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Edited by C. J. Pycock
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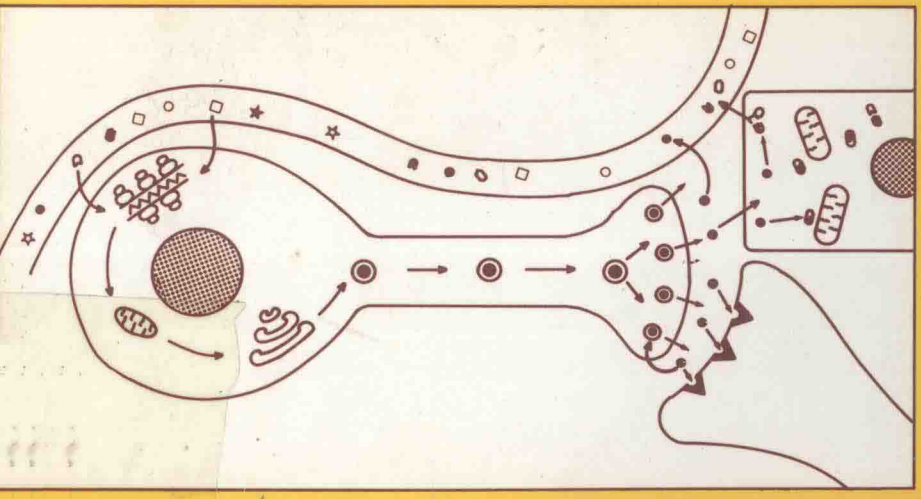

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PREFACE

The concept of chemical transmission in the central nervous system has taken some time to be generally accepted, but an increasing number of compounds are now being recognized as having a transmitter role in the brain. The acetylcholine system was the first to be discovered in the periphery and its characteristic features of storage of transmitter in vesicles in the nerve terminal, its electrically-evoked release and rapid extraneuronal breakdown were considered to be necessary criteria for any neurotransmitter candidate. The subsequent elucidation of the noradrenergic system made it apparent that rapid enzymatic breakdown was not essential for a released transmitter, and the possibility of high-affinity re-uptake processes became established as an alternative means of terminating the synaptic actions of a transmitter.

With the eventual acceptance of the amino acids as excitatory or inhibitory transmitters, the requirement for a transmitter to be present in a low concentration overall (although locally concentrated in specific terminals) also had to be discarded. This necessitated the additional concept of specific metabolic pools with different functions being located in different cells or within different regions of the same cell. Some localization of glutamate and aspartate remote from excitable membranes is clearly essential since their overall brain concentrations would be sufficient to maximally depolarize the majority of neurones in the brain. The concept of separate metabolic pools has been supported by studies on turnover rate (see Chapter 5).

The recent discovery of the presence of opiate receptors and endogenous short chain peptides with potent neurophysiological and behavioural effects has necessitated the further relaxation of the limiting criteria applied to potential transmitter candidates.

Having decided that a wide range of endogenous chemicals in the brain, ranging from the very small (e.g., glycine) to the very large (e.g., β-lipotropin) have significant neurophysiological and behavioural effects, the pharmacological manipulation of their synthesis and breakdown is one of the commoner research approaches used to investigate their function in the brain. The measurement of brain levels of putative transmitters is usually the first avenue of approach, and even this basic measure can present practical difficulties in the case of the more labile compounds such as acetylcholine and the enkephalins.

However, if a particular neurone or group of neurones employing a specific transmitter alters its activity (that is, firing rate), this will not necessarily result in a change in transmitter concentration. For example, the ensuing change in

the rate of synthesis may well be immediately matched by equivalent changes in the rate of release and catabolism. Alternatively, a pre-existing store of transmitter may be released and retaken up at a new, but steady rate. In both cases the overall level of transmitter will not be observed to change although its turnover rate has been markedly altered. In other words, it is the turnover of a transmitter which is more likely to reflect the functional activity of the neurones for which it is acting rather than its overall concentration.

The primary aim of this book, therefore, is to examine, for each major transmitter system, the methods that have been employed to estimate the turnover rate. There are a large number of methods available; some are only applicable to particular transmitters, and virtually all of them make important assumptions that are not always evident in the text of papers that have described such methods. The relative merits of each method will be compared and it is up to the individual to decide where the balance between convenience, expedience and validity lies. Estimates of turnover obtained by alternative methods for given transmitters are purposely included in order to demonstrate the variation in the results presented in the literature. It should soon become apparent that, in many cases, the turnover rate measured is a function of the method used, and where the effects of drugs or lesioning on a system are being considered, it is essential to compare the values obtained with their own controls.

The inclusion of a chapter on peptides reflects the new interest in their role as potential transmitters, and illustrates the difficulties inherent in estimating the turnover of a single species of peptide. The contribution of the new technologies of mass spectrometry linked to gas liquid chromatography and high performance liquid chromatography has made turnover studies feasible in fields where previously no satisfactory methodology existed. It is hoped that this volume will encourage more studies on transmitter turnover as a means of elucidating the biological role of specific groups of neurones in the brain.

The book has been divided into five main chapters which cover the major groups of neurotransmitters, namely, acetylcholine, catecholamines, 5-hydroxytryptamine, amino acids and peptides. The introductory chapter provides a general overview of all the systems and compares the available methods for studying turnover in each system. Finally, we have included some short research papers which illustrate the application of some of the previously described techniques to specific areas of research.

C.J.P.
P.V.T.

Bristol, June 1980

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C.J.P.
P.V.T.

SECTION I

TURNOVER OF CENTRAL NEUROTRANSMITTERS

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J. Korf

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1.1 TERMINOLOGY

In order to function, all living organisms take up exogenous substances for use in the various metabolic processes required to maintain their integrity. Metabolites are interconverted and eventually degraded to yield products which can be excreted. The total quantity of a metabolite in a cell is often referred to as the metabolic pool of that substance and the amount that is transported or metabolised, the turnover (Zilversmit *et al.*, 1943). Often the term turnover rate is used instead of turnover although both expressions have the same dimensions of mass.time^{-1}. In some cases (e.g. glutamic acid in nervous tissue), it has been recognised that the metabolic pool is not homogeneous and that different turnover rates apply to different proportions of the metabolite. Under these circumstances the metabolite is said to exist in different pools or compartments. A compartment is defined as a quantity of metabolite having uniform and distinguishable kinetics of transformation or transport. The term (fractional) rate constant (with the dimension of time^{-1}) is also used in estimates of turnover (Robertson, 1957; Atkins, 1969) and refers to the ratio of the turnover of a compartment to the size of that compartment.

In turnover studies on central neurotransmitters it is usually assumed that either the transmitter is in a steady state: rate of synthesis equals 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 of depletion or accumulation of transmitter can be determined as an estimate of turnover.

The synthesis of neurotransmitters is dependent on the precursors entering the neurones from their environment. Moreover, degradation products of neurotransmitters can leave the brain. We can therefore consider the kinetics of the neurotransmitters under normal circumstances as that of open systems.

To describe the kinetics of substances in the body models are used and in the present context mainly theoretical models will be considered. To evaluate such a system a mathematical model may be derived which is the set of equations that describe the concentrations and amounts of the substance involved as a function of time. In subsequent sections we shall discuss general methods to determine the turnover of a neurotransmitter which are based on either steady state or non-steady state kinetics. For the first methods radioactive tracers are used to label the substance. In the other methods the turnover of a substance under steady state conditions is estimated from the kinetics of the compound, or its precursors, or metabolites, after perturbing the steady state with drugs. The major central neurotransmitters to be considered are 5-hydroxytryptamine (5HT), dopamine, noradrenaline, acetylcholine (ACh) and γ-aminobutyric acid (GABA).

1.2 MODELS AND METHODS

1.2.1 *Models for Dopamine and 5HT*

Of the transmitter substances, the kinetics of 5HT and of dopamine are the most simple, at least as far as the theoretical models are concerned. The kinetics can be represented as depicted in Figure 1.1, where A and B are the precursors:

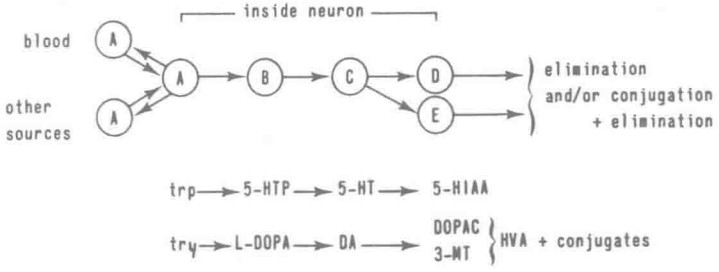

Figure 1.1 Theoretical model for the synthesis and degradation of dopamine and 5HT in the central nervous system (for further details see text).

(L-tryptophan (trp) and 5HTP for 5HT: L-tyrosine (tyr) and DOPA for dopamine respectively). C is the active amine and D and E are metabolites, which can be formed inside as well as outside the neurone. In the dopamine system D and E are DOPAC (3,4-dihydroxyphenylacetic acid) and 3-MT (3-methoxytyramine). In the case of 5HT both D and E represent 5HIAA (5-hydroxyindoleacetic acid). The turnover of the metabolites should be the same as that for the amine or the precursor of type B, which is formed from the precursor A by a neurone specific enzyme (tyrosine or tryptophan hydroxylase). The source of the precursor A is not well established, and it may be derived from blood, from the surrounding nerve tissue or from both. The transmitter amines are degraded to products which leave the brain in a free or conjugated form. 5HT is degraded to 5HIAA which can leave the brain. Dopamine is converted to DOPAC and 3-MT, which can be further metabolised to HVA (homovanillic acid) or conjugates which are eventually eliminated from the brain.

1.2.2 *Methods for Dopamine and 5HT*

The following methods have been used for the measurement of the turnover of dopamine and 5HT.

a) *Estimation of the rate of accumulation of 5HT or dopamine + 3-MT after blockade of monoamine oxidase.* Inhibitors of monoamine oxidase (MAO) which have been used in studies to estimate the turnover of dopamine and 5HT include pargyline and

pheniprazine (Neff & Tozer, 1968; Morot-Gaudry *et al.*, 1974; Glowinski, 1975). This method works only if it is assumed that the block of MAO has a rapid onset and is complete, that the amine does not leak out the brain, that the synthesis of the amine is not disturbed by the inhibitor or end product inhibition and that the accumulation of the amine is due exclusively to the inhibition of MAO.

Several authors have indicated that MAO inhibitors such as pargyline and pheniprazine cause more than 95% inhibition of the enzyme within 5 min after administration (Javoy *et al.*, 1973; Morot-Gaudry *et al.*, 1974; Glowinski, 1975). Evidence of leakage of the amine out of the brain is not apparent. A possible difficulty with the use of inhibitors of MAO may be their interference with the synthesis of the amine because of a feedback inhibition (see *e.g.* Carlsson *et al.*, 1972; Hamon & Glowinski, 1974; Glowinski, 1975). Such an inhibition does occur at time intervals of more than 15 min after exposure to the inhibitor, but may be absent at earlier time intervals (Morot-Gaudry *et al.*, 1974; Schutte, 1976). A serious source of error is in the small *post mortem* changes of the amines. In the case of 5HT a *post mortem* decrease is prevented, at least in part, after inhibition of MAO (Table 1.1) (Van Wijk & Korf, unpublished). Such *post mortem* effects may lead to an overestimation of the turnover rate of 5HT when MAO inhibitors are used. The apparently rapid increase of the amine following inhibition of MAO is prevented when the animal is killed with microwave irradiation (Table 1.1).

The methods with MAO inhibition have also been applied to dopamine but contradictory results have been reported. A rapid accumulation of the amine, pointing to high turnover values, was reported by Javoy *et al.* (1973), but this was not observed by Kehr (1976). The reason for this discrepancy is uncertain, but *post mortem* effects may also be involved here (Wiesel & Sedvall, 1974; Le Roy Blank *et al.*, 1979). 3-MT accumulates rapidly following inhibition of MAO, presumably because the formation of DOPAC is prevented. From the initial rapid increase of 3-MT which, in the absence of MAO activity is slowly metabolised or eliminated, the rate of turnover of DA may be estimated (DiGiulio *et al.*, 1978).

b) *Decline of the amine after inhibition of synthesis.* This method has not widely been applied for 5HT as the available inhibitors of tryptophan hydroxylase have not sufficiently rapid action, or have additional effects on 5HT metabolism (such as the central decarboxylase inhibitors, which are also inhibitors of the MAO). For dopamine the most often used drug is α-methyl-*p*-tyrosine. This drug acts slowly and a substantial blockade of the synthesis is not obtained earlier than 30 min after its administration (Costa *et al.*, 1975; Moleman & Bruinvels, 1976). The decline of the level of dopamine at earlier time intervals has therefore been attributed in part to

Table 1.1 *POST MORTEM* CHANGES OF 5HT AND 5HIAA LEVELS IN THE MOUSE FOREBRAIN

Post mortem treatment:	5HT		5HIAA	
	no	yes	no	yes
Controls alone	706 ± 23	634 ± 17**	314 ± 15	406 ± 11**
Controls and pargyline	753 ± 9	743 ± 18	327 ± 16	325 ± 16
Microwave Fixation (M)	794 ± 23	810 ± 20	334 ± 14	356 ± 17*
M + pargyline	858 ± 26	897 ± 34	334 ± 15	341 ± 19

Effect of inhibition of monoamine oxidase and microwave fixation on values of 5HT and 5HIAA levels in mouse brain. Mice were killed and the brains divided into two halves. One side underwent *post mortem* procedure which included dissection of the tissue to pieces of about 5 mg, exposure to air at room temperature for 6 min and freezing until analysis: the other half of the brain was dissected rapidly and immediately frozen. Control animals were killed by cervical dislocation: others were killed by microwave fixation (M). Pargyline (75 $mg.kg^{-1}$, i.v.) was administered to half of the animals in each of these two groups 2 min before killing.

5HT and 5HIAA concentrations are expressed as $ng.g^{-1}$ tissue ± s.e.m.; the mean of 8-12 experiments. Statistical differences between sides with or without *post mortem* treatment shown by * $p < 0.05$, ** $p < 0.001$ (results from Van Wijk & Korf, 1980).

the displacement of dopamine by metabolites of the inhibitor (Doteuchi *et al.*, 1974). The latter effects may be responsible for the high estimated turnover rates of dopamine (Javoy & Glowinski, 1971) calculated from the interval part of the decline curves of dopamine following the administration of α-methyl-*p*-tyrosine.

c) *Labelling of amines with radioactive precursors.* In contrast to the above mentioned methods the technique of labelling the neurotransmitter amines with small quantities of radioactive precursors does not perturb the steady state conditions. To calculate the turnover of the amine with a radioactive precursor the labelling pattern of the immediate precursor, *i.e.* 5HTP or DOPA, should be known.

There is a study on the turnover of dopamine where this has been reliably measured in the striatum of the rat (Costa *et al.*, 1975). It appeared that the time course of the specific

activity of DOPA followed that of striatal tyrosine. In virtually all studies on 5HT turnover it has been assumed that the labelling pattern of 5HTP is the same as that of plasma or total brain tryptophan, thus neglecting other possible sources of tryptophan in serotonergic neurones. If this is not the case then the reported turnover values of 5HT will be erroneous.

d) *Accumulation of DOPA and 5HTP.* Administration of centrally acting decarboxylase inhibitors produces a rapid accumulation of the immediate precursors of the amines 5HTP and DOPA. It is usually assumed that the block of the decarboxylases involved is rapid and complete. The drugs used are also often inhibitors of monoamine oxidase and it is known that these drugs may influence the rate of accumulation of amines by feedback control (Carlsson *et al.*, 1972). Moreover, decarboxylase inhibitors may compete with the uptake of precursor amino acids in the brain. Considering the suggested uncertainties it is not surprising that relatively low values of the rates of synthesis of the amines are found.

e) *Decline of metabolites after inhibition of monoamine oxidase.* After inhibition of MAO there is a rapid decline of the acid metabolites due to elimination and/or conjugation processes. This approach is useful when the MAO inhibitor acts rapidly and produces a complete block of enzyme activity. Drugs such as pargyline and pheniprazine satisfy these requirements. However, it is not known whether these drugs influence the elimination and/or conjugation processes (see *e.g.* Dedek *et al.*, 1979). A possible source of error with this method are *post mortem* artefacts. We have observed that after dissection of the brain an increase in the levels of 5HIAA may occur and that this effect is prevented after pretreatment with pargyline (Table 1.1). If such *post mortem* changes take place too high turnover rates will be found with this method.

Of the DA-metabolites only DOPAC disappears mono-exponentially from the striatum after inhibition of MAO (*i.e.* described by $d/dt[DOPAC] = -k_1[DOPAC]$). Disappearance curves for HVA became mono-exponential when, in addition to MAO, catechol-O-methyltransferase (COMT) activity was blocked by tropolone (Westerink & Korf, 1976, see Fig. 2.6). This approach was also applied to estimate the turnover of the conjugates of the acidic metabolites. The theoretical model of Figure 1.2 was evaluated mathematically (Dedek *et al.*, 1979). The major conclusions were, that the contribution of 3-MT to the formation of HVA in the rat striatum was small and that about 33% of DOPAC and 66% of HVA were conjugated in the striatum of the rat. According to this model the turnover of DOPAC approximates to that of dopamine. Although DOPAC is converted to at least two metabolites (HVA and the sulphate conjugate of DOPAC), the decline of DOPAC appeared to be monophasic after the inhibition of MAO. This is to be expected only if all DOPAC is subject to a rate-limiting process (such as diffusion) before further

metabolism occurs or that all DOPAC is confined to a single pool. A similar reasoning has to be applied when the mono-exponential decline of HVA following treatment with tropolone and pargyline is considered (Dedek *et al.*, 1979).

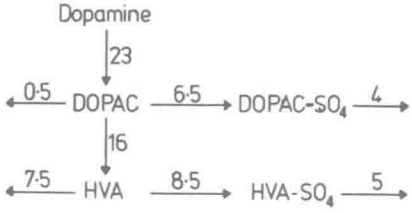

Figure 1.2 Sequence of formation of dopamine metabolites in the rat striatum. Numbers refer to the conversions (in nmol. $g^{-1}hr^{-1}$) in the rat striatum, calculated from disappearance rates after pargyline (100 mg.kg^{-1}, i.p.) alone or in combination with tropolone (100 mg.kg^{-1}, i.p.). (Data from Dedek *et al.* 1979).

f) *Accumulation of metabolites following inhibition of egress.* In this procedure probenecid is commonly used. The following assumptions have to be made: the drug does not interfere with the synthesis of the accumulated metabolites and should cause a rapid and complete blockade of the egress. The first factor complicates its use for 5HIAA turnover measurements, as probenecid produces a substantial change in the distribution of tryptophan in the body, which increases the synthesis of 5HT and presumably of its metabolite (Schubert, 1974; Van Wijk & Korf, 1980). Probenecid at high dosages influences the conjugation (Pennings *et al.*, 1976) which may lead to an underestimation of conjugate formation *in vivo* (Dedek *et al.*, 1979). This method can be used for the estimation of dopamine synthesis in the rat if both the free and conjugated acid metabolites are taken together (Dedek *et al.*, 1979), but it appears to be species dependent. In the rat the accumulation of HVA following probenecid treatment gives turnover rates of 25% of that of dopamine. In contrast, the probenecid method may be more useful in the guinea-pig (Spano & Neff, 1972). In the rabbit HVA does not accumulate following probenecid treatment (Werdinius, 1967; Extein *et al.*, 1973).

1.3 5HT TURNOVER

The turnover values for 5HT with various methods from different laboratories vary widely. The study of Morot-Gaudry *et al.* (1974) has investigated various methods to estimate 5HT turnover in mice. Their results are summarized in Table 1.2 (and Table 3.1). The turnovers measured differ by at least a factor