required as work to-date suggests that it has several dose dependant actions.

The calculation of turnover rates and turnover times provided a different series of problems from the investigation into the action of oxypertine. The initial methodology of Brodie et al (1966) is well documented and the calculation of turnover rates and times using their method is relively simple. Problems arise in the units in which final results are presented, and the methods used in calculating the final

results. Final results have been presented in various forms, ie. ng/g of wet tissue, ng/mg of protein and nmol/g wet tissue. This makes it difficult to compare absolute levels between different groups of workers and therefore difficult to compare calculated results between groups. It may be aiming for utopia to suggest that one standard form for the results be adopted, but until some unification in the way the result are presented, is adopted, comparisons between results from different groups will remain difficult.

The analysis of the calculated turnover rates and times also varies between different groups and further complicates the interpretation of results. Results are presented in the form devised by Brodie et al (1966) (just as turnover rate and time) the form of Hohn and Wuttke (1978), (SEM of turnover rate and time calculated assuming variable independence) and in the form of Rance et al (1981) (SD of turnover rate calculated assuming variable dependance). This was further complicated recently by Hallman and Jonsson (1984) and Anderson et al (1985) who was presented their results in the form, half life of t, ie t 1/2. The experiments in chapter 4 were undertaken in an attempt to establish a method for the calculation of SEM of turnover rates and times and it is hoped that the method present provides a simple to use mathematical model for the calculation. Problems arise though out of the results that these calculations produce. In chapter 4 the SEMs of turnover rate and turnover time for the amines are relatively small and therefore the turnover rates and times calculated are relatively accurate. In chapter 6 however when the effect of oxypertine on amine turnover was studied the calculated results proved less informative. By

introducing another compound that effects amine storage along with MPT and pCPA into the experiment and varying the times used in the calculation the SEM of the turnover rate and turnover time increased. This tells us that the calculated turnover rates and times are less precise than before, so numerically large differencies in turnover rate and times may not be statistically significant. In many cases the SEM of turnover rate is larger than the calculated turnover rate showing how imprecise the calculations are. The SEM of turnover times in general provides a more accurate estimate than the SEM of turnover rates, so it may be a more useful calculation to undertake. The problem of calculation of turnover rates and times may be further aggravated by the existence of at least two neuronal amine pools. The calculations are based on single compartment kinetics but as has been shown there exists at least two neuronal amine pools. The rate at which these pools are affected by drugs differs and the rates at which they themselves synthesis the amines differs. Attempts to monitor the turnover rate and time of the fast synthesis non-vesicular pool of amines gave results with very large SEMs of turnover time, showing that this pool activity can vary tremendously. It may therefore be impossible to calculate accurately turnover rates and times for various pools until the importance of these pools has been established. It appears that the calculations presented for the SEM of turnover rate and time are useful when single compartment models are being examined, but may need reexamined when two or more compartment models are being examined.

The investigations mentioned above were made possible through the development of the new HPLC-ECD method developed for this study. The combination of improved sample preparation and chromatographic separation help provide a fuller analysis of the biogenic amines and metabolites composition of the samples. It should be remembered though that the chromatographic conditions used were optamized for these investigations and therefore may not fulfill the conditions required for other investigations. Indeed, to suggest that further improvements in the analysis of biogenic amines and metabolites is not possible would be foolhardy. Improvements in the sample preparation may be possible so that the biogenic amine precursors as well as metabolites may be analysed, and also reduce the interference of analysis caused by the solvent front. Improvement in chromatographic hardware, ie. pumps, column packing, and detector systems means that there is unlimited scope for the improvement of the chromatographic separations.

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