THE PROPERTIES OF 5-HYDROXYTRYPTAMINE AND DOPAMINE CONTAINING NEURONES IN PULMONATES

by

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A thesis presented for the Degree of Doctor of Philosophy of the University of Southampton

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Snail

'I'm sorry I can't put you up'—
The snail said to her friend,
'For 'tho' the house is all my own
And - as you know - I live alone,
One room is all there is inside
And that, of course,
is occupied!

John Tompkins
ACKNOWLEDGEMENTS.

I wish to thank my supervisor, Professor Gerald A. Kerkut, for his help, guidance and witty remarks. I would also like to thank other members of the department and in particular Dr. J.D.C. Lambert, Dr. R.J. Walker, Dr. Lynda V. Wilton and Dr. G.N. Woodruff for their help in various ways.

I am grateful to Professor K.A. Munday for the use of the facilities of the department and to the Science Research Council for financial support.

Last, but not least, I wish to thank my parents for their help, interest and encouragement during my years of study.
ABBREVIATIONS

The following abbreviations have been used in this thesis:

DA - dopamine (3-hydroxytyramine)
5-HT - 5-hydroxytryptamine
NA - noradrenaline
ACh - acetylcholine
Dopa - 3,4-dihydroxyphenylalanine
5-HTP - 5-hydroxytryptophan
MAO - monoamine oxidase
COMT - catechol-O-methyl transferase
PCPA - para-chlorophenylalanine
AP - action potential
ILD - inhibition of long duration
EPSP - excitatory post synaptic potential
IPSP - inhibitory post synaptic potential
CILDA - cell showing inhibition of long duration
EEG - electro-encephalogram
g, mg, µg, ng - gram, 10^-3 gram, 10^-6 gram, 10^-9 gram
M, mM - Molar, millimolar
µl - 10^-6 litre (microlitre)
µ - micron = 10^-6 metres
Å - Angstrom unit = 10^-10 metres
s - second
kΩ, MΩ, GΩ - 10^3, 10^6, 10^9
V, mV - volt, 10^-3 volt
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ABSTRACT

FACULTY OF SCIENCE

PHYSIOLOGY AND BIOCHEMISTRY

Doctor of Philosophy

THE PROPERTIES OF 5-HYDROXYTryptAMINE AND DOPAMINE CONTAINING NEURONES IN PULMONATES.

by Janet Elsie Loker.

Much work has been carried out to locate the positions of monoamine-containing cells in invertebrates but little work has been done to identify these cells by their electrical activity and responses to specific stimuli. The aim of the present study was to identify some of the monoamine containing cells in the pulmonates Helix aspersa and Planorbis corneus using electrical activity, responses to the addition of drugs and to nerve stimulation as the criteria for identification.

The formaldehyde-fluorescence histochemical technique for the localisation of catecholamines and 5-hydroxytryptamine was applied to the central ganglia of these two pulmonates in order to determine the positions of monoamine-containing neurones that would be suitable for making intracellular recordings. In Helix large 5-hydroxytryptamine containing neurones were located on the dorsal surfaces of the right parietal and visceral ganglia that were suitable for making such recordings.

Intracellular recordings were made from cells of their spontaneous electrical activity and their response to specific stimuli (the addition of acetylcholine, dopamine or 5-hydroxytryptamine and the stimulation of 4 main nerve trunks). Procion dye was injected intracellularly as a marker and the ganglia were subsequently exposed to formaldehyde to determine whether the cells tested contained monoamines. In the right parietal ganglion out of 6 cells tested, 3 were found to contain 5-hydroxytryptamine and in the visceral, 5 out of 6 cells contained 5-hydroxytryptamine. The 5-hydroxytryptamine containing cells in Helix had a similar
pharmacology. They were all depolarised by 5-hydroxytryptamine and acetylcholine and hyperpolarised by dopamine. 5-hydroxytryptamine cells in the right parietal ganglion showed an inhibitory response to stimulation of the four nerve trunks, while the visceral cells showed an excitatory response to nerve stimulation.

In Planorbis a large dopamine containing cell and some large 5-hydroxytryptamine cells were located on the ventral surface of the pedal ganglia. Seven large neurones were identified using electrical activity and the responses of the cells to the addition of acetylcholine, 5-hydroxytryptamine, dopamine and glutamate as the criteria for identification. Of the 7 cells tested, 3 contained 5-hydroxytryptamine and 1 contained dopamine. The responses of the 5-hydroxytryptamine containing cells to acetylcholine and to dopamine was a depolarisation and was therefore similar to the 5-hydroxytryptamine containing cells in Helix. The Planorbis dopamine containing cell was hyperpolarised by acetylcholine, depolarised by 5-hydroxytryptamine and hyperpolarised by dopamine and glutamate. The acetylcholine response in this cell was thought to be mediated at least in part by chloride ions.

The pharmacology of the 5-hydroxytryptamine containing cells found in this study has been compared with other 5-hydroxytryptamine containing cells whose pharmacology has been determined.
CHAPTER 1

GENERAL INTRODUCTION

PART A: SOME ASPECTS OF THE VERTEBRATE NERVOUS SYSTEM

1.1 Transmitters in the Vertebrate Nervous System

The concept of chemical junctional transmission arose in 1921 from Otto Loewi's classical experiments. He showed that the perfusate from a stimulated frog heart could transfer the effects of nerve stimulation to an unstimulated frog heart.

Since this time many compounds have been suggested as candidates for chemical transmitters in the central nervous system (CNS) as well as the peripheral nervous system. Among these compounds are the biogenic monoamines dopamine (DA), noradrenaline (NA) and 5-hydroxytryptamine (5-HT).

It is generally accepted that various criteria have to be satisfied before a compound can have a transmitter role ascribed to it. These criteria have largely arisen from work done on the peripheral nervous system. These requirements can be listed as follows:

1. The transmitter should be present.
2. Synthesising enzymes should be present.
3. Precursors should be present.
4. Inactivating enzymes should be present.
5. There should be a specific release mechanism.
6. The transmitter should be detectable in the extracellular fluid following nerve stimulation.
7. The proposed transmitter and the natural transmitter should have identical actions.
8. The proposed and the natural transmitters should have an identical
pharmacology.

Werman (1966) has discussed the validity of these criteria in a review. The identification of central transmitters presents technical problems that do not occur in the peripheral systems. In particular it is difficult to get access to the synaptic loci in the CNS.

The requirement for an inactivating enzyme mainly arose from studies on acetylcholine esterase. However, enzymatic 'degradation' is not the only method by which it is possible to inactivate a molecule. Other possibilities include an increase in molecular weight, a change in steric structure (either spontaneous or catalysed), diffusion away from the site of action and breakdown elsewhere (for example in the blood or another organ) or uptake by the presynaptic endings. GABA and glutamate are suspected inhibitory and excitatory transmitters at the crustacean nerve-muscle junction (Takeuchi and Takeuchi, 1965) and degradative enzymes for these compounds are present but are located inside the cytoplasm of the nerve and muscle and it is unlikely that they are accessible enough to account for the rapid disappearance of the transmitter activity.

For a transmitter to be collected from the tissue it is necessary for excess quantities of the transmitter to be released, and for the inactivating system to be inefficient (if it has not been blocked for the purposes of the experiment). A drug that specifically blocks the inactivating system may not be available (ie. for glutamate).

The most important requirement is that of identical action of the proposed transmitter and the natural transmitter. With the advancement of electrophysiological methods it is possible to measure more parameters than for example a simple muscle contraction or relaxation. Parameters that should be identical are postsynaptic ionic permeability changes, dose-response effects, membrane potential and resistance changes and reversal potentials. The requirement for identical
pharmacology can be considered as an extension of identical action, provided the drugs are affecting the transmitter at the postsynaptic membrane.

If these last two criteria are completely fulfilled it may not be necessary to satisfy all the others before a compound can be reasonably considered to be a transmitter.

1.2 Dopamine

The role of DA as a precursor to NA and adrenaline has been well established (Blaschko, 1960; Schumann, 1960). The sensitivity and specificity of methods available for measuring compounds in tissues imposes a limit on the extent to which they can be studied. DA was originally measured using bioassays where it was usually less active than NA and adrenaline. Chemical methods have been developed that are specific, sensitive and quantitative for DA. For example Carlsson and Waldeck (1958) reported a fluorimetric method for measuring DA. A combination of bioassay and chemical methods is often used to give more positive results.

DA had been found in urine and was subsequently found in a variety of tissues. Montagu (1957) found a third catecholamine in brain tissue in addition to adrenaline and NA and suggested it might be DA. This was confirmed by Carlsson et al (1958).

There is only a small amount of DA in chromaffin tissue where it functions as a precursor for NA and adrenaline but in adrenergic tissue there is as much DA as NA and this led Blascho (1957) to suggest that DA may have a separate role in addition to that of precursor to NA.

The concentrations of DA and NA in whole brain were found to be approximately equal but their regional distributions were found to be different (Bertler and Rosengren, 1959a, 1959b; Carlsson, 1959). These authors found that nearly all the brain's DA was present in the
basal ganglia (caudate nucleus and putamen) whereas the highest NA concentrations was in the brain stem, in particular the hypothalamus. The levels recorded in the caudate nucleus were 0.1 µg/g for NA and 10 µg/g for DA. In NA rich areas the concentration of DA was about 10% that of NA.

This uneven distribution would not be expected if DA was present solely as a precursor to NA. Carlsson (1959) suggested that DA could be involved in motor functions for the following reasons:

1. The distribution of DA did not mirror that of NA.
2. Large amounts of DA were present in the corpus striatum which is an important part of the extrapyramidal motor system.
3. Reserpine has extrapyramidal actions and was shown to deplete DA from the corpus striatum (Carlsson et al., 1958).
4. Dopa (dihydroxyphenylalanine) has the ability to reverse the effects of reserpine (5-hydroxytryptophan is ineffective in this respect) and has been shown to cause an increase in DA levels while not greatly affecting the NA levels.
5. Treatment with Dopa alone causes a hyperactivity.

1.3 Metabolism of Dopamine

a. Biosynthesis

The formation of DA from tyrosine is on the same pathway as the synthesis of adrenaline from tyrosine. The first step is the hydroxylation of tyrosine to form 3,4-dihydroxy phenylalanine (Dopa). The second step is the decarboxylation of Dopa to form 3-hydroxytyramine (dopamine). Dopa decarboxylase the enzyme required for this step has been shown to be present in nervous tissue (Anden et al., 1964; Anden, 1965).

The synthesis of NA from C-tyrosine in isolated guinea-pig heart was demonstrated by Spector et al. (1963). This meant that it was
Figure 1.1

The degradation of DA to homovanillic acid and the degradation of NA to vanillinmandelic acid by the enzymes monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT).
unnecessary to postulate that the heart obtained its NA by removing it from the blood. The rate-limiting step in the pathway in the isolated guinea-pig heart was found by Levitt et al (1965) to be the step from tyrosine to Dopa which is catalysed by the enzyme tyrosine hydroxylase. Thus, under normal circumstances there is no accumulation of Dopa because its decarboxylation, by Dopa decarboxylase, to DA occurs freely.

b. Inactivation

The major breakdown product of DA in the brain is homovanillic acid (HVA). Two enzymes are involved in the production of this metabolite, monoamine oxidase (MAO) and catechol-O-methyl transferase (COMT).

MAO has been found in all vertebrates tested and in some but not all invertebrates. It has been found in a variety of tissues (e.g. brain, liver, kidney and intestine) and has been given several different names in the past. Blaschko et al (1937) have demonstrated that adrenaline oxidase, 'tyramine oxidase' and 'aliphatic amine oxidase' were all the same enzyme which is now known as monoamine oxidase.

Certain metabolic pathways for catecholamines were found to occur in vitro but not in vivo. COMT was shown to be present in brain and to be involved in the metabolism of catecholamines in vivo (Axelrod, 1959). The degradation of DA to HVA by MAO and COMT is outlined in figure 1.1. All the enzymes necessary for the metabolism of DA are present in the brain (Seiler, 1969).

1.4 Cellular Localisation of Dopamine in the CNS

An important step in the study of DA as a transmitter in the CNS was the development of a fluorescence histochemical technique for the localisation of catecholamines in tissue. This method was devised by Eranke (1955b) and developed for use on the nervous system by Falck and coworkers (see for example Falck, 1962).

The basis of the method is the formation of a highly fluorescent
compound as the result of the condensation of a monoamine with formaldehyde. Freeze-dried tissue is exposed to formaldehyde vapour under specific reaction conditions. Primary amines will react under milder conditions than are necessary for secondary amines. Sections of the tissue are examined under UV light and a bright fluorescence can be seen at the storage sites of the amines. The method is discussed in more detail in chapter 2.

One of the main difficulties encountered with this method is that DA and NA produce fluorophores with identical fluorescence characteristics. In order to differentiate between these two catecholamines various methods have been employed:

a. Pharmacological methods

Drugs can be employed which affect the NA and DA levels to different degrees. Recently Aghajanian and Roth (1970) have found that gama-butyrolactone causes an increase in brain DA but not in NA. Gama-butyrolactone is converted in the body to gama-hydroxybutyrate (GHB).

b. Direct chemical estimations

Regional levels of the two amines may be determined and compared with the histochemical results. For example the corpus striatum has been shown to contain much larger concentrations of DA than of NA so it would be reasonable to suppose that the majority of the specific catecholamine fluorescence developed in this region was due to DA and only a small part to NA.

c. Chemical methods

Chemical methods for distinguishing between DA and NA fluorophores have been developed in model systems using solutions of the amines in albumen (Corrodi and Jonsson, 1965). These methods have since been applied to tissues (Bjorklund et al, 1968). The fluorophores are exposed to HCl vapour for short periods of time. The excitation maxima of the fluorophores before and after treatment are compared. The main
excitation peak for both fluorophores is at 410nm with a smaller peak at 330nm. After a short exposure to HCl vapour the NA fluorophore has its major excitation peak at 330nm. The excitation peak for the DA fluorophore shifts from 410 to 370nm. Eranko and Eranko (1971) have reported that for longer exposures to HCl vapour (10 minutes) the NA excitation peak shifts back again to 430nm.

The combined use of chemical, pharmacological and fluorescence methods has established that DA is present in neurones in the CNS. Catecholamine containing cell bodies and nerve terminals have been demonstrated in mammalian CNS by Dahlstrom and Fuxe (1964) and Fuxe (1965a).

Pharmacological and electrophysiological criteria suggest that the striatum contains DA and 5-HT nerve terminals. Cell bodies were absent or only faintly fluorescent in the striatum (Carlsson et al, 1962; Dahlstrom and Fuxe, 1964). The nerve terminals in the striatum are very fine and the fluorescence has a diffuse appearance as a consequence. DA containing cell bodies were mainly found in the substantia nigra.

1.5 Morphology of Mammalian Catecholamine-containing Neurones

Fluorescence studies on the mammalian CNS showed the following results. The catecholamine fluorescence was not evenly distributed throughout the neurone. The cell body contained a moderate fluorescence which was confined to the cytoplasm. The nucleus was always non-fluorescent. The axons and processes were smooth and only weakly fluorescent. The nerve terminals were intensely fluorescent with varicosities along their lengths. These varicosities were strongly fluorescent. The catecholamines are thought to be stored within the varicosities.

Reserpine depletes the fluorescence of the cell bodies and the terminals. During recovery from reserpine the first fluorescence to return
is around the nucleus.

The central neurones that contain monoamines have similar properties to peripheral adrenergic neurones. This supports the view that they are monoamineergic, i.e. they release amines from synaptic terminals.

1.6 Mapping the Dopamine Containing Neurones

Much work has been done to trace the pathways of the central monoamine containing neurones. Fuxe (1965b) has described the distribution of the monoamine nerve terminals. Most DA nerve terminals were found in the caudate nucleus and putamen. Anden et al (1964a) presented the following evidence to show that the DA containing cell bodies in the substantia nigra had their terminations in the neostriatum:
1. Electrolytic lesions in the substantia nigra caused a decrease in fluorescence in the caudate and putamen.
2. Removal of the neostriatum caused an increase in fluorescence in the substantia nigra cells and the proximal axons.

Further evidence for the existence of a nigro-neostriatal pathway was presented by Anden et al (1965a) and the substantia nigra cells were shown to be multipolar.

Hornykiewicz (1966) has noted that the fluorescence area of the striatum is large compared with the pars compacta in the substantia nigra which contains the cells that supply the terminals.

1.7 Subcellular Distribution of Dopamine

Laverty et al (1963) have studied the subcellular distribution of DA and ACh (acetylcholine) in the caudate nucleus. They found most of the DA in the soluble fraction and the rest in the nerve endings particles (NEPs). ACh was mostly bound. This evidence suggests that DA in the caudate nucleus terminals either exists in a free state or
1.8 Storage and Release of Dopamine

Hokfelt (1968) has demonstrated that small granular vesicles are used to store DA, 5-HT and NA. McLennan (1964) demonstrated a release of DA from the caudate above the resting level during stimulation of the centromedial nucleus of the thalamus. Stimulation of control areas produced no increased release of DA. A response could be recorded in the caudate after a single shock in the centromedial nucleus.

1.9 Central Actions of Dopamine

Curtis and Davis (1961) were the first to show that iontophoresed DA suppressed orthodromic excitation in the cat lateral geniculate nucleus. DA was more potent in this action than NA. In the caudate nucleus, iontophoresed DA has been shown to inhibit spontaneous activity and L-glutamate induced activity, (Bloom, 1965)

1.10 Drugs Affecting DA Levels

a. L-Dopa

L-Dopa is the immediate precursor to DA. Injection of L-Dopa into animals causes an increase in motor activity, alertness and irritability, (Carlsson, 1959). There is an increase in brain DA which is proportional to the dose of Dopa. A proportional decrease in 5-HT levels is also measured. There is an increase in the concentration of 5-hydroxyindole acetic acid (5-HIAA) indicating an increase in release and metabolism of 5-HT. Concentrations of NA are virtually unaffected (Everett and Borchering, 1970).

b. Reserpine

Reserpine is known to deplete the brain of its DA content. The behavioural effects of reserpine include a reduction in motor activity and a general tranquillizing effect. 5-HT levels are also lowered by
reserpine but the behavioural effects of reserpine can be reversed by
the injection of Dopa whereas the injection of 5-HTP alone is ineffective
in this respect. This indicates that the motor effects of reserpine are
due to changes in the brain DA levels. However, the possibility cannot
be ruled out that a change in NA levels plays some part.

1.11 Parkinsonism and Brain Dopamine

Reserpine has an action on the extrapyramidal system producing
akinesia and rigidity in animals. Parkinsonism is characterised by
extrapyramidal symptomatology such as rigidity, akinesia and tremor.
Lesions that may be associated with Parkinsonism include a degeneration
in the substantia nigra, a DA deficiency in the striatum, a decreased
ability to form DA and an impaired ability to store DA (suggested by an
increased HVA : DA ratio). Treatment with L-Dopa can relieve the
symptoms in people suffering from Parkinsonism.

1.12 Noradrenaline

Noradrenaline (NA) was found to be present in tissue by von Euler
(1946) who identified it as the transmitter in the post ganglionic
sympathetic nerve fibre (von Euler, 1948).

1.13 Metabolism of Noradrenaline

a. Synthesis of noradrenaline

NA is synthesised from DA by the enzyme dopamine-β-hydroxylase.
The synthesis of DA from tyrosine has been described in section 1.3.

b. Inactivation of noradrenaline

The inactivation of NA can be effected in several ways:

(i) Enzymes MAO is thought to be mainly intraneuronal while
COMT is responsible for extracellular enzymatic destruction. A lack
of change of metabolite concentrations following nerve stimulation
indicates that enzymes do not play a major role in inactivating released transmitter.

(ii) **Diffusion** Released NA may diffuse away from the synaptic cleft and into the blood stream. Increased levels of NA have been found in the blood after hard exercise. This may be an 'overflow' mechanism when the re-uptake mechanism is saturated. The NA that has entered the blood in this way may go on to play a hormonal role before it is metabolised by COMT in the liver.

(iii) **Re-uptake into nerve endings.** This mechanism is thought to be important for normal conditions of NA release. Released transmitter is actively taken up into the nerve endings by a membrane pump. This pump can be blocked by cocaine (Hillarp and Malmfors, 1964).

1.14 **Cellular Localisation of Noradrenaline**

Fluorescence microscopy has been used extensively in the localisation of NA within neurones. Using this technique the existence of NA nerve terminals and cell bodies has been demonstrated. Although the fluorophores of DA and NA have the same characteristics they may be distinguished by a number of methods that have been described in section 1.4.

The neurones in the brain that contain NA are very similar to those containing DA. Formaldehyde induced fluorescence is seen in nearly all parts of the neurone. The strongest fluorescence is seen in the nerve terminals where intensely fluorescent varicosities are found. The cell body is moderately fluorescent and it has been calculated that the concentration of NA in the cell body is $4 \times 10^{-4}$ ng while in the terminal net it is 300 times greater (Dahlstrom and Haggendal, 1966).

1.15 **Subcellular Localisation of NA**

The fluorescence seems to be associated with granules which are
synthesised in the cell body and are transported down the axon. Ligatures around these axons lead to an accumulation of granules above the ligature.

1.16 Mapping of Noradrenaline Containing Neurones

Carlsson and others (1964) have presented evidence for the existence of NA containing neurones in the spinal cord. Cell bodies containing NA were demonstrated by Dahlstrom and Fuxe (1964). These cell bodies were mainly located in the lower brain stem. Their axons have been traced by various methods including lesions to decrease fluorescence and drug treatment to affect the fluorescence. Many workers have contributed to the elucidation of the pathways described below.

a. Bulbospinal neurones

NA cell bodies (and 5-HT cell bodies) in the medulla oblongata descend and have their terminals in the ventral, dorsal and lateral horns of the grey matter in the spinal cord.

b. Lower brain stem neurones

Some NA neurones have their cell bodies and their terminals in the lower brain stem.

c. Ascending fibres

The medial forebrain bundle contains some ascending NA containing fibres that have their terminals in the hypothalamus, parts of the limbic system and the cortex (Anden et al, 1966b). There is evidence to suggest that one neurone may send axons to the cortex, cerebellum and the spinal cord (Anden et al, 1966a).

1.17 Release of Noradrenaline

The demonstration of release of possible transmitters in response to stimulation is difficult in the CNS. Difficulties are found both in collecting the compound and in stimulating a specific area. Release of
general amino acids has been shown in response to non-specific stimulation.

Anden et al (1965b) have demonstrated a release of NA after stimulation of the spinal cord.

1.18 5-Hydroxytryptamine

5-hydroxytryptamine (5-HT) was first shown to be present in mammalian CNS using bioassays. Twarog and Page (1953) used the Venus heart preparation which has a sensitivity of $10^{-9}$ to $10^{-8}$ M. Amin et al (1954) used the rat uterus preparation. 5-HT was also shown to be the vasoconstrictor present in serum when blood clots (this substance had previously been referred to as thrombocytin and vasocytin) and was named serotonin by Page (1954). Erspamer (1954) independently showed that the substance from enterochromaffin cells in the gastric mucosa (called enteramine) was 5-HT.

The distribution of 5-HT within the CNS was found to be non-uniform (Amin et al, 1954; Paasonen and Vogt, 1956). Paasonen et al (1957) have shown that cortical structures with high levels of 5-HT were all associated with the limbic system, e.g. the hippocampus, medial and lateral pyriform cortex and entorhinal cortex. In general the distribution of 5-HT was similar to that of NA with a few exceptions. The caudate nucleus and the putamen have low levels of NA but high 5-HT levels.

The development of a specific and sensitive fluorimetric assay for the measurement of 5-HT by Udenfriend et al (1955) enabled its presence in the CNS to be confirmed by chemical methods (Bogdanski et al, 1956). 5-HT has not been found in peripheral nerve.

1.19 Metabolism of 5-HT in Nervous Tissue

a. Synthesis of 5-HT

There is a blood-brain barrier to the entry of 5-HT into the
nervous system so there is a need for the synthesis of this indole-amine in nervous tissue. Tryptophan is taken up into the cells by an active process and hydroxylated in the 5 position by tryptophan hydroxylase to form 5-hydroxytryptophan (5-HTP). This enzyme is present in brain tissue and this reaction is the rate-limiting step in the synthesis of 5-HT (Jequier et al, 1967; Udenfriend et al, 1957). Para-chlorophenylalanine (PCPA) is a specific inhibitor of tryptophan hydroxylase (Koe and Weissman, 1966; 1968). It combines irreversibly with the enzyme.

The second step in the synthesis of 5-HT is the decarboxylation of 5-HTP by the enzyme 5-HTP decarboxylase to give 5-HT. This enzyme is found in various tissue including the brain. The distribution of the enzyme in the brain has been found to mirror that of 5-HT with a few anomalies (Bogdanski et al, 1957). The highest levels of decarboxylase were found in the caudate nucleus. In brain and other tissue homogenates the decarboxylase was found to be present almost entirely in the soluble fraction.

5-HTP decarboxylase and Dopa decarboxylase appear to be identical enzymes and are sometimes referred to as amino acid decarboxylase.

b. Inactivation of 5-Hydroxytryptamine

Sjoerdsma et al (1955) investigated the metabolism of 5-HT and concluded that MAO metabolism was the major pathway for the degradation of 5-HT in mammals. Bogdanski et al (1957) investigated the distribution of MAO within the brain and found that it was relatively evenly distributed throughout the grey matter. The hypothalamus had an exceptionally high MAO activity. The product of the deamination is 5-hydroxyindolacetaldehyde which is oxidised further to 5-hydroxyindolacetic acid (5-HIAA) as the major metabolite of 5-HT.

A presynaptic uptake mechanism similar to that for NA has also been indicated for the inactivation of 5-HT.
1.20 Cellular Localisation of 5-HT in the CNS

The histochemical fluorescence technique developed by Hillarp and co-workers has provided much information on the neuronal localisation of 5-HT as well as the catecholamines (see sections 1.4 and 1.14). This information has helped in the elucidation of the functions of 5-HT in the CNS.

When fluorescence microscopy was first applied to the CNS the 5-HT fluorescence was located within fibres which were thought to represent the terminal parts of the axons. Heller et al. (1962) provided some of the first evidence that 5-HT may be involved in specific neuronal pathways by showing that destruction of the medial forebrain bundle produced a fall of 5-HT in the brain compared with control lesions. Carlsson et al. (1963) measured a decrease in 5-HT levels below a transection in the spinal cord and suggested that descending 5-HT fibres were present in the spinal cord. Magnusson and Rosengren (1963) presented similar evidence for the existence of descending NA fibres. Further evidence for the existence of descending 5-HT and NA containing fibres in the spinal cord was presented by Carlsson et al. (1964). Using fluorescence microscopy they demonstrated a disappearance of fluorescence below a transection of the spinal cord. The green fluorescence was specifically depleted by m-tyrosine and methyl-m-tyrosine.

Dahlstrom and Fuxe (1964) directly demonstrated the presence of 5-HT containing cell bodies in the CNS. These cell bodies were located in the Raphe nuclei of the lower brain stem. As with the catecholamine neurones the fluorescence of 5-HT containing neurones is most intense at the varicose nerve endings (Fuxe, 1965a), the cell body is much less fluorescent and the proximal part of the axon contains very little, if any, fluorescence.

Dahlstrom and Fuxe (1964) were able to increase the fluorescence in the cell bodies and axons by pretreating with a combination of
nialamide and reserpine. A possible mechanism of the action of this combined drug treatment is that reserpine prevents the storage of 5-HT in granules and allows it to spread throughout the cytoplasm where the action of MAO has been inhibited by nialamide. In addition to this the bright green fluorescence of the catecholamines is depleted by reserpine and this improves the contrast between the yellow 5-HT fluorescence and the background. Treatment with reserpine alone depletes both green, catecholamine and yellow, 5-HT fluorescence. During recovery from reserpine treatment the fluorescence returns around the nucleus first.

1.21 Mapping of 5-HT containing neurones

Much work has been published on the localisation of 5-HT containing neurones and the demonstration of their pathways. The results have been discussed and summarised by many authors (see for example Fuxe, 1965b; Hillarp et al, 1966; Fuxe et al, 1968).

5-HT containing terminals are found scattered throughout the CNS. The highest concentration is found in the lumbar enlargement and sacral part of the spinal cord. In the brain nerve terminals have been found in the brain stem, hypothalamus and even the cerebral cortex. There is no evidence for 5-HT in vertebrate peripheral nerves (cited by Vogt, 1969).

In general, biochemical levels of 5-HT and the numbers of terminals observed using the fluorescence method are in agreement. In cases where they do not agree the biochemical data generally indicates higher levels of 5-HT than the terminal numbers suggest, this situation occurs in the striatum. A possible explanation of this is that the fibres are very fine and are below the resolution of the light microscope. In areas where the DA is also present the bright green catecholamine fluorescence masks the yellow 5-HT fluorescence. This problem can be improved with nialamide and reserpine treatment (see section 1.20). In some cases NA and 5-HT nerve endings have been seen to make close contact with the
same nerve cell (Dahlstrom and Fuxe, 1965; Fuxe, 1965a).

Electrolytic lesions have been employed in the mapping of 5-HT containing neurones. Lesions in the area of the cell bodies cause a depletion in the fluorescence of the nerve terminals. The 5-HT cell bodies in the Raphe nuclei give rise to descending fibres in the spinal cord. Ascending fibres in the telencephalon and diencephalon are thought to have their cell bodies in the caudal mesencephalon. No cell bodies that contain 5-HT have been found in the spinal cord, the telencephalon or diencephalon.

1.22 Subcellular Localisation of 5-HT

Whittaker (1958, 1959) has demonstrated the presence of bound 5-HT in a brain fraction containing pinched off nerve endings (NEPs). These results were confirmed using a fluorimetric technique (Michaelson and Whittaker, 1962). They were unable to separate the ACh and 5-HT nerve endings. Later work (Michaelson and Whittaker, 1963) showed the ACh to be associated with lighter nerve endings than 5-HT but there was a lot of overlap in the fractions.

The development of the histochemical fluorescence method for the demonstration of catecholamines and 5-HT enabled these monoamines to be visualised at their storage sites. Masuoka (1965) combined this fluorescence technique with the isolation of NEPs from the brain stem. He found that bright fluorescent spots were found in the same fraction as the NEPs (the B fraction). These fluorescent spots were mainly green and were the same size as the varicosities observed in tissue sections.

Fuxe et al (1967) extended these results and demonstrated fluorescent dots associated with the B fraction of homogenates from brain stem, spinal cord and caudate nucleus. Electron microscopy showed that NEPs were also associated with the B fraction. This fraction from
the spinal cord contained mostly yellow fluorescent dots (1-2µ) while the caudate + putamen fraction contained mostly green fluorescence. The brain stem fraction contained a large number of green dots 0.3 - 2.0 µ and also some yellow dots. Treatment with nialamide increased the number of yellow dots. Fuxe et al (1967) have presented evidence to suggest that the yellow fluorescent dots represent 5-HT containing varicosities and the green dots represent the catecholamine varicosities.

1.23 Storage and Release of 5-Hydroxytryptamine

a. Storage

Using electron microscopy, Hokefelt (1968), has demonstrated that small-type granular vesicles provide storage for the three monoamines 5-HT, DA and NA. Reserpine depletes the biogenic amines apparently by preventing these vesicles from storing the amines.

b. Release

One criterion for transmitter action has been the demonstration of the release of transmitter after nerve stimulation. Anden et al (1964b) have measured an increase in release that was above the resting level of 5-HT by the spinal cord on stimulation. No release was observed without pretreatment with a MAO inhibitor (e.g. nialamide).

A reduction in the fluorescence of 5-HT in varicose nerve terminals after stimulation of neurones in the medulla oblongata was demonstrated by Dahlstrom et al (1965). Aghajanian et al (1967) found that stimulation of the Raphe nuclei (where most of the 5-HT cell bodies are found) produced an increase in 5-HIAA and a decrease in 5-HT of the forebrain. This also strengthens the evidence for the existence of ascending pathways.

Farnebo (1971) examined the release of 5-HT from terminals in field-stimulated slices of cerebral cortex by fluorescence microscopy and by isotope measurements. The results from both techniques indicated
a release of 5-HT from nerve terminals.

1.24 Central Actions of 5-HT

Curtis and Davis (1961) tested several compounds and found 5-HT to be the most potent blocker of single orthodromic spikes in the lateral geniculate nucleus of cat produced in response to a train of spikes in the optic nerve. 5-HT was shown to depress synaptic firing of the lateral geniculate neurones (Curtis and Davis, 1962). Many LSD derivatives also depressed orthodromic excitation while having no effect on antidromic excitation.

Bloom et al (1972) have reported that iontophoretic applications of 5-HT produce effects in all regions of the brain. The effect was sometimes excitatory and sometimes inhibitory. There is evidence to suggest that the 5-HT nerve terminals synapse onto the granule cells which are always excited by 5-HT. (The action of 5-HT on Purkinje cells is always depressant).

Cells in the Raphe nuclei are the principle 5-HT containing neurones of the brain. Aghajanian (1972) tested certain drugs (applied parentally) for their effects on the firing of the Raphe nuclei cells. He found that LSD caused an inhibition of firing. MAO inhibitors and L-tryptophan increased the fluorescence in the cells and also depressed their firing. Neither the increase in fluorescence nor the decrease in firing was blocked by PCPA (a specific inhibitor of tyrptophan hydroxylase). The author suggested that 5-HT might not be the only amine present in the cells. (Tryptophan and 5-HT could be detected after tryptophan loading). He concluded that the firing rate was affected by compounds structurally similar to 5-HT and by compounds affecting 5-HT's metabolism.
1.25 Drugs Affecting 5-HT Levels

a. Reserpine

Reserpine lowers brain 5-HT levels but the behavioural changes which it induces are thought to be due to a reduction in DA rather than of 5-HT.

b. Reserpine + Nialamide

This combination treatment of reserpine and a MAO inhibitor increases the fluorescence in 5-HT containing neurones.

c. Iproniazid

This drug is a monoamine oxidase inhibitor and increases the level of 5-HT in the brain. The increased levels have the same location, subcellularly, as the normal 5-HT (Michaelson and Whittaker, 1963).

d. para-Chlorophenylalanine

This compound has been shown to be a specific inhibitor of tryptophan hydroxylase (the rate limiting enzyme in the synthesis of 5-HT). It causes a reduction in 5-HT levels and various behavioural changes which will be mentioned in section 1.26.

1.26 Functions of 5-HT in the CNS

a. Transmitter Action

There is considerable evidence to suggest that 5-HT functions as a transmitter in the mammalian CNS:

1. It is present in the brain tissue.
2. Enzymes are present for the synthesis and inactivation of 5-HT.
3. There is evidence for its release on nerve stimulation.
4. Neurones in the regions of 5-HT terminals are sensitive to 5-HT.
5. There is evidence for the existence of specific neuronal pathways containing 5-HT.

The possible physiological roles of 5-HT have been outlined in reviews by Bloom (1969) and Seiler et al (1971).
b. Temperature regulation

5-HT has been implicated in temperature regulation. The mechanism suggested is that NA and 5-HT are released in the hypothalamus in response to temperature changes detected by thermosensitive cells in the area. Environmental changes in temperature are known to influence the turnover rates of NA and 5-HT.

Injections of NA and 5-HT into the third ventricle lead to changes in body temperature. There is some discrepancy of results which may be due to a species difference. For example 5-HT injected into the hypothalamus of sheep and rabbits produces hypothermia whereas in other animals e.g. cat, dog, and monkey it has the opposite effect and produces high fever (Feldberg and Myers, 1964; 1965; Cooper et al, 1965). Stimulation of the caudal Raphe region in rat can produce hyperthermia. Pretreatment with reserpine --or PCPA abolishes this effect although PCPA alone does not produce hypothermia (see Bloom, 1969).

c. 5-HT and Sleep

Loss of 5-HT from the Raphe nuclei by either ablation (Jouvet, 1969) or by PCPA causes insomnia which takes the form of a decrease in slow-wave sleep. Recovery from PCPA is accompanied by a return in 5-HT levels. Injection of 5-HP reverses the effect of PCPA by by-passing the blocking action of this specific inhibitor. Increased 5-HT levels (brought about by treatment with MAO inhibitors, 5-HP etc.) give an EEG with more 'slow wave sleep' than normal. The circadian levels of 5-HT could be the cause or the effect of sleep.

d. Behaviour

One of the most useful tools in the study of 5-HT and behaviour has been the introduction of PCPA by Koe and Weissman (1966) as a specific depletor of 5-HT. Previously drugs which would deplete 5-HT also depleted the catecholamines. Behavioural changes induced by PCPA depletion of 5-HT include an increase in sexual activity in rats.
(Tagliamonte et al, 1969) an increase in the aggressive behaviour of rats towards mice (Sheard, 1969) and a decrease in aggressive behaviour in mice (Welch and Welch, 1968).

Stevens (1967) has shown a facilitation of learning in a brightness avoidance task in rats. There was no facilitation of learning in a position avoidance task. This may be due to an increased reactivity to painful stimuli. This has been demonstrated to electric shock by Tenen (1967).

Aprison and Hingten (1972) have recently reviewed the relationship between 5-HT and behaviour.

1.27 Dopamine, Noradrenaline and 5-Hydroxytryptamine as Central Transmitters

It has not always been possible to fulfil all the rigorous criteria for a neuro-transmitter that have evolved from the study of peripheral nerves but several important points can be mentioned:

1. The presence of monoamines in synaptic structures has been demonstrated. Large quantities of monoamines are found within varicosities at nerve endings. These nerve endings have been shown to be in close contact with cell bodies and dendrites.

2. All the enzymes required for the synthesis of the amines have been shown to be present in brain tissue. There is evidence to suggest that some of the enzymes are located within the neurones.

3. The precursors required for the synthesis of the amines are present in the CNS.

4. The enzymes necessary for the inactivation of the amines are present in brain tissue. MAO is thought to be largely responsible for the intracellular oxidation of the amines and COMT is thought to be located extracellularly. A presynaptic uptake mechanism is thought to be important in the inactivation of transmitters from aminergic neurones.

5. Release of the amines following specific stimulation has been
demonstrated.

6. The amines have been shown to have specific actions on the CNS. Certain drugs which affect the levels of the amines have been shown to cause similar behavioural changes to those found in specific physiological conditions that are known to be accompanied by changes in these amines.

In view of this and other evidence it seems likely that DA, NA and 5-HT function as chemical transmitters in the CNS.
1.28 The Occurrence of 5-HT in Invertebrates

5-HT is widely distributed in Nature. It is found in bananas, the venoms of wasps, spiders and scorpions and in toads and frogs. It is also contained in the intestine wall enterochromaffin cells, blood platelets and mast cells. Its presence in the nervous system was not clearly demonstrated until the 1950's.

Florey and Florey (1954) and Welsh (1954) were the first to find 5-HT in the nervous system of invertebrates. Since then the presence of 5-HT has been reported in a representative from each of the major phyla of the animal kingdom. The development of a specific and sensitive fluorescence assay for 5-HT has been largely responsible for this advancement.

The first suggestion of a transmitter role for 5-HT in invertebrates was in the heart of Venus (Mercenaria mercenaria) where ACh was known to be the inhibitory transmitter (Prosser, 1940) but the excitatory mechanism was unknown. Erspamer and Ghiretti (1951) had showed that extracts of salivary glands from Octopus vulgaris and Eledone mochata had a powerful excitatory effect on the hearts of Helix and Octopus. They suggested that the excitatory substance was 5-HT. Welsh (1953) had demonstrated that 5-HT had an excitatory effect on Venus heart at concentrations as low as $10^{-10}$ M. Electrical stimulation of the heart regulatory nerves had a similar effect. Welsh therefore suggested that 5-HT could be the transmitter for the excitatory nerves of this heart. 5-HT was found to be present in Venus ganglia (Welsh, 1954; Welsh, 1957).

The sensitivity of the Venus heart preparation to 5-HT has been used as a bioassay for 5-HT in tissue, including the mammalian CNS (Twarog and Page, 1953). The distribution of 5-HT in a wide variety of invertebrates was investigated by Welsh and Moorhead (1960). They
analysed isolated nervous tissue where possible but for small species and those with a diffuse nervous system they used whole animals. The only phyla of the eleven they tested where 5-HT was not found were the protozoa and the sponges (Porifera). The most primitive animal in which they found 5-HT was Hydra (Coelenterata). It is possible that the 5-HT was present in the nematocysts in the tentacles of Hydra where it could function as a venom. The highest levels of 5-HT were found in the ganglia of the bivalve molluscs (40 µg 5-HT/g tissue was reported for Venus ganglia). More highly developed molluscs contain less 5-HT. The annelids Lumbricus terrestris and Hirudo medicinalis have large amounts of 5-HT in their nervous systems (10.4 and 6.9 µg/g tissue respectively). The arthropods tested had only low levels of 5-HT with the exception of parts of the body that contained a sting apparatus. Echinoderms also had only low levels of 5-HT.

With the introduction of the fluorescence histochemical technique for the localisation of 5-HT and catecholamines in tissue (Falck, 1962) further advances could be made. The distribution of 5-HT in the nervous tissue of animals has been reviewed by Welsh (1968).

1.29 The Occurrence of Catecholamines in Invertebrates

Catecholamines (adrenaline, NA and DA) have been detected in representatives from several invertebrate phyla. In many cases the catecholamines have been located within the nervous system and, using the fluorescence histochemical technique for localising monoamines, they have been located within neurones. Molluscs in particular have a high concentration of catecholamines, especially DA, within their ganglia. One of the highest levels of DA reported is in the ganglia of Venus where Sweeney (1963) found concentrations of between 137 and 405 µg/g. He found no evidence for NA in the ganglia.

Cottrell and Laverack (1968) have compiled a list of inverte-
brate species where catecholamines have been detected. At one time DA was thought to be the only catecholamine occurring in certain molluscan ganglia, for example *Helix aspersa*, but recently more sensitive assays have shown small concentrations of NA present in some of these ganglia. Osborne and Cottrell (1970a) have reported 80 ng NA/g tissue in *Helix* ganglia.

DA and NA have been demonstrated to have effects on individual molluscan neurones. Kerkut and Walker (1961) have shown that some *Helix* neurones will respond to concentrations of DA as low as $10^{-11}$ g/ml, and have suggested a role as a neurohumour for DA in this mollusc. In general molluscan neurones are less sensitive to NA but Glaizner (1967) has demonstrated a cell that responded to NA and not to DA. Where NA and DA are both present it is not easy to distinguish between their fluorophores. The differentiation of these two fluorophores has been discussed in sec. 1.4.

In insects DA is present as a precursor to N-acetyldopamine which is involved in the tanning of the cuticle. It seems likely that the DA present in insect neurones has a transmitter function.

1.3 The Cellular Localisation of 5-HT, DA and NA in the Major Phyla

a. Protozoa

Janakidevi et al (1966) have shown the presence of 0.11 - 0.13 µg 5-HT/g tissue in the flagellate, *Crithidia fasciculata* and 0.6 µg/g in the ciliate, *Tetrahymena pyriformis*. He used two different assay procedures and obtained a good correlation between them. 5-HT could be involved in the mechanism of contraction of the cilia and flagella.

b. Sponges

It is uncertain whether sponges have a nervous system but Lentz (1966) has reported the presence of 5-HT in multipolar and some bipolar cells that resemble neurones in *Sycon ciliatum*. The 5-HT was depleted
by reserpine and α-methyl Dopa. Lentz has suggested that these cells could be neurosecretory and that nerve cells may have evolved as glandular cells.

Lentz also found NA and adrenaline in bipolar and multipolar cells of *Sycon*. NA was found in more bipolar than multipolar cells and the converse was true for adrenaline. A histochemical method was used for the detection of the amines.

c. Coelenterates

These animals have the most primitive type of organised nervous system. 5-HT has been shown to be present in whole *Hydra* and parts of sea anemone (Welsh and Moorhead, 1960). Wood and Lentz (1964) demonstrated the presence of 5-HT in the nervous system of *Hydra* using a histochemical method, this 5-HT was depleted by reserpine. In the sea anemone they found 5-HT present in the tentacles, peristome, pharynx and the nematocysts. They have also reported the presence of NA and adrenaline in the nervous elements of *Hydra* and the sea anemone.

Dahl et al (1963), using Falck's fluorescence histochemical technique for the demonstration of certain monoamines, examined the sea anemones *Metridium senile* and *Tealia felina*. In these two species they found nervous structures displaying fluorescence within the tentacles, the fluorescence was bright green. Cell bodies were found in the ectodermal epithelium which each sent a fibre to the surface. These fibres usually possessed varicosities and a terminal expansion. Branched fibres also passed from the cells to the base of the epithelium. In both species there was a basi-epithelial network of fluorescent fibres which was more dense at the distal end of the tentacles. In *Tealia* the fluorescent cells were less numerous than in *Metridium*. The fluorescence was not positively identified but was thought to be due to the presence of a primary catecholamine. Applications of 5-HT, DA
NA and ACh to the column and sphincter of *Metridium* did not have any effect. It was proposed that the neurones are sensory receptors. It is possible that they may also have motor functions. A third species examined (*Hydractinia echinata*) did not show any fluorescence.

d. **Platyhelminthes** (Flatworms)

These unsegmented worms have an organised nervous system with ganglia. Welsh and Moorhead (1960) reported concentrations of 5-HT up to 3.4 µg/g for *Dugesia* (Turbellaria) using whole animals. Further analysis, however, revealed that the tail portion of this planarian often had a greater concentration of 5-HT than the head portion, which contained the greatest proportion of nervous tissue.

Using fluorescence microscopy Welsh (1968) and Welsh and Williams (1970) have examined the small white planarian, *Phagocata oregosa*. The cerebral ganglia were found to contain 2-4 pairs of 5-HT containing cells. The ventral nerve cord also contained some 5-HT cells. In addition to these central cells there were also some 5-HT cells with processes penetrating the epidermis, these cells could have a sensory or cilia-regulatory function. Also in the brain of this planarian there were 4 pairs of catecholamine containing cells, 2 pairs were monopolar, 1 pair was bipolar and 1 pair was multipolar. Many fine catecholamine fibres were present in the neuropile. No fluorescent cells were found associated with the eyes of planaria. Another species *Procotylea fluviatilis* also had four pairs of CA cells in the brain but had in addition some smaller blue cell bodies.

Both 5-HT and catecholamine systems were present in the flatworms examined. The catecholamine is most likely to be a primary amine, either DA or NA or a mixture of the two. Large cells were up to 20µ in size, small cells could only be distinguished from varicosities by the presence of a nucleus. Both the catecholamine and the 5-HT cells
could have a sensory function. In other groups only catecholamine cells appear to be sensory. The 5-HT cells in the flatworms may have a cilia-regulatory function, this is supported by the observation that reserpine produces a decrease in movement.

e. Echinoderms

This group includes the starfish, the brittlestar and sea urchin. Very little 5-HT was found in the species of this phyla that have been analysed by Welsh and Moorhead (1960). Cottrell and Penreath (1970) were unable to detect any 5-HT by fluorescence histochemistry in the starfish, Asterias rubens and the brittlestar, Ophiothrix fragilis.

Cottrell (1967) determined the concentrations of NA and DA in the radial nerves of the sea urchin, Echinus exulentus and the starfish, Asterias rubens. The levels of DA which he found were 2.5 - 7.0 µg/g for Echinus and 3.0 - 8.0 µg/g for Asterias. The NA levels were lower in both animals than the DA levels, 1.5 - 3.5 µg/g in Echinus and 0.5 - 2.0 µg/g in Asterias. Asterias that had been immersed in sea water containing reserpine (3µg/ml) for 5 days showed a greater decrease in DA than in NA levels. These starfish had a slower righting reflex.

Cobb (1969) has studied the localisation of monoamines in a starfish (Patiriella calcar) and a sea urchin (Heliocodaris erythrogramma) using formaldehyde-induced fluorescence. He found that neither the nerves innervating the skeletal and visceral muscles nor the sensory nerves contained monoamines. The radial nerve of the sea urchin was made up of hundreds of fine fluorescent fibres, 0.2-1µ in diameter, that were only just visible under the light microscope. Fluorescent cell bodies were found in the epithelial cells surrounding the nerve. In the starfish there were only a few fluorescent cell bodies. The nerve is divided into two parts, hyponeural and ectoneural tissue and all the fluorescent fibres were found in the latter.
Three main types of vesicles were found in the nervous system of echinoderms:

(i) small granular vesicles (up to 700 Å)
(ii) large granular vesicles (up to 2000 Å)
(iii) small agranular vesicles (up to 500 Å)

The fluorescence in the tissue appeared to coincide with the presence of small granular vesicles (from electron microscopy).

Cottrell and Penreath (1970) also studied the distribution of DA and NA using fluorescence microscopy in Asterias rubens and Ophiothrix fragilis. They found similar results to those reported by Cobb (1969). The fluorescence was located exclusively in ectoneural tissue (sensory -association and possibly internuncial neurones). A few fluorescent cell bodies were located in the ectodermal layer but not enough to account for all the fluorescent axons.

It is possible that the cell bodies of these fluorescent axons contain a very low concentration of amine and are not detected. Cobb and Cottrell and Penreath have suggested that fluorescent cell bodies in the stomach wall are interneurones. Cottrell and Penreath also found the fluorescence to be associated with the granular vesicles.

Reserpine has been shown to deplete the levels of DA and NA in Asterias, the amine fluorescence disappeared almost completely in animals kept in reserpinised sea-water. These animals became sluggish, lost their righting reflex and there was some indication that co-ordination of the tube feet was lost. Ophiothrix kept in reserpinised sea-water did not show a marked reduction of fluorescence nor was there any marked behavioural change. The difference in response is thought to be due to a possible difference in permeability barriers.

f. Annelids (Segmented Worms)

5-HT is present in the isolated nerve cords of several species of
annelids. High concentrations were found in the nerve cords of *Lumbricus terrestris* and *Hirudo medicinalis* (10.4 and 6.9 µg/g respectively). Several polychaetes also had high levels of 5-HT in their nerve cords.

Clark (1966) made a study of the monoamine fluorescence in the polychaete *Nephtys*, where she found both types of fluorescence present. 5-HT containing neurones predominated in the ventral nerve cord where there were only three pairs of green neurones while green neurones predominated in the supra-oesophageal ganglia (the brain). There was a repetitive pattern of neurones in successive segments in the nerve cord. The neuropile contained fluorescent varicose fibres.

There were four segmental nerves of which three carried fluorescent fibres. All fluorescent cells in the periphery were green with the exception of some 5-HT cells in the wall of the intestine. A few of these green cells were located in peripheral ganglia while the rest resembled bi-polar epidermal sensory cells found in other polychaetes. Yellow fibres were found peripherally at points of muscle insertions onto basement membranes. These were the only yellow peripheral fibres and could represent end plates of motor fibres.

The morphology and distribution of the two types of fluorescence indicates that the green system may have a sensory function and the yellow system a motor function.

(i) *Lumbricus terrestris* Welsh and Moorhead (1960) reported 10.4 µg 5-HT/g tissue present in the nerve cord of the earthworm. Rude (1966) and Kerkut, Sedden and Walker (1967a) have examined the distribution of fluorescence following treatment with formaldehyde. Most of the fluorescent cells were aggregated together in ganglia of the ventral nerve cord where 7 groups of fluorescent cells have been described by Rude. A few fluorescent cells were found scattered throughout the nerve cord. The majority of the fluorescence was due to 5-HT and only a few cells contained catecholamine fluorescence. A pair of green fluorescent
tracts ran the length of the nerve cord. Some yellow cells were thought to be motor neurones but there was no evidence of fluorescent fibres innervating the muscles. These fibres could be below the resolution of the light microscope. The presence of yellow varicosities in the neuropile suggests an interneurone function for some of the yellow cells. It seems likely that there is a sensory catecholamine system in the body wall that sends axons into the nerve cord.

Myhrberg (1967) has extensively discussed the location of monoamines in Lumbricus and has reported the presence of many 5-HT containing cells in the ventral nerve cord. In the cerebral ganglia he reported large numbers of both types of fluorescent neurones as well as both types of fluorescent fibres in the neuropile. Myhrberg also reported that the green fluorescence was most likely to be due to primary catecholamines because prolonged exposure to formaldehyde did not produce any increase in fluorescence as would be expected for a fluorescence due to secondary amines. Analysis of the catecholamine content indicated that NA was the dominant catecholamine and only traces of DA were present. Rude (1969a) using thin layer chromatography has estimated that the nerve cord of the earthworm contains twice as much DA as NA.

Ehinger and Myhrberg (1971) have extended the fluorescence studies of Rude (1966) and Kerkut et al (1967a). They were unable to find cells in the nerve cord which contained specific green fluorescence. They confirmed the presence of green fluorescent cells scattered throughout the epithelium and described them as receptor cells. The highest numbers of these cells were found in the buccal cavity and prostomium. They send a distal process to the cuticle. Proximal fibres divide and form a basi-epithelial network. This network is connected to nerve rings within segments.

Microspectrometry on the yellow cells showed that they had an emission peak at 530-550 nm and the green cells had an emission peak.
at 480-485 nm. Exposure of the sections to HCl vapour revealed that the green cells were of two types. The fluorescence in the green cells had two excitation peaks, one at 370 nm and the other at 320 nm. The ratio of the two peaks i.e. 370 : 320 for the green cells fell into two groups:

1. In this group the ratio was 1.3-1.5. Ehinger and Myhrberg have suggested that this fluorescence was due to the presence of DA. Cells with this type of fluorescence were found in the epithelium, small cells in the cerebral ganglia and in axons of the cerebral ganglia, ventral nerve cord and the basi-epithelial nerve net.

2. This type of fluorescence had a value of 0.7-1.1 for the ratio, 370 : 320. This value is higher than would be expected for cells containing purely NA but it seems probable that some NA is present in these cells. A mixture of NA and DA could be present in these cells. Some large neurones in the cerebral ganglion and some nerve fibres show this type of fluorescence. The possible presence of some interfering substance has not been excluded.

In Lumbricus the DA, NA and 5-HT appears to be stored in small granular vesicles in the nerve fibres. It seems unlikely that neurosecretory neurones contain monoamines (Myhrberg, 1972).

(ii) Hirudo medicinalis Welsh and Moorhead (1960) estimated the amount of 5-HT in the nerve cord and sinus of leech to be 6.9 µg/g.

Kerkut, Seddon and Walker (1967a) have described the presence of six yellow fluorescent cells in the ganglia of leech, of which two cells are the giant Retzius cells (60-80µ).

The central nervous system of the leech consists of 34 ganglia. The brain consists of two fused ganglia, four ganglia are fused to form the suboesophageal mass, the anal ganglionic mass is formed from 7 fused ganglia. The remaining ganglia are unfused. Each ganglia contains approximately 350 cells grouped into six follicles.
Most of the ganglia of the central nervous system have an identical content and arrangement of monoamine containing cells. There are seven yellow fluorescent cells within the ganglia and one pair of blue-green fluorescent cells outside the ganglia in the anterior roots. Fluorescent fibres in the neuropile were mainly varicose but occasional smooth fibres of both types of fluorescence were found. The brain contained 2 pairs of blue-green fluorescent cells but no yellow cells. (Marsden and Kerkut, 1969b; Rude, 1969b).

The fluorescence in the yellow cells had the same characteristics as 5-HT fluorescence. The identification of 5-HT in the Retzius cells has been confirmed by microspectrophotometry and chromatography (Rude et al., 1969).

The Retzius cells are known to send axons down the lateral nerves and to innervate muscles in the body wall. ACh causes contractions of the body wall which are inhibited by 5-HT. It has been suggested that 5-HT is an inhibitory motor transmitter onto these muscles. The catecholamine neurones may have a sensory or an interneurone function. A sensory function for catecholamine containing neurones has been suggested for other invertebrates (Myhrberg, 1967; Rude, 1966; Dahl et al., 1963).

g. Arthropoda

(i) Crustacea Welsh and Moorhead (1960) found very little 5-HT in the nervous systems of the crustacea they tested. The horseshoe crab (Limulus polyphemus) contained 0.1 - 0.3 µg/g but in other species 5-HT was undetectable or present only in trace amounts.

In a histochemical fluorescence study of the crayfish Astacus, Eloffson et al. (1966) reported 2 large yellow fluorescent cells in the 'brain'. In the gut of the crayfish Eloffson et al. (1968) described two plexi containing catecholamine fibres innervating the intestine. This fluorescence was depleted by reserpine and preliminary evidence suggested
that the fluorescence was due to NA. Fibres containing specific catecholamine fluorescence were also found running between muscle fibres in the gut. Elofson and Klemm (1972) described the presence of fluorescent strata in the visual pathways and in the optic ganglia which were characteristic of the species. The dominant catecholamine was DA.

Monoamine fluorescence (probably due to catecholamines) was reported in cell bodies and the neuropile of the stomatogastric ganglion of the lobster *Homarus vulgaris* by Osborne and Dando (1970). This ganglion contains 30-35 neurones. Most of the cells are motor and innervate the stomach muscles. The authors have suggested this ganglion as a simple model for study.

Cottrell (1967) was able to detect DA in the nervous tissue of the crab *Carcinus maenus* but he was unable to detect either DA or NA in the spider crab, *Hyas araneus*. Goldstone and Cooke (1971) have applied the fluorescence histochemical technique to the crab, *Carcinus maenus*. Because marine species are difficult to freeze-dry they chose estuarine species and adapted them to diluted sea water before use. This treatment did not appear to affect the green fluorescence but under these conditions no yellow fluorescence was visible. They were able to localise catecholamine fluorescence in neurones and neuropile of the eyestalk ganglia, brain ventral ganglia, circumoesophageal connectives and ganglia. Yellow fluorescence that appeared to be due to 5-HT was found in neurones of the brain and ventral ganglia.

(ii) **Insecta** Ostlund (1954) using whole animals has shown that insects contain high levels of catecholamines particularly of DA. Welsh and Moorhead (1960) had found that there was very little 5-HT present in the nervous systems of insects that they had tested.

The levels of NA and DA in the brain of the cockroach, *Periplaneta americana*, have been estimated by Frontali and Haggendal (1969) and
were found to be 0.37 μg NA/g tissue and 2.5 μgDA/g tissue. The high level of DA suggests that it has a separate role and is not present purely as a precursor of NA. The cellular localisation of catecholamines in cell bodies and varicose terminals has been demonstrated by Frontali and Norberg (1966) using fluorescence histochemistry.

Frontali (1968) has described the localisation of 14 recognisable groups of fluorescent cells in the brain of the cockroach. Treatment with reserpine caused a sedation of the animals and the fluorescence in the neuropile could be depleted after one day. Prolonged treatment with reserpine was necessary to deplete the fluorescence in the cell bodies with the exception of two groups of cells that appeared resistant to reserpine. No 5-HT fluorescence was found.

Smalley (1970) has demonstrated an uptake of labelled DA by midline median nerve cells in the abdominal ganglia of the cockroach. Axons from these cells pass down the median nerve and end in the neurohaemal organ. Their endings appear to contain electron transparent vesicles which are not characteristic of typical catecholamine containing vesicles. This suggests that these cells may be neurosecretory.

Klemm (1968, 1971) has described the occurrence of catecholamine containing fibres and cell bodies in Trichoptera (Anabolia nervosa, the caddis fly). Catecholamine containing cell bodies were located in the optic lobe, protocerebrum, α lobes, caudal pars intercerebralis and oesophageal ganglia.

Klemm and Bjorklund (1971) analysed whole heads of this insect for NA, DA and 5-HT. The concentration of DA was about 3.4 μg/g wet weight and that of NA was about 0.3 μg/g wet weight. They were unable to detect any 5-HT. The ratio of DA : NA for the head region was similar to that found for brain in the cockroach (Frontali and Haggandal, 1969).

Microspectrometry indicates that the fluorescence in many nerve
terminals and in some cell bodies in the cerebral ganglia of Trichoptera was due to DA. They were only able to record a NA type of spectrum from nerve terminals but only cell bodies with a high fluorescence intensity could be analysed by this technique.

DA is known to be involved in sclerotisation of the cuticle but it is unlikely that such a high intraneuronal concentration of DA would be needed for this purpose.

h. Molluscs

The ganglia of bivalve molluscs contain the highest levels of 5-HT found in nervous systems. It was first shown to be present in Venus ganglia by Welsh (1954). The ganglionic mass of Venus (Mercenaria mercenaria) has an average of 40 μg 5-HT/g tissue (Welsh and Moorhead, 1960). Welsh and Moorhead have also shown 5-HT to be present in the nervous systems of gastropods and cephalopods. Mercenaria mercenaria was shown to have an average concentration of 216 μg DA/g by Sweeney (1963) and he found that DA was the only detectable catecholamine in a number of lamellibranch and gastropod species. Kerkut and Walker (1961, 1962) had suggested an inhibitory role for DA in the brain of the snail Helix aspersa. Cardot (1963) showed DA to be present in the snail Helix pomatia. Kerkut, Seddon and Walker (1966) showed DA to be present in the ganglia of Helix aspersa at a concentration of 5.5 μg/g. The DA content was lowered by treatment with reserpine and raised by pretreatment with Dopa. MAO and O-methyl transferase inhibitors had no effect on brain DA content.

(i) Gastropods The presence of monoamine containing neurones in molluscs was first demonstrated by Dahl et al (1962) using fluorescence microscopy on Helix pomatia and Anodonta piscinalis. Further investigations on these monoamine containing neurones showed that in general their morphology was similar to those in vertebrates. The cell body
contains moderate concentrations of monoamines, the smooth preterminal
axons have lower concentrations and the varicose nerve terminals have a
high concentration and are intensely fluorescent. The fluorescence could
be depleted by treatment with reserpine (Dahl et al., 1966).

The localisation of the monoamine containing neurones has been
described by Seddon, Walker and Kerkut (1968). Medium-large neurones
showing a yellow fluorescence were found in the cerebral, pedal, visceral
and right parietal ganglia. Smaller cells showing green fluorescence
were found in the cerebral and pedal ganglia. The neuropile in all the
ganglia contained fibres and varicosities of both types of fluorescence.

Marsden and Kerkut (1970) measured the monoamine content of the
circumoesophageal ganglia of the water snail Planorbis corneus and found
9.53 μg DA/g and 3.34 μg 5-HT/g. They were unable to detect any NA.
They described the distribution of formaldehyde-induced fluorescence in
Planorbis and compared it with the fluorescence in Helix. The distrib-
ution of fluorescence in both snails was similar with one notable excep-
tion. A large cell (up to 300 μ) containing green fluorescence was found
in the right pedal ganglion of Planorbis, there was no such cell in Helix.
Microspectrometry indicated that this fluorescence was due to DA. The
neuropile of the ganglia contained numerous fibres and varicosities of
both types of fluorescence. Green fluorescent cells with smooth processes
were found in the oesophagus wall and a sensory function has been
suggested for them. Yellow dots found in the musculature of the oesoph-
Agus wall could be terminals of 5-HT motor neurones.

The levels of 5-HT in Helix are subject to a seasonal variation.
Cardot (1971) has shown two peaks in 5-HT concentration in the brain of
Helix pomatia. One of the peaks occurred in January and the second in
the June-August period. 5-HT levels in the heart followed a similar
pattern. Jurio and Killick (1972) have also reported a seasonal
variation in the levels in Helix ganglia and heart.
For some time it was thought that all the primary catecholamine fluorescence present in certain molluscan ganglia was due to DA as no NA was detected in chemical assays of the ganglia. Recently, however, it has been shown that small quantities of NA are also present in some of these molluscs. Osborne and Cottrell (1970a) have reported 80 ng NA/g in the brain of Helix aspersa. A.G. Rammage (1972, personal communication) has detected NA in the brain of Helix aspersa using radiochemical methods. Jurio and Killick (1972) have reported a concentration of NA 1/60 that of the DA concentration (70 ng NA/g tissue). It is therefore possible that some of the catecholamine neurones contain NA either as the only amine or in addition to DA. It is possible that NA could act as a transmitter in Helix ganglia. Glaizner (1967) has reported a neurone in the brain of Helix aspersa that responds to NA but not to DA.

Further tests to distinguish between the two types of catecholamine fluorescence in Helix are required.

(ii) Lamellibranchs (Bivalves) Zs-Nagy (1967a) has suggested a neurosecretory role for 5-HT in Anodonta cygnea because it is present in the majority of nerve cells but no fluorescent axons were found. After treatment with iproniazide the fluorescence in the neuropile was located in the cytoplasm of the glia. A few cells were found to contain DA. Recovery after DA depletion by reserpine was accompanied by all the cerebral cells becoming green. All the green fluorescent axons showed a positive pseudoisocyanine reaction (PSI) indicating the presence of elementary neurosecretory granules. In addition some non-fluorescent axons also gave a positive PSI reaction. (Zs-Nagy, 1967b)

The bivalve Sphaerium sulcatum has been shown to contain 13.4 ng 5-HT/animal; 5.2 ng DA/animal and 3.5 ng NA/animal (Sweeney, 1968). The distribution of these monoamines was also examined. Monoamine containing cells were located in the foot, syphons, mantle, statocysts and gill filaments. These cells probably have a sensory function.
Monoamine neurones were also found in the ganglia. The fluorescent cells in the visceral ganglia were all catecholamine cells, in the pedal ganglia the fluorescent cells all contained 5-HT fluorescence and both types of fluorescence was found in the cerebral ganglia. There was no evidence for peripheral monoamine innervation which suggested that these ganglionic neurones do not have a motor function. They could function as inter-neurones within or between the ganglia.

(iii) Cephalopods Cottrell (1967) has detected DA and NA in extracts of nervous tissue from the octopus Eledone cirrhosa using a technique described by Belle and Somerville (1966) of paper chromatography followed by exposure to formaldehyde vapour. The concentration of DA in the brain of this octopus was greater than that of NA. Jurio (1971) and Jurio and Killick (1972) have measured the concentrations of DA, NA and 5-HT in three octopods: O. vulgaris, O. macropus, Eledone moschata and in two decapods: Loligo vulgaris and Sepia officinalis. All three amines were present in each species. The highest concentrations were found in the optic and buccal lobes. The concentrations of amines was higher in the octopods than the decapods.

Acid metabolites of DA and 5-HT were found in cephalopods but not in gastropods. MAO inhibitors increased the concentrations of amines in O. vulgaris but not in Helix.

1.31 Metabolism of 5-HT in Invertebrates (in particular the Molluscs)

a. Synthesis

Enzymes for the conversion of 5-HTP to 5-HT are present in molluscan nervous tissue (Kerkut and Cottrell, 1963). Cottrell and Powell (1971) have shown that the giant serotonin cells (GSC) in the cerebral ganglia of Helix are able to convert 5-HTP to 5-HT when isolated. Non-serotonin containing cells were isolated and used as controls. Non-synthesis of 5-HT could be detected from these cells.
Colhoun (1963) has shown a qualitative conversion of 5-HTP to 5-HT by neural tissue of the cockroach.

b. Inactivation

Blaschko et al (1937) were unable to find any MAO activity in Helix aspersa. It was present in certain other invertebrates, for example Patella vulgata. Jurio and Killick (1972) have demonstrated its presence in O. vulagaris. MAO was found in non-nervous tissue of molluscs by Blaschko and Hope (1957). It seemed from these results that another inactivating mechanism must be considered for Helix and some other invertebrates.

Marsden and Kerkut (1970) have reported a slight increase in yellow fluorescence in Planorbis after treatment with nialamide, a MAO inhibitor, this suggests an inhibition in the breakdown of 5-HT. Marsden (1972) has been able to measure the presence of 5-HIAA in the nervous system of Planorbis corneus. Pretreatment with 5-HTP produces an increase in the concentration of 5-HIAA.

1.32 Metabolism of Catecholamines in Invertebrates (especially Molluscs)

a. Synthesis

Injection of Dopa into Helix aspersa causes a rise in ganglionic DA levels. α-Methyl Dopa reduces the content of DA in the nervous system. This suggests that DA is formed from Dopa by decarboxylation (Kerkut, Sedden and Walker, 1966). Homogenates of Helix pomatia ganglia and hearts can convert Dopa to DA (Cardot, 1963).

The synthetic pathway of DA would appear to be the same as for vertebrates with Dopa as the immediate precursor to DA. In insects some DA is used to form N-acetyl dopamine which is incorporated into the cuticle.

b. Inactivation

Kerkut et al (1966) found that MAO inhibitors and O-methyltrans-
Ferase inhibitors had no effect on DA levels in *Helix aspersa*. The yield of DA from Dopa using homogenates of *Helix* ganglia was not increased by the addition of a MAO inhibitor (Cardot, 1963) MAO inhibitors have been shown to increase the levels of amines in *O. vulgaris* (Jurio and Killick, 1972). From this it would seem that in certain gastropods MAO is not important in the metabolism of DA.

Blaschko and Hope (1957) have reported the presence of MAO in molluscan nervous tissue. Osborne and Cottrell (1970a) have detected deamination and O-methylation metabolites of DA and NA in *Helix* heart and brain. In the heart the metabolites of NA (ie. 3,4-dihydroxymandelic acid and vanillinmandelic acid) predominated while in the brain the metabolites of DA (ie. 3,4-dihydroxyphenylacetic acid and homovanillic acid) predominated.

In *Lumbricus* treatment with nialamide caused an increase in the monoamine fluorescence (Myhrberg, 1967).

1.33 Storage of 5-HT

Using formaldehyde-induced fluorescence to localise monoamines it has been shown that in the nervous systems of many invertebrates these monoamines occur intraneuronally. The distribution of monoamines within the neurones is similar to that of the vertebrate systems. That is the fluorescence in the cell body is moderate to strong, the first part of the axon contains weaker fluorescence and the varicose terminal regions of the neurone are intensely fluorescent.

Rude et al (1969) have suggested that the 5-HT in the giant Retzius cells of the leech ganglia is contained within small dense cored vesicles seen under E.M. These vesicles were absent from control cells that did not contain 5-HT.

Osborne and Cottrell (1970b) demonstrated an accumulation of small granular vesicles (30-150 nm) above a ligature of the visceral nerve in
Helix pomatia. The accumulation of granules was accompanied by an increase in fluorescence and 5-HT concentration. A decrease in fluorescence was observed below the ligature. They have concluded that 5-HT and catecholamines are transported bound to small granules from the CNS along the visceral nerve to peripheral nerve terminals.

Cottrell and Osborne (1970) estimated the amount of 5-HT in the GSC of the slug Limax maximus to be 0.7 ng/cell. After making allowances for the nucleus this gave a concentration of $4 \times 10^{-3}$ M in the cytoplasm. They investigated the subcellular localisation of this 5-HT and concluded it was contained in small granules, the number of which could be reduced by reserpine.

In Mercenaria nervous tissue Cottrell (1966) found that 70% of the 5-HT and NA could be sedimented, suggesting it was particle bound. 5-HT was associated with a denser fraction than ACh. Further examination of the fractions (Cottrell and Maser, 1967) have indicated that 5-HT was not associated with dense-cored particles ($1,000-2,000 \, \AA = 100-200\,\text{nm}$).

Evidence for the existence of 5-HT containing granules in Venus neurones has been presented by Welsh (1958). Zs-Nagy et al (1965) examined fractions of homogenates from the CNS of 2 lamellibranchs, Anodonta piscinalis and Anodonta cygnea for the presence of 5-HT. 5-HT was found in several fractions but the results indicated that it was associated with endoplasmic reticulum. Because of this they have suggested a regulatory function for 5-HT rather than a transmitter role.

1.34 Storage of Dopamine

Cottrell (1968) suggested that DA in Spisula solida was associated with small granular vesicles (300-1,000 \, \AA). These vesicles were particularly numerous in the neuropile. The possibility that 5-HT or NA could also be associated with the particles was not ruled out.
In the cockroach Maricini and Frontali (1970) have demonstrated the presence of two types of terminal fibres in the β lobes:

**Type 1 endings** have small clear vesicles and larger semi-dense vesicles. Reserpine had no effect on these vesicles.

**Type 2 endings** contain larger and denser vesicles. These were only partly affected by reserpine but evidence indicates that the dense-core vesicles of the type 2 endings are the storage sites for catecholamines.

Cobb (1969) presented evidence to suggest that the catecholamine fluorescence in the starfish was associated with small (up to 700 Å) granular vesicles.

### 1.35 Release of Monoamines

S-Róza and Perenyi (1966) have demonstrated a release of 5-HT in *Helix* heart after stimulation of the extra-cardial nerve. They were able to excite an unstimulated heart with the perfusate from the stimulated heart.

Chase et al (1968) have demonstrated a release of radioactivity from *Aplysia* heart and ganglia loaded with tritiated 5-HT in response to field stimulation. Release was reduced in Ca²⁺ sea water. No release was observed in connective nerves. Release of radioactivity from heart in response to nerve stimulation was also demonstrated. LSD caused a stimulation of the heart and a release of 5-HT.

### 1.36 The Action of 5-HT and Dopamine on Invertebrate Neurones

5-HT has been shown to excite molluscan neurones. Gerschenfeld and Tauc (1961) have described the effect of 5-HT on two types of neurones in *Aplysia*:

(i) **D cells**, those excited by ACh ie. depolarised.

(ii) **H cells**, those inhibited by ACh ie. hyperpolarised.

5-HT had an effect on both these types of cells but was ten times more
potent on the D cells.

Kerkut and Walker (1961, 1962) have demonstrated an excitatory effect for 5-HT on Helix aspersa neurones. DA was also shown to affect Helix neurones where it inhibited spontaneous firing at concentrations as low as $10^{-11}$ g/ml. There is considerable evidence for the existence of specific DA receptors in invertebrates. A particular study of those in Helix has been made by Woodruff (1971) who has also reviewed the evidence for DA receptors in other species.

1.3.7 Drugs Affecting Monoamines in Invertebrates

a. Reserpine

In general reserpine depletes monoamine stores in invertebrates. The doses required for this depletion are usually high, Sedden (1967) used 5 mg/animal for Helix aspersa. Five hours after reserpine treatment the snails were unable to crawl or to maintain an upright position. A depletion of 5-HT from sponges was demonstrated by Lentz (1966). Cottrell (1967) noted a slow righting reflex and a depletion of DA from starfish (Asterias) that had been kept in sea water containing 3 mg/ml reserpine. In earthworm reserpine caused a depletion of monoamine fluorescence but this was greatest at the site of injection and diffusion between segments was very slow (Myhrberg, 1967).

Reserpine has been shown to cause a depletion of monoamine fluorescence or monoamine levels in various other species, for example: in the lobster (Osborne and Dando, 1970), in the cockroach (Frontali, 1968) and in the water snail (Marsden and Kerkut, 1970).

b. Imipramine

Cottrell (1971) has shown that imipramine (a compound which is known to block 5-HT uptake in blood platelets and has been shown to inhibit the uptake of labelled 5-HT into the brain of Helix pomatia by 80%) potentiated the transmission between the giant serotonin cell in the cerebral ganglia and the buccal cell with which it makes contact in
Helix. 5-HT is thought to be the transmitter between these two cells (Cottrell, 1970b). Transmission between these two cells is impaired by reserpine and blocked by LSD-25 (Cottrell, 1970a).

1.38 5-HT as a Transmitter in Invertebrates

There is reasonable evidence that 5-HT serves as a transmitter in many invertebrate species.

a. Central Molluscan Neurones

5-HT has been shown to have an excitatory effect on neurones in molluscan central ganglia (Kerkut and Walker, 1961, 1962; Gerschenfeld and Tauc, 1961) Gerschenfeld and Stefani (1965) proposed 5-HT as a transmitter onto CILDA cells in Crypton aspersa, the Argentinian land snail, with the following reasons:

1. Iontophoresed 5-HT affected specific cells.
2. 5-HT specifically increased membrane conductance in these cells.
3. The action of 5-HT was blocked by LSD and BOL.
4. Repeated applications of 5-HT caused tachyphylaxis (this was similar to that shown for ACh on motor end plates).
5. 5-HT had earlier been shown to be present in this ganglia.
6. Synthesising and degradation mechanisms had also been reported by other workers in molluscs.
7. The 5-HT receptors on these cells were located on the axon hillocks and the ACh receptors were located all over the soma and axon hillock.
8. There was no cross desensitisation between the two types of receptor (Gerschenfeld and Stefani, 1966).
9. The pharmacology of the two receptors was different (Stefani and Gerschenfeld, 1969).

The evidence for 5-HT as the chemical transmitter responsible for the slow excitatory post-synaptic potentials of these CILDA neurones has been reviewed by Gerschenfeld and Stefani (1968). They have
proposed diffusion as a possible mechanism of inactivation of 5-HT.

An elegant study of a giant serotonin cell (GSC) in the metacerebral ganglion of *Helix pomatia* and similar cells in the corresponding ganglia of *Helix aspersa* and the slug *Limax maximus* has been made by various workers. The input organisation of the cell has been determined by Kandel and Tauc (1966). The GSC makes contact with a non-serotonin containing cell in the buccal ganglion. The evidence that 5-HT is the transmitter between these two cells is good (Cottrell, 1970b):

1. Fluorescence microscopy and microspectrometry work indicated that these cells contained 5-HT (Cottrell and Osborne, 1970).
2. The presence of 5-HT in these cells has been confirmed by Briel, Neuhoff and Osborne (1971) using microchromatography of dansylated compounds (They have also noted that in cells dissected with the aid of Methylene blue staining they were unable to demonstrate 5-HT).
3. Formation of 5-HT from 5-HTP in these cells has been demonstrated by Cottrell and Powell (1971) and Osborne (1971).
4. Transmission between the two cells is impaired by reserpine and LSD (Cottrell, 1970a).
5. Directly applied 5-HT produces a decrease in the membrane resistance.
6. Imipramine inhibits the uptake of 5-HT into the molluscan brain by 80%. After treatment with imipramine transmission between the GSC and the buccal cell is facilitated. The mechanism is thought to be an increased rate of depolarisation (Cottrell, 1971).

b. 5-HT as an Excitatory Transmitter on the Molluscan Heart

The cardio-regulatory nerves of *Venus* were thought to contain inhibitory and excitatory nerve fibres. Stimulation of the nerve produced an inhibition which was shown to be mediated by ACh. Welsh (1953) found that low concentrations of 5-HT applied to *Venus* heart caused an excitation. He suggested that 5-HT might be the normal mediator of excitation. The presence of 5-HT in the ganglia of *Venus*
was demonstrated by Welsh (1957).

Loveland (1963) used the ACh antagonist benzoquinonium to separate the inhibitory and excitatory effects of stimulation. When benzoquinonium-treated preparations were stimulated there was an excitatory response. If these preparations were made tachyphylactic to 5-HT then nerve stimulation failed to cause excitation. Pretreatment with reserpine abolishes the excitatory effect of stimulation. Reserpine has been shown to lower the concentration of 5-HT in Venus ganglia.

Excitation due to nerve stimulation was blocked by methysergide (Wright et al, 1962) which also blocked the excitation due to 5-HT.

These observations provide good evidence that 5-HT is the normal mediator of excitation in Venus heart (see also Welsh, 1971). 5-HT is probably the excitatory transmitter in other invertebrate hearts. Release of 5-HT after cardiac nerve stimulation has been shown in Helix heart (S-Rozsa and Perenyi, 1966) and in Aplysia (Chase et al, 1968).

1.39 Dopamine as a Transmitter in Molluscan Central Neurones

The possibility of a transmitter role for DA in molluscs is supported by many workers. These views have been discussed by Tauc (1967) and include the following points:

1. DA has been shown to be present in the neurones of many molluscan species.

2. The CNS of Helix aspersa has been shown to convert Dopa to DA (Cardot, 1963). Injection of Dopa increases DA concentration in Helix aspersa ganglia (Kerkut, Sedden and Walker, 1966).

3. DA modifies neuronal activity in some molluscan neurones. It is usually more potent than NA.

4. Labelled Dopa is taken up by neurones in Helix and Aplysia.

5. Dense-core vesicles similar to those shown to contain catecholamines in vertebrates have been observed in molluscan presynaptic structures.
Walker, Ralph, Woodruff and Kerkut (1971) have presented evidence for an inhibitory post-synaptic potential (IPSP) in the brain of Helix aspersa being caused by presynaptic release of DA. The evidence included:

1. Ergometrine, a specific DA antagonist, reversibly antagonised the IPSP and the DA response.
2. Prolonged application of DA reduced both the IPSP and the DA response.
3. There was an increase in the permeability of the postsynaptic membrane to K⁺ ions associated with both the IPSP and the DA response.
4. Pretreatment with reserpine reduced the height of the IPSP.
5. Pretreatment with Dopa increased the height of the IPSP.

The properties of the DA receptors in the snail have been reviewed by Woodruff (1971).

1.40 The Functions of Monoamine Neurones in Invertebrates

In the lower groups of invertebrates, the anatomy of the catecholamine containing structures gives the main clue as to their functions.

a. Sponges

Catecholamines and 5-HT were found in multipolar and bipolar cells that could be neurones.

b. Coelenterates

Catecholamine containing fibres form a network in the tentacles and these fibres, which are denser at the tip, are thought to have a sensory function. 5-HT has been demonstrated in Hydra in various cells and in the nematocysts.

c. Platyhelminthes

Four pairs of primary catecholamine containing cells were found in the brain of a planarian. There were also fine fluorescent fibres in the neuropile. A sensory function has been suggested for these
catecholamine containing neurones.

5-HT cells have been reported in planaria cerebral ganglia and ventral nerve cord. Processes containing 5-HT were found that penetrated the epidermis and these are thought to have a sensory function.

d. Echinoderms

There was very little 5-HT present. In most species the predominant catecholamine was DA but significant quantities of NA were also present. An interneurone function has been suggested for these catecholamine containing neurones. A few catecholamine cell bodies were found in epithelial tissue surrounding the radial nerve. The ectoneural tissue of the radial nerve contained all the fluorescent fibres. It is thought that the catecholamine cells are most likely to be interneurones as no fluorescence is found at muscle innervation or in sensory nerves.

e. Annelids

There are considerable numbers of 5-HT containing cells in the nervous systems of the annelids. A motor function has been suggested for these cells. In the case of some cells there is evidence that they are inhibitory, for example the Retzius cells in the leech ganglia.

Many catecholamine containing cells have been found in the body wall of Lumbricus terrestris with axons running into the ventral nerve, it seems that these cells have a sensory function. Other catecholamine containing neurones have had an interneurone function suggested for them.

f. Arthropods

(i) Crustacea Very little 5-HT has been found in crustacea, although two large yellow fluorescent cells have been found in the brain of crayfish.

Catecholamine neurones appear to innervate the intestine in some crustacean species, e.g. the lobster Homarus vulgaris where catecholamine neurones are found in the stomatogastric ganglion and appear to innervate the stomach muscles. Catecholamine fluorescence was also
associated with the visual pathways of several crustacean species.

(ii) Insecta

Very little 5-HT has been found in the nervous systems of insects that have been tested. DA is the predominant catecholamine present in the nervous system of the insect species examined. Several recognisable groups of fluorescent cells have been demonstrated in the brain of the cockroach. These cells have a variable sensitivity to reserpine.

g. Molluscs

5-HT containing cells have been demonstrated in ganglia of various molluscan species. They are generally thought to have a motor function. In species where the excitatory transmitter onto the heart has been shown to be 5-HT it is probable that some 5-HT neurones in the central ganglia send axons to the heart.

DA is the predominant catecholamine in most molluscan ganglia but NA is also present in small amounts in some species. In molluscs the catecholaminergic neurones are thought to have a sensory or interneurone function.

1.41 The Aim of the Present Study

It can be seen from section B of this introduction that much information has been published on the localisation of monoamine containing neurones in invertebrates but only in very few cases has the pharmacology of the neurones been determined. It was the aim, therefore, of the present study to locate the monoamine containing neurones in the CNS of the pulmonate Helix aspersa and then to determine the responses of these neurones to drugs added to the bath and to stimulation through the nerve trunks. The pharmacology of some monoamine containing neurones in the central ganglia of another pulmonate, Planorbis corneus, were also determined.

The characteristics of the monoamine containing cells determined
in this study could then be compared with the characteristics of monoamine containing cells that have been reported in the literature for common features.
CHAPTER 2

THE LOCALISATION OF DOPAMINE AND 5-HYDROXYTRYPTAMINE IN THE BRAIN OF HELIX ASPERELA

2.1 Plan of Experiments

The fluorescence histochemical technique for the localisation of monoamines was applied to the circumoesophageal ganglionic mass of Helix aspersa with the following aims:

1. To localise formaldehyde-induced fluorescence in the ganglia and to distinguish it from any non-specific fluorescence present.
2. To differentiate between the 5-HT and the catecholamine fluorophores.
3. To determine the positions of single neurones and groups of neurones that were recognisable from one preparation to another. Of particular interest were large cells on the surface of the ganglia that would be:

   (i) identifiable in a whole brain
   (ii) suitable for making intracellular recordings.

2.2 Methods used for Examining the Structure of Helix Brain

To make the location and the identification of the fluorescent cells easier, the structure of the snail brain was studied in some detail. The methods employed to accomplish this were as follows:

1. Direct observation of the dissected brain.
2. Macrophotography of the dissected brain (for example, figure 2.1).
3. Examination of haemotoxylin and eosin stained sections. Figure 2.2 shows a transverse section through the suboesophageal ganglia of Helix.
4. Reconstruction from serial sections.
5. The construction of a scale model to which the identified nerve cell bodies could be added at a later date. Figures 2.3, 2.4 and 2.5.
Figure 2.1

A. is a photograph of the dorsal surface of the suboesophageal ganglia of *Helix aspersa* after the connective tissue had been removed. A microelectrode can be seen in one of the neurones.

B. is an outline drawing of the photograph in A. D and F are the left and right parietal ganglia, E is the visceral ganglion, 3 is the intestinal nerve and 4 is the right pallial nerve.
Figure 2.2

A. Is a photograph of a transverse section through the suboesophageal ganglia of *Helix aspersa*, stained with haematoxylin and eosin.

B. Shows a diagram of the photograph in A, where C and G are the pleural ganglia, D and F are the left and right parietal ganglia, E is the visceral ganglion and H and I are the pedal ganglia. S is a statocyst and np is neuropile.
Figure 2.3

Photograph of the scale model of the brain of Helix aspersa. Some of the neurones in the brain are represented by beads. See figure 2.4 for a labelled drawing of this photograph.
Figure 2.4
An outline drawing of the model Helix brain (see figure 2.3).

Ganglia: A and B, cerebral ganglia; C and G, left and right pleural ganglia; D and F, left and right parietal ganglia; E, visceral ganglion; H and I, pedal ganglia.

Nerves: 1. left pallial nerve; 2. anal nerve; 3. intestinal nerve; 4. right pallial nerve.
2.3 Structure of the Snail Brain

The brain of *Helix aspersa* is made up of nine ganglia. Two supra-oesophageal or cerebral ganglia and seven suboesophageal ganglia. The supra- and suboesophageal ganglia are connected by two pairs of commissures. Figure 2.4 shows a drawing of the model snail brain that had been constructed from plasticine. The ganglia are labelled A-I and the four main nerve trunks are numbered 1-4. A and B are left and right cerebral ganglia, C and G are the left and right pleural ganglia, D and F are the left and right parietal ganglia, E is the visceral ganglion and H and I are the pedal ganglia. The four main nerve trunks, numbered 1-4, are 1 and 4 the left and right pallial nerves, 2 is the anal nerve and 3 is the intestinal nerve.

2.4 Cerebral Ganglia

The cerebral ganglia are symmetrical in *Helix* and each can be divided into three regions: The proto-, the meso- and the meta-cerebrum. The metacerebrum is further subdivided three lobes: the pedal, the pleural and commissure lobes. The metacerebrum has a large sensory input and is considered to be a region of integration.

2.5 The Commissures

There are four commissures joining the supra-oesophageal and the suboesophageal ganglia. These are the two cerebro-pedal commissures and the two cerebro-pleural commissures.

2.6 The Suboesophageal Ganglia

The arrangement of these ganglia is shown in a photograph of the plasticine model of the *Helix* brain, figure 2.3. There are seven ganglia lying below the oesophagus. Lying ventrally are a pair of symmetrical pedal ganglia above which are the right and left parietal
Figure 2.5

A. Photograph of the model Helix brain viewed from the right hand side.

B. A diagram of the photograph in (A).

C. Photograph of the model Helix brain viewed from the posterior end.

D. A diagram of the photograph in (C).

Ganglia: D and F are the left and right parietal ganglia; E is the visceral ganglion; H and I are the left and right pedal ganglia.

Nerves: 1 and 4 are the left and right pallial nerves; 2 is the anal nerve; 3 is the intestinal nerve.
ganglia. This arrangement can be seen in figure 2.5. Between the parietal ganglia and partly fused to the left parietal ganglion is the visceral ganglion. Anterior to the parietal ganglia are the two smaller pleural ganglia. The pleural and the pedal ganglia are connected to the cerebral ganglia by two pairs of commissures. There are also connections between the pleural and the pedal ganglia. There is a connective tissue sheath surrounding the entire ganglionic mass.

2.7 The Nerve Supply

There are several nerves leaving the cerebral ganglia. They make connections with the eyes, the olfactory organs, the buccal ganglia, the penis and the retractor muscle. Three pairs of nerves run from the pleural ganglia to the columella muscles and pharyngeal retractor muscle. From the parietal ganglia three pairs of nerves pass to the musculature of the body wall and to the mantle. The main nerve trunks leaving these ganglia are the left and right pallial nerves, they are labelled 1 and 4 in figure 2.4. Two large nerve trunks leave the visceral ganglion, the anal and the intestinal nerves (labelled 2 and 3 in figure 2.4). These nerves supply the heart, the viscera and the reproductive apparatus. Ten pairs of nerves run from the pedal ganglia to the muscles of the foot.

2.8 Introduction to Fluorescence Histochemistry

Eranko (1955a) observed a fluorescence in certain cell groups of the adrenal medulla that had been fixed in formaldehyde. This fluorescence was not present in tissue that had not been treated with formaldehyde. Analysis of these cells using chemical techniques showed that the fluorescent cells contained NA. NA and formaldehyde were shown to produce a highly fluorescent compound and so Eranko (1955b) proposed treatment with formaldehyde as a histochemical method for the localization of NA.
One of the major problems to be overcome was diffusion of the amine away from its storage site. NA in the adrenals was detected despite this problem because of the large concentrations present.

Freezing the tissue stops the diffusion of chemicals away from their storage sites and also prevents or reduces metabolism by enzymes. It is possible to dry tissue whilst keeping it frozen by holding it in a vacuum. If the tissue is held below the triple point for water then any ice present will sublime. Various apparatus has been devised to 'freeze-dry' tissue in this way.

Once the tissue had been dried it was necessary to react the amines with formaldehyde without allowing a subsequent diffusion away from their storage sites. To do this the tissue was exposed to formaldehyde in the vapour state. When paraformaldehyde is heated it depolymerises to produce gaseous formaldehyde.

It was independently observed by several workers (Eräkko, 1961, unpublished; Falck and Torp, 1961; Lagunoff et al, 1961) that aromatic monoamines in freeze-dried tissue exposed to formaldehyde vapour became intensely fluorescent.

For some time demonstrations of monoamines in the nervous system proved elusive. The first workers to report a successful demonstration of catecholamines in nervous tissue were Falck, Hillarp and their associates (Carlsson et al, 1962; Falck, 1962; Falck et al, 1962).

This method enabled possible transmitter substances to be localised in nervous tissue, providing valuable information on their physiological roles. For a review of the methodology see Corrodi and Jonsson (1967).

2.9 Outline of the Method

The tissue to be studied is rapidly frozen in precooled isopentane or directly in liquid nitrogen after being coated with talc. The tissue
Reactions of Monoamines with Formaldehyde

Catecholamines

\[
\text{Dopamine } R = \text{H} \\
\text{Noradrenaline } R = \text{OH}
\]

\[
\begin{align*}
\text{HCHO} & \quad \text{Protein} \\
\text{6,7 dihydroxy 1,2,3,4 tetrahydro isoquinoline} & \quad \text{6,7 Dihydroxy 3,4 dihydroisoquinoline}
\end{align*}
\]

Indole amines

\[
\begin{align*}
\text{5 Hydroxytryptamine} & \quad \text{HCHO} \\
\text{β Carboline} & \quad \text{Protein}
\end{align*}
\]

\[
\begin{align*}
\text{3,4 Dihydro β Carboline} & \\
\text{Quinoidal form}
\end{align*}
\]

Figure 2.6

The reaction of monoamines with formaldehyde vapour in the presence of dry protein.
is then dried under vacuum whilst still frozen. In this way the mono-
amines are held at their storage sites. The dried tissue is then exposed
to formaldehyde vapour at +80°C for 1-3 hours. The water content of the
paraformaldehyde is controlled to give the maximum possible reaction
which does not allow diffusion of the product away from the storage site
(Hamberger et al, 1965). The monoamines condense with the formaldehyde
in the manner described in section 2.10 to give highly fluorescent
products. The treated tissue is then embedded in wax, sectioned and
examined under a fluorescence microscope (Falck, 1962; Falck and Owman,
1965).

2.10 The Reaction

Using model systems where the tissue protein is replaced by a
suitable amino acid or albumen solution the conditions of the reaction
and the structural requirements of the compounds have been analysed
(Falck et al, 1962; Ritzen, 1966; 1967).

a. Catecholamines

Primary or secondary amines with hydroxyl groups in the 3
and 4 positions are required for intense fluorescence (with the exception
of α-methyl dopa).

The first step in the reaction is a condensation between form-
aldehyde and the catecholamine to form a 6,7-dihydroxy 1,2,3,4-tetra-
hydroisoquinoline (See figure 2.6). These compounds are non-fluorescent.
This reaction will take place in solution and requires only relatively
mild conditions (+20°C). The second step is a protein catalysed
dehydrogenation reaction to form the corresponding 6,7-dihydroxy
3,4-dihydroisoquinoline. The isoquinolines are in a pH dependent
equilibrium with the quinoidal form. Both forms are intensely fluorescent
in the solid state at pH 7-8. In solution or at acid pH the intensity
of their fluorescence is reduced.
b. **Indoleamines**

The 5 (or 6) hydroxy (or methoxy) tryptamines give rise to a highly fluorescent compound after exposure to formaldehyde. The reaction is outlined in figure 2.6. The 5-HT and 5-HTP fluorophores are indistinguishable.

The first reaction is a condensation between the indole amine and formaldehyde to produce 6-hydroxy-1,2,3,4-tetrahydro-β-carboline which then undergoes a dehydrogenation to form the fluorescent compound 6-hydroxy-3,4-dihydro-β-carboline.

2.11 **The Fluorophores**

Both fluorophores absorb light in the ultra violet (UV) region of the spectrum and emit light in the visible region. The two principal excitation peaks for the CA fluorophore are at 320 and 410 nm. The principal excitation peaks for the 5-HT (5-HTP) fluorophore are 385 and 415 nm. It is the 410 and the 415 nm absorption peaks that are made use of as they most nearly correspond to a line in the emission spectra of the mercury lamp used in the fluorescence microscope.

The emission characteristics of the two fluorophores are different and this difference can be used to distinguish between the two. The catecholamine fluorophore has an emission peak at 480nm and the 5-HT (5-HTP) fluorophore has a peak at 520-530nm (Caspersson et al, 1966).

Methods of distinguishing between the 5-HT and catecholamine fluorophores (section 2.24), primary and secondary catecholamines (section 2.25) and between NA and DA (section 2.26) will be discussed in the sections indicated.

The apparent colour of the catecholamine fluorophore has been reported by different workers as blue or green and yellow when present in high concentrations. The 5-HT fluorophore is almost invariably reported as appearing yellow.
Figure 2.7
Diagrammatic outline of the histochemical treatment of tissue for the cellular localisation of monoamines.
METHODS

2.12 The Preparation and its Dissection

For this study the circumoesophageal ganglia of the common garden snail *Helix aspersa* was used. Specimens were collected locally or obtained from a dealer and kept in a room at 10°C or at room temperature. This temperature did not appear to have any effect on the later fluorescence of the brain. The animals tended to go into a state of hibernation whether kept at room temperature (20°C) or at 10°C.

To remove the central ganglia from *Helix* the shell was removed by means of coarse forceps. The foot of the snail was then made to extend by squeezing the body. This was easier if the covering membrane had not been damaged when removing the shell. The extended foot was then pinned to a wax block. A cut was made through the dorsal skin of the foot from anterior to posterior and the skin pinned back. This exposed the gut and the reproductive apparatus which were carefully removed. With the tissue cleared away from the ganglia it was possible to trace the paths of the nerves away from the brain. Nerves required for the experiment were traced back as far as possible before cutting. The remaining nerves were cut, the ganglia gently lifted up (usually with curved forceps between the supra- and suboesophageal ganglia) and any remaining tissue was freed.

The histochemical procedure has been described in detail by Marsden and Kerkut (1969a) and is represented diagrammatically in figure 2.7.

2.13 Freezing the Tissue

a. Tissue for Sections

The isolated tissue was rapidly frozen in isopentane that had been cooled in liquid nitrogen. Rapid freezing of the tissue was essential to prevent the growth of large ice crystals which can cause
damage within the tissue. The use of isopentane allows a more rapid quenching of the tissue than can be achieved with liquid nitrogen alone. An alternative to isopentane is to coat the surface of the tissue in talc (H\textsubscript{2}Mg\textsubscript{2}(SiO\textsubscript{3})\textsubscript{4}) before immersing in liquid nitrogen.

b. Tissue for Whole Mounts

The outer connective tissue over the surface of the ganglia was removed using fine pointed forceps. The ganglia were then orientated on a piece of paper before being frozen in the same way as in paragraph a.

2.14 The Tissue Dryer

An Edwards-Pearse tissue dryer was used throughout this work. The drying chamber of the freeze-dryer consisted of a water-cooled baseplate covered by a Pyrex glass cover of the type used on dessicators. An 'O' ring between the baseplate and the cover sealed the chamber when it was under vacuum. Mounted centrally on the baseplate was the specimen carrier. The temperature of the specimen carrier was controlled by means of two thermoelectric elements supplied by a low voltage D.C. current from a variable transformer. A thermocouple was used to measure the temperature of the carrier.

Small trays containing fresh phosphorus pentoxide to act as a dessicant were placed on the baseplate surrounding the specimen carrier. The chamber was evacuated by means of a 'Speedivac' two stage gas ballast rotary pump and the pressure measured using a Pirani gauge head type M6A and a Pirani gauge meter. Pressures between 0.5 torr and 0.001 torr could be measured (1 torr = 1 mm Hg).

2.15 Freeze-drying

The principle of freeze-drying is to hold the tissue below the triple point of water. Under these conditions water exists in either
the solid or the vapour state. If the pressure is low enough then ice in the tissue will sublime. The water vapour formed is removed from the atmosphere of the chamber by a dessicant.

The dissicant trays were filled with fresh $P_2O_5$ and the specimen carrier was cooled to $-40^\circ C$. The frozen tissue was placed onto the specimen carrier and the chamber evacuated. The pressure usually attained was just below 0.01 torr. The tissue was kept at $-40^\circ C$ and 0.01 torr until drying was complete.

**Typical Tissue Drying Times**

<table>
<thead>
<tr>
<th>Thickness</th>
<th>Temperature</th>
<th>Time</th>
</tr>
</thead>
<tbody>
<tr>
<td>2 mm</td>
<td>$-20^\circ C$</td>
<td>2-3 hours</td>
</tr>
<tr>
<td>2 mm</td>
<td>$-40^\circ C$</td>
<td>5 hours</td>
</tr>
<tr>
<td>5 mm</td>
<td>$-20^\circ C$</td>
<td>10 hours</td>
</tr>
<tr>
<td>5 mm</td>
<td>$-40^\circ C$</td>
<td>20 hours</td>
</tr>
</tbody>
</table>

In this work the tissue was left overnight and for up to 45 hours. At the end of the drying time the tissue carrier was allowed to come slowly to room temperature and was then heated to $+40^\circ C$ and held at this temperature for 15 minutes. Warming the tissue in this way prevented moisture from the atmosphere condensing onto the surface of the tissue when it was removed from the freeze-dryer.

2.16 **Histochemical Treatment**

The amount of water contained in the paraformaldehyde was critical for the success of the reaction. The higher the relative humidity of the paraformaldehyde the stronger the reaction and also the greater the diffusion of the compounds away from their true positions. An optimum relative humidity had to be found that allowed the strongest possible reaction with little or no diffusion. The best humidity for each type of tissue was determined by trial and error.
Table 2.1  Sulphuric Acid Solutions for the Control of Humidity*

*Modified from Solomon (1952)

<table>
<thead>
<tr>
<th>R.H. % at 25°C</th>
<th>Weight % (g H₂SO₄/100 g soln.)</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>0</td>
<td>0.999</td>
</tr>
<tr>
<td>90</td>
<td>17.91</td>
<td>1.126</td>
</tr>
<tr>
<td>80</td>
<td>26.79</td>
<td>1.196</td>
</tr>
<tr>
<td>70</td>
<td>33.09</td>
<td>1.248</td>
</tr>
<tr>
<td>60</td>
<td>38.35</td>
<td>1.292</td>
</tr>
<tr>
<td>50</td>
<td>43.10</td>
<td>1.334</td>
</tr>
<tr>
<td>40</td>
<td>47.71</td>
<td>1.377</td>
</tr>
<tr>
<td>30</td>
<td>52.45</td>
<td>1.423</td>
</tr>
<tr>
<td>20</td>
<td>57.76</td>
<td>1.478</td>
</tr>
<tr>
<td>10</td>
<td>64.45</td>
<td>1.551</td>
</tr>
</tbody>
</table>

Table 2.2  Relative Humidities and Ratio of Stock Solution to Water

<table>
<thead>
<tr>
<th>R.H. %</th>
<th>WATER</th>
<th>STOCK SOLN.</th>
</tr>
</thead>
<tbody>
<tr>
<td>30</td>
<td>0.324</td>
<td>1.0</td>
</tr>
<tr>
<td>40</td>
<td>0.509</td>
<td>1.0</td>
</tr>
<tr>
<td>50</td>
<td>0.729</td>
<td>1.0</td>
</tr>
<tr>
<td>60</td>
<td>1.010</td>
<td>1.0</td>
</tr>
<tr>
<td>70</td>
<td>1.415</td>
<td>1.0</td>
</tr>
<tr>
<td>80</td>
<td>2.11</td>
<td>1.0</td>
</tr>
</tbody>
</table>
2.17 Preparation of Paraformaldehyde to a Constant Relative Humidity

The relative humidity of the paraformaldehyde was controlled by storing dried paraformaldehyde over sulphuric acid of various concentrations in a sealed container. The ratio of water to sulphuric acid required to produce a specific relative humidity was determined from the relationship between the relative humidity, the weight % (g sulphuric acid/100 g solution) and the density of the acid at that relative humidity.

For this work it was not necessary to know the exact relative humidity of the solution and so the values for the specific gravity and the weight % of the concentrated acid could be taken from the label on the bottle. A stock solution was made up of one part water to one part concentrated acid. This avoided excessive heat production at subsequent diluting operations.

The weight % of this stock solution was:

\[
\frac{1.84 \times 98}{1.84 + 1} = 63.49 \text{ weight } \% 
\]

where 1.84 was the specific gravity and 98% was the weight % of the concentrated acid.

Using a table compiled by Solomon (1952) the density corresponding to this weight % was read off (see table 2.1). For this case the density of the stock solution was 1.54. To calculate the ratio of stock solution to water for a particular humidity, for example 70%, the corresponding weight % was read off table 2.1. For 70% it was 33.09%.

The required volume was:

\[
1 \text{ part stock solution : } V \text{ parts distilled water}
\]

where

\[
V = d \left( \frac{s}{A} - 1 \right)
\]

and

\[
d = \text{density} = 1.54
\]

\[
s = \text{weight } \% \text{ stock solution} = 63.49
\]

\[
A = \text{weight } \% \text{ solution required} = 33.09
\]
therefore \[ V = 1.54 \left( \frac{63.9}{33.09} - 1 \right) \]

\[ V = 1.415 \]

Thus: 1 ml stock solution : 1.415 ml water will give a solution of relative humidity equal to 70%. For this work 15 ml of stock solution were used so 21.23 ml distilled water were added to produce a relative humidity of 70%.

The paraformaldehyde was stored over phosphorus pentoxide for about 1 week to dry it thoroughly. The dry paraformaldehyde was then stored at the required humidity for about 1 week before it was used. A range of humidities was prepared (60, 70, 80 and 90%). A suitable container was made from a polythene food container (Marsden, 1970). The sulphuric acid was contained in a 50-100 ml beaker in the bottom and a wire gauze shelf was placed half-way up to support a Petri dish containing the paraformaldehyde.

2.18 Exposure of Tissue to Formaldehyde Vapour

The dried tissue was taken from the freeze-dryer and placed in small bags made from a circle of fine net or muslin and drawn up with cotton. The bag containing the tissue was then hung inside a sealed glass jar containing 3-5 g of paraformaldehyde at the required humidity. The volume of the jar used was about 200 ml. The jar plus the tissue was then heated at +80°C for 1-3 hours.

A reaction temperature of +80°C was used routinely although the primary catecholamines will react at +60°C and develop maximum fluorescence within 1 hour. Secondary catecholamines require higher energy for the dehydrogenation and so for adrenaline 3 hours at +80°C would be necessary for maximum fluorescence. The time taken for 5-HT fluorescence to develop is reported to be 1 hour by some workers (Falck and Ovman, 1965) but 3 hours by other workers.
To increase the intensity of the 5-HT fluorescence the tissue was first exposed to paraformaldehyde at 70\% relative humidity, for 1-2 hours and then at 90\%, relative humidity, for the remainder of the reaction time. Control tissues were heated in the presence of P_2O_5 for the same times as the experimental tissues.

2.19 Preparation of Sections

a. Embedding

After exposure to formaldehyde the tissues that were to be sectioned were impregnated with degassed wax (M.P. 56°C) under vacuum on the freeze-dryer at +60°C.

The wax was heated in small specimen holders on the thermomodule of the freeze-dryer to just above its melting point. The chamber was then evacuated and any gas dissolved in the wax bubbled out. When bubbling had ceased the thermomodule was allowed to cool and the wax hardened whilst still under vacuum. Small holes were made in the hard wax and the tissue was placed in these holes. The chamber was re-evacuated and allowed to equilibrate for 10 minutes after which time the temperature was raised to just above the melting point of the wax. The tissue was left in the molten wax under vacuum for 10-15 minutes before being removed and embedded in fresh wax.

b. Sectioning

The tissue was serially sectioned using a Cambridge Rocking Microtome. Sections were normally 10μ thick. Water or aqueous solutions could not be used to flatten the sections as the fluorescence is quenched by moisture. Several alternative methods can be employed to flatten the sections:

(i) Liquid paraffin (Falck and Owman, 1965)

(ii) Acetonitrile (Sweeney, 1968)

(iii) Heating.
The heating method was normally used and was found to be satisfactory. For this method the sections were laid onto a clean glass slide and gently flattened with a camel hair brush, the slide was then placed onto a hotplate just above the temperature at which the wax melts. As the wax melts the sections stretch and flatten. Heating for excess periods of time was avoided as this can cause an increase in the autofluorescence in the sections. The slides were cooled rapidly by placing them on cold metal or glass.

Routinely the slides were examined under the fluorescence microscope without a coverslip. For high power examination the sections were mounted in Fluormount (Gurr's).

2.20 Preparation of Whole Mounts

After the tissues had been exposed to formaldehyde vapour they were cleared in liquid paraffin under vacuum on the freeze-dryer at room temperature for about 15-30 minutes. They were then viewed under the fluorescence microscope using epi-illumination.

2.21 The Fluorescence Microscope

A Zeiss Photomicroscope II was used for the examination and photography of sections by transmitted light. The light source for this microscope was a separate high pressure mercury lamp. A mirror, silvered on the outside, was used to shine the light up through the condenser. This method of illumination gave a more intense light than the attached lamp system of the Zeiss large fluorescence microscope. For whole mounts it was necessary to use epi-illumination. The large Zeiss fluorescence microscope was used for this. The excitation
Figure 2.8 Transmission characteristics of the excitation and barrier filters.

A. Transmission of the excitation filters; B. Transmission of the barrier filters.
light could be directed down the objective and onto the surface of the preparation. Reflected and emitted light passed up through the objective and to the eyepieces in the normal manner.

The mercury vapour lamp used with the microscope had an energy distribution that corresponded to that of the mercury vapour spectrum. The catecholamine fluorophores have excitation maxima at 320 and 410 nm and the 5-HT fluorophore has excitation maxima at 385 and 415 nm. The line at 405 nm in the mercury spectrum is mainly used.

a. Excitation Filters

A heat absorbing filter was inserted immediately after the lamp to protect the other filters against excessive heat. A minus red filter BG 38 was also always in position. Exciter filters BG 12, BG 3, UG 1 and UG 5 were available. The characteristics of these filters are shown in figure 2.8. The purpose of the excitation filters was to remove the visible light and the UV light with shorter wavelengths than required.

b. Barrier Filters

These filters are 'cut off' filters and they filter out light of a shorter wavelength than their specification. For example barrier filter 50 only allows light with a wavelength longer than 500 nm to pass through. Five barrier filters with values between 41 and 53 were available.

The function of these filters is to remove the UV light that has passed through the specimen. This could damage the eyes. They also remove light from the blue-violet end of the spectrum which interferes with the fluorescence. The characteristics of these filters are shown in figure 2.8.

2.22 Photography

Using the photomicroscope it was possible either to use the
automatic exposure system when taking a photograph or to operate a shutter manually. The manual shutter had the advantage that all the light fell onto the film whereas for the automatic system the light was split up and only a part fell onto the film. This was a disadvantage for fluorescence studies as a certain amount of fading occurred on exposure to UV light.

Photographs of the whole mounts were taken on the Zeiss large fluorescence microscope. The body of a single lens reflex camera was fitted onto the upright tube of a triocular head.

For black and white photography Kodak Tri X pan film (ASA 400) was used. Exposure times varied between 5 and 180 seconds. The film was developed in D.76 for 10 minutes and fixed in F.5 for 5-10 minutes. Prints were made on Kodak bromide paper no. 2, 3, or 4 using paper developer D.163 diluted to 25% with water. The paper was fixed for 10-15 minutes in F.5.

It was difficult to obtain a true colour representation in colour transparencies. Kodachrome II colour film was found to be unsuitable for the blue fluorescence. Kodak High Speed Ektachrome (ASA 160) gave the best representation of colour. Exposure time for this film varied between 2 and 4 minutes.

2.23 Differentiation of Specific and Autofluorescence

Not all the fluorescence in the tissues was specific fluorescence formed by the reaction between formaldehyde and a monoamine. It was necessary to distinguish between the specific monoamine fluorescence and the autofluorescence. To do this several methods can be employed. A combination of the tests was usually used.

a. Control Tissues

Fluorescence in tissues that were heated to +80°C for the same time as the experimental tissue but in the absence of formaldehyde
vapour, was assumed to be autofluorescence.

b. Colour

The autofluorescence was often a different colour from the specific fluorescence. Some autofluorescence is bright red or orange.

c. Photodecomposition

Specific fluorescence was usually less stable to UV light than the autofluorescence. This was particularly true for the 5-HT fluorophore.

d. Pharmacology

Pretreatment of the animals with monoamine precursors e.g. Dopa or 5-HTP increased the levels of specific fluorescence whilst having no effect on the non-specific fluorescence. Other pharmacological criteria have been employed by Sedden (1967). These included the injection of reserpine to deplete the specific fluorescence and the use of MAO inhibitors to increase monoamine fluorescence.

e. Chemical method

A chemical method for distinguishing the two types of fluorescence was originally developed by Corrodi et al (1964) and has been modified by Sedden (1967) and Marsden and Kerkut (1969a).

The principle on which the method is based is that the fluorescent 3,4-dihydro compounds are reduced to the non-fluorescent tetrahydro compounds by sodium borohydride. These compounds can be reconverted to the fluorescent dihydro compounds by a second exposure to formaldehyde. The method used was as follows:

1. The sections were mounted on slides using albumen to help adhesion.
2. The sections were checked for fluorescence.
3. The sections were dewaxed with dry xylene.
4. They were then put through absolute isopropanol and into 80%, 90% or 95% alcohol and extracted for 10 minutes.
5. Following this they were dehydrated again, mounted in xylene and
6. Where the fluorescence had resisted the 80 or 90% alcohol adjacent sections were treated with 80 or 90% isopropanol containing 0.03% and 0.01% sodium borohydride for 2-3 minutes. The sections were then well rinsed, dehydrated and examined. For the less resistant fluorescence 0.01-0.03 sodium borohydride in 95% isopropanol was tried.

7. The dried sections were re-exposed to formaldehyde vapour at +80°C for 1-3 hours and re-examined.

If the fluorescence was absent after the borohydride treatment but reappeared after exposure to formaldehyde it was considered specific. Fluorescence that disappeared on treatment with borohydride but did not return after further formaldehyde treatment may be specific or non-specific.

2.24 Differentiation between Catecholamine and 5-HT Fluorescence

The colour of the 5-HT fluorophore has been reported to be yellow and that of the catecholamines has been reported to be green (see for example Caspersson et al., 1966). At the beginning of this work difficulty was found in distinguishing between the two fluorophores. All the specific fluorescence in the tissues appeared yellow. The filter system that was used was that described by Marsden and Kerkut (1969a).

The light from the Hg lamp passed through 2 filters, a minus red filter BG 38 and an exciter filter BG 12. The light from this set of filters had a peak at 405 nm. The two barrier filters used were 47 and 50. This combination of filters will be called filter system 'A'.

Earlier chemical analysis of the snail brain using a fluorimetric assay (Ansell and Beeson, 1968) had indicated that there were approximately equal quantities of 5-HT and DA present in the ganglia of Helix, 5.34 µg 5-HT/g wet weight tissue and 5.01 µg DA/g wet weight tissue.
It was therefore expected to find two types of fluorescence present in
the formaldehyde treated ganglia.

Various approaches were tried in order to determine the identity
of the specific fluorescence.

a. Model Systems

Model systems were used to study the characteristics of the
5-HT and the DA fluorophores under the conditions used for the tissue
fluorescence. The model spots were prepared by dissolving various
concentrations of 5-HT or DA in a 2% aqueous solution of bovine serum
albumen. The albumen solution provided a protein catalyst for the
dehydrogenation reaction. Solutions of 1, 10, 100 and 1,000 µg/ml
were used. Some spots were prepared containing a mixture of amines.
1 µl quantities of these solutions were spotted onto clean glass slides
and air-dried. The spots were then exposed to formaldehyde vapour at
70% relative humidity and +80°C for 1-3 hours. Controls were prepared
by either omitting the amine from the solution or by omitting the
formaldehyde from the histochemical treatment. The model spots were
examined under the fluorescence microscope using filter system 'A',
described in paragraph 2.24.

b. Measurement of the Fluorescence in the S.P.F.

To investigate the wavelength of the fluorescence in the model
spots they were eluted with 96% isopropanol, acidified with 0.05 N HCl
and both the emission and the excitation wavelengths were scanned on an
Aminco-Bowman spectrophotofluorimeter.

c. The Filter Systems

The use of a barrier filter at 500 nm, as was the case in filter
system 'A', meant that any light below that wavelength would be absorbed.
The emission maximum for the DA fluorescence was 480 nm and that for
the 5-HT fluorescence was 525 nm. The presence of a barrier filter
at 500 nm meant that the real difference of 480-525 nm between the maxima of the two types of fluorophores was reduced to an apparent difference of 500-525 nm, making differentiation between the two difficult. With excitation filters BG 38 and BG 12 the illumination was very intense and a filter at 500 nm was necessary to remove excess UV light before observation. To try and improve the difference between the two fluorophores various combinations of filters were experimented with.

d. Concentration Effect

Caspersson et al (1956) have reported that at high concentrations the catecholamine fluorophore appears yellow instead of green. This was more marked in model systems than in tissue. It is caused by the tendency of the eye to shift intense colour towards the yellow region. The peak of the fluorophore when measured on a spectrophotometer remains unchanged.

Some snails were pretreated with Dopa ½-1½ hours before dissection to increase the concentration of stored DA.

e. Photodecomposition

The 5-HT fluorophore fades more rapidly than the DA fluorophore on exposure to UV light. The model spots and tissue fluorescence were continuously exposed to UV light for periods up to 10 minutes and the effects noted. A method for continuously recording the intensity of the fluorescence is described in paragraph 2.24.

2.25 Differentiation between Primary and Secondary catecholamines

The main difference between the primary and the secondary amines as detected by fluorescence microscopy lies in the time taken for the development of the maximum fluorescence. Adrenaline takes 3 hours at 480°C for maximum fluorescence to develop, whereas noradrenaline and dopamine need only 1 hour. A lower temperature can be used for the
primary amines but $+80^\circ\text{C}$ was used routinely.

2.26 Differentiation between DA and NA

These two amines are not easy to distinguish. A method has been developed in model systems by Corrodi and Jonsson (1965) and modified by Bjorklund, Ehinger and Falck (1968). The method is based on a shift in the excitation spectra of NA from 380 to 330 nm after the fluorophore has been exposed to HCl vapour and thionyl chloride ($\text{SOCl}_2$). The excitation spectra of the DA fluorophore only shifts to 370 nm.

Until recently attempts to detect NA in gastropod brains had been unsuccessful. Osborne and Cottrell (1970a) and A.G. Ramage (Personal communication, 1972) have recently shown that Helix brain contains a small amount of NA (80 ng/g tissue).

In the present work no tests have been carried out to check whether the catecholamine fluorescence could be due to NA. It is likely that most of the catecholamine fluorescence present is due to DA because of the relative quantities of the amines present. The snail brain contains 5.01 µg DA/g tissue compared with 80 ng NA/g tissue. The possibility cannot be ruled out that the fluorescence of a small group of cells or nerve endings could be due to NA.
RESULTS OF THE MONOAMINE FLUORESCENCE IN HELIX ASPEREA BRAIN

2.27 Fluorescent Cells

All the specific fluorescence found within the neurones was confined to the cytoplasm. The nucleus of the cell was always non-fluorescent. Figure 2.9, photograph A shows a cell with fluorescent cytoplasm and non-fluorescent nucleus. The fluorescence in the cytoplasm usually continued down the axon although it was less intense than in the soma. The axon could be traced using this fluorescence as a marker until it merged with axons of a similar fluorescence. Figure 2.9, photograph B shows a group of fluorescent Helix neurones with non-fluorescent nuclei. Photograph C shows a group of fluorescent cells, fluorescent axons can be seen leaving some of the cells and running towards the neuropile. When filter system 'B' was used (see paragraph 2.29) it was possible to follow axons further into the neuropile because the DA fluorescence then appeared blue while the 5-HT fluorescence remained yellow. With this filter system it was possible to distinguish tracts of axons containing one type of fluorescence passing from one ganglion to the next.

2.28 Autofluorescence

Using one or more of the criteria of specific fluorescence that have been listed in paragraph 2.23 it was found that some of the cells in the ganglia contained autofluorescent granules. The autofluorescence was usually of a different colour from the specific fluorescence, see for example figure 2.9 (A). The non-specific fluorescence was readily distinguishable from the specific fluorescence by omitting the formaldehyde from the histochemical treatment. Autofluorescent granules have been indicated in photograph B of figure 2.9.
A. Section through a \textit{Helix} neurone after exposure to formaldehyde, (150\textmu). Monoamine fluorescence can be seen within the cytoplasm. The nucleus is non-fluorescent. The specific fluorescence was yellow and had an emission peak at 530 nm. \((AF = \text{autofluorescence})\)

B. Fluorescent cells with non-fluorescent nuclei in the right parietal and visceral ganglia of \textit{Helix}. \(AF\) indicates the presence of autofluorescent granules in the cytoplasm of a non-fluorescent cell. The large cells are 100\textmu in diameter.

C. A group of fluorescent neurones in \textit{Helix}. Fluorescent axons can be seen running towards the neuropile.
2.29 Colour Difference between 5-HT and Catecholamine Fluorescence

a. Results from model systems

The 5-HT fluorescence in the model spots appeared yellow under UV light with filter system 'A'. The DA spots varied in colour with concentration and were either yellow - green or yellow. The most concentrated DA spots (1 mg/ml) were yellow. When the spots dried they always did so with a ring of concentrated amine around the edge. In the DA spots this ring appeared more yellow than the centre where the amine was less concentrated.

b. Results of SPF studies

The excitation/emission maxima were found to be 415/480 nm for the DA fluorophore and 415/520 nm for the 5-HT fluorophore. The difference in the emission spectra of the two fluorophores was 40 nm. This should easily be distinguishable as a colour difference.

c. Results of filter systems

It was found that by substituting exciter filter UG 5 for the BG 12 filter and still keeping the minus red filter BG 38 in position the intensity of the light was considerably reduced. The barrier filter with its edge at 500 nm (filter 50) could be omitted and filter 47 used on its own. This filter has its edge at 470 nm and so did not interfere with the 480 nm peak of the catecholamine fluorescence.

This filter system i.e. filters UG 5, BG 38 plus barrier filter 44 has been called filter system B. The light that passed through the excitation filters BG 38 and UG 5 had a peak at 380 nm.

(i) Filter system 'A' consisted of excitation filters BG 38 and BG 12 plus barrier filters 47 and 50. With this filter system the model spots containing 5-HT had a yellow fluorescence at all the concentrations tested, (1-1,000 µg/ml). The spots containing DA varied slightly in colour but were generally a yellow-green to yellow colour. Although there was a slight colour difference between the 5-HT and DA
Figure 2.10

A. Photomicrograph of the pedal ganglia taken with Filter system 'A', (see text 2.29). All the specific fluorescence in the neurones and the neuropile is yellow.

B. Photomicrograph of the pedal ganglia taken with Filter system 'B', (see text 2.29). The larger neurones appear yellow but the neuropile and some of the small neurones (BN) appear blue.
spots it was not large enough to make a confident identification of a spot of unknown content and concentration.

Using this filter system the formaldehyde-induced fluorescence found in the tissue sections all had a yellow colour and therefore it was not possible to distinguish between the catecholamine and 5-HT fluorescence. Photograph A in figure 2.10 shows the colour of the specific monoamine fluorescence in a section viewed under filter system 'A'.

(ii) Filter system 'B' was comprised of excitation filters BG 33 and UG 5 plus barrier filters 47 or 44. In the model spots the 5-HT fluorescence appeared to be the same yellow colour as with system 'A'. The DA spot, however, appeared to change from a yellow-green colour to a pale blue colour. Using the new filter system 'B' there was a marked colour difference between the DA and the 5-HT fluorophores and the identity of unknown model spots of various concentrations could now be determined.

The specific fluorescence in the tissue sections had all appeared yellow under filter system 'A'. When the filters were changed to system 'B' some of the fluorescence appeared to change colour from yellow to a blue or blue-green. The rest of the specific fluorescence remained yellow. Some of the blue fluorescence was found in the cytoplasm of small cells but most of this type of fluorescence was located in axons running through the neuropile which had previously appeared a yellow or yellow-green colour. Figure 2.10 shows a photograph (A) of a section taken with filter system 'A' and a photograph (B) of a section taken with filter system 'B', note the colour differences in the neuropile and some of the fluorescent cells. It was possible to trace individual axons into the neuropile for a distance by their difference in colour from neighbouring fibres.
d. Effects of increased concentrations of amines

The model spots always dried with a ring of concentrated amine around the edge however carefully the drying was carried out. In the most concentrated spots (1 mg/ml) there was a tendency for the fluorescence to appear yellow, especially in this ring of more concentrated amine.

No colour changes were noticed in identified blue cells after pretreatment with Dopa to increase the levels of DA in the ganglia. A slight colour change was sometimes noticed when the magnification of the microscope was changed.

2.30 Photodecomposition of the Fluorescence

The 5-HT fluorophore faded more rapidly than the DA fluorophore in the model systems. It was also noticed that the yellow fluorescence in the tissue sections faded more rapidly than the blue. The fading can be measured quantitatively using a microspectrometer, see section 2.34.
2.31 Introduction

By using a different filter system it had been possible to distinguish between the DA and 5-HT fluorescence in the model systems by a colour difference. In the tissue sections fluorescence of two different types had been observed. The colour of the yellow fluorescence corresponded to the colour of the 5-HT fluorescence in the model spots and the blue fluorescence appeared to correspond to the DA fluorescence in the model spots.

From fading experiments the rate of photodecomposition of the yellow tissue fluorescence was found to be greater than that of the blue fluorescence. This also corresponded with the results found in the model spots.

To confirm the initial observations that the yellow tissue fluorescence was due to 5-HT and the blue fluorescence in the tissues was due to DA a microspectrometer was used. This enabled the emission spectra to be measured and the peak determined for the fluorescence in the model systems and the tissue sections. These values could then be compared. The rate of fading of the intensity of the fluorescence could also be measured and recorded for both the model systems and the tissue.

For this purpose the Zeiss large fluorescence microscope was adapted (MPM). It was not possible to measure the excitation wavelength because a xenon lamp with a continuous spectral light distribution would be needed and one was not available.

2.32 The Microspectrometer

To measure the wavelength of the fluorophores the Zeiss large fluorescence microscope plus the Zeiss MPM microscope photometer was...
Figure 2.11

Diagram of the Zeiss large fluorescence microscope adapted for microspectrometry. The path of the light during measurements is shown. The light may be diverted for observation through the normal eyepieces or through the photometer head.
used. A diagram of the set-up is shown in figure 2.11. The light source was a high pressure mercury lamp (HBO 200 W) with a stabilised power supply. The light passed through a light modulator containing a chopper system that will allow for subtraction of the dark current. This gives a stable zero point. The light then passed through the exciter filters (F1) and via a system of mirrors up through the condenser and onto the specimen. The transmitted light then passed through the barrier filters (F2) and into the photometer head. In the photometer head there were four mirrors arranged on a revolving disc. Three of these have central holes (5.0, 3.2 and 2.0 mm in diameter). The holes allow a selected area of light to pass into the monochromator for measurement. Very small areas of the specimen can be selected for measurement and using a suitable objective areas down to 1μ can be measured. The fourth mirror does not allow any light to pass into the monochromator but enables observation of the whole field of the specimen.

Observation of the specimen was through a viewing magnifier which has a shutter that prevents the influx of extraneous light during measurements.

Light which passed through a central hole in one of the mirrors entered a Hilger-Watts prism monochromator (200-1000 spectral band) and light of the selected wavelength passed into an E.M.I. 6256 B photomultiplier where the light signal was converted into an electrical signal and passed to a stabilised display unit. Pen recordings were obtained by feeding a signal from the display unit into the y axis of a Servoscribe pen recorder. Two separate controls were available on the display unit for amplifying the voltage to the photomultiplier and the current from the photomultiplier. The effect of this was to increase the sensitivity of the photomultiplier.

The wavelength of the monochromator could be continuously changed in either direction by means of a small motor attached to the monochromator.
2.33 Measurement of the Spectra

The method used to measure the spectrum of the fluorescence in model systems or tissue sections was as follows.

The fluorescent cell or area of fluorescence was located in the visual field of the microscope using the normal eyepieces and viewing system. The light was then diverted into the photometer head and the microscope was refocussed for observation through the viewing magnifier. The mirror without a central hole was used at this stage so that the whole field was visible. The mirror with the appropriate sized hole was then swung into position allowing light from that area to pass into the monochromator and then into the photomultiplier. The shutter in the viewing magnifier was closed.

The sensitivity control on the display unit was set to allow a suitable amplification of the signal from the photomultiplier. The wavelengths required were scanned while the signal from the display unit was fed into the y axis of the pen recorder. This signal was proportional to the intensity of light passing into the photomultiplier.

The spectra of the fluorescence in the model spots and that in sections of ganglia were measured using both filter system 'A' and filter system 'B'. The results were compared.

2.34 Rate of Photodecomposition of Fluorescence

To measure the rate of fading of the fluorescence in both the model systems and the tissue the wavelength of the monochromator was set to the peak value of that particular fluorescence and the intensity of the fluorescence was continuously recorded against time. This indicates the rate of fading of the fluorescence. The photodecomposition was compared for the different types of fluorescence in both the model and the tissue fluorescence.
Figure 2.12
The fluorescence characteristics of model systems containing DA, 5-HT and a mixture of DA and 5-HT.
Figure 2.13

The fluorescence characteristics of blue and yellow cells in the ganglia of Helix aspersa.
RESULTS OF THE MICROSPECTROMETRY STUDIES

2.35 Measurement of the Spectra

a. Filter system 'A'

No significant differences could be measured in the fluorescence maxima of the DA and the 5-HT model systems. The peaks in all cases were between 500 and 525 nm. In the tissue fluorescence no distinction could be made in the specific fluorescence of cells that were expected to contain DA or 5-HT.

b. Filter system 'B'

Using this filter system the DA model spot fluorescence had a maximum at 480 nm and that of 5-HT had a maximum at 525 nm. There was a clear difference between the two peaks which can be seen in figure 2.12. The tissue fluorescence that appeared blue with this filter system had a maximum at 480 nm and the yellow fluorescence had a maximum at 530 nm. These values corresponded well with the fluorescence peaks for the model systems. Figure 2.13 shows the fluorescence characteristics of some blue cells measured between 420 and 600 nm, there is a peak at 480 nm. Measurement of the fluorescence characteristics of some yellow cells between 470 and 620 nm showed that there was a peak at 530 nm.

Some model spots were prepared that contained a mixture of 5-HT and DA. These were measured on the microspectrometer to see if the presence of two amines could be detected. The solution used contained 1 mg/ml of each amine. When one spectra had been run it was repeated using the same area of fluorescence. For the first spectra of the mixed spot two peaks were observed, one at 480 nm and the other at 520 nm, see figure 2.12. Further measurements of the same area produced only one peak with a value somewhere between the two peaks. Rapid fading of the 5-HT fluorophore probably explained the disappearance of the
Figure 2.14
The effect of UV light on the intensity of the 5-HT and the DA fluorescence.
separate peaks and the appearance of one peak that was dominated by the DA fluorophore.

2.36 Results of the Measurement of Photodecomposition

The measurement of fading of the tissue fluorescence is shown in figure 2.14. After 10 minutes exposure to UV light the intensity of the blue fluorescence had fallen by about 10% of the original value whereas that of the yellow fluorescence had fallen by just over 50%. Similar results were obtained from fading experiments in the model spots, the 5-HT fluorescence faded rapidly but the DA fluorescence was more resistant to exposure to UV light.

2.37 Conclusions from the Microspectrometry

The characteristics of the fluorophores that is the wavelengths of their maxima, their appearance, fading properties and the conditions for their development indicated that the tissue fluorescence in Helix ganglia was probably due to either 5-HT or to DA. The tissue fluorescence that appeared blue and had its peak at 480 nm corresponded to the DA fluorescence in the model spots. The yellow fluorescence in the tissue corresponded to the 5-HT fluorophore in the model spots. No cells were found that gave two peaks in their fluorescence spectra. The blue fluorescence could be due to NA and/or DA but from the relative quantities of the two catecholamines present in the snail brain it was more likely to be due to DA. A more accurate identification of the catecholamine fluorophore would be possible if the excitation wavelengths were measured before and after exposure to HCl vapour, see paragraph 2.26.
Figure 2.15

Diagram to show the positions of the cells containing monoamine fluorescence in *Helix* ganglia.
THE LOCATION OF THE FLUORESCENT CELLS IN THE BRAIN OF HELIX ASPERSA

Two types of fluorescence have been identified in the cells of Helix aspersa. The location of these cells is described below, and is also shown in figure 2.15.

2.38 Supra-oesophageal Ganglia

a. Cerebral ganglia

Situated on the ventral surface of each metacerebrum was a giant yellow fluorescent cell. These two cells were symmetrical and up to 150μ in diameter. On one side of each giant cell there was a cluster of small blue-green fluorescent cells. The fluorescence of the giant yellow cells tended to fade rapidly when they were exposed to UV light but the intense fluorescence of the small cells was more stable to UV light. Figure 2.16 shows a photograph of a section through both the large yellow cerebral cells. The group of small blue cells is only present on the right hand side of this section. The large area of uniform fluorescence is due to a fold in the section which reflects the light. The tracing below the photograph indicates the connective tissue which contains a considerable amount of autofluorescence.

Axons which leave the small blue-green cells run together until they enter the neuropile. It was not possible to follow the path of the giant cells very far but their paths have been elucidated using antidromic stimulation by Kandel and Tauc (1966). They found that one branch from each cell divided and passed into the ipsilateral cerebro-buccal connective and a second branch passed into the contra-lateral cerebro-buccal connective.

There are groups of small fluorescent cells in the cerebral ganglia situated near the edge of the neuropile. Some of these groups contained yellow cells while others contained blue-green cells. A few blue cells appear throughout the ganglia. Most of these single cells
Figure 2.16
Photograph and drawing of the cerebral ganglia. The large yellow cell (YC) can be seen in each ganglion, some of the small blue cells (bc) can also be seen. The shaded area in the diagram represents connective tissue. The area of intense fluorescence is a fold in the section.
were slightly larger than those in the groups.

The yellow fluorescence was probably due to the presence of 5-HT and the green-blue fluorescence was probably due to the presence of DA.

b. The commissures

The right cerebro-pedal commissure was encased in a layer of yellow fluorescent cells of about 40\( \mu \) in diameter. The left commissure does not have any such cells.

2.39 The Suboesophageal Ganglia

a. Pedal ganglia

The arrangement of the fluorescent cells in the pedal ganglia is shown in figure 2.18. This diagram is a composite sectional diagram of the pedal ganglia. The cells shown in the diagram were not all found at the same level in the ganglia. The ganglia were symmetrical and contained a large number of fluorescent cells. The majority of these cells were medium sized (40-50\( \mu \)) and were yellow. They were located over the anterior surfaces and between the two ganglia. In the right pedal ganglion these cells continued over the surface of the commissure. A few large yellow cells of 100-120\( \mu \) diameter were also present. The photograph in figure 2.17 shows the arrangement of the medium sized yellow cells and also the presence of a large yellow cell.

The neuropile was situated centrally inside a cortex of cells and was intensely fluorescent. This fluorescence was a mixture of blue and yellow but the blue fluorescence was predominant. Where the pedal nerves enter the ganglia the neuropile extended to meet it and was associated with a large area of very intense blue fluorescence.

Small blue fluorescent cells were found along the outside edge of the neuropile, they were oblong and 15-20\( \mu \) long. One end of the cell usually extended into the neuropile. Further away from the neuropile towards the outside edge of the ganglia groups of small cells were found.
Figure 2.17

Longitudinal section through the pedal ganglia and part of the right cerebro-pedal commissure of *Helix aspersa*. Note the large number of medium-sized (40-50\(\mu\)) yellow fluorescent cells present in the ganglia and surrounding the commissure.
Figure 2.10 shows a group of small blue cells in photograph B. These cells appeared yellow under filter system 'A' but blue under filter system 'B'. The fluorescence maximum for these blue cells was 480 nm, see figure 2.13 which corresponded to the fluorescence maximum for the DA model spots, see figure 2.12.

On the inside of the neuropile there were two groups of small yellow cells. These cells were of about the same size as the blue cells and were intensely fluorescent.

There were connectives between the two pedal ganglia and axons containing both types of fluorescence could be seen passing through these connectives.

b. Pleural ganglia

These two small ganglia were situated at the base of the cerebro-pleural commissures and dorsal to the pedal ganglia. These ganglia did not normally contain any fluorescent cells. The neuropile contained both fluorescent and non-fluorescent fibres. The majority of the fluorescent fibres were blue.

c. Left parietal ganglion

This ganglion was smaller than the right parietal ganglion and was partly fused to the visceral ganglion. There were not normally any fluorescent cells in this ganglion. As with the pleural ganglion the neuropile contained fluorescent as well as non-fluorescent fibres.

d. Right parietal and visceral ganglia

These two ganglia contained the largest of the fluorescent cells. These cells were of the greatest interest because they were the ones used for electrophysiological studies. There were about 7-10 medium-large yellow fluorescent cells on the surface of each ganglion. The largest of these cells were found along the border between the two ganglia. The size of these cells was 100-200µ in diameter. Each ganglia had a group of 20-30 small, intensely fluorescent, yellow cells on the
Diagram to show the arrangement of the fluorescent cells in the pedal ganglia of *Helix aspersa*. (The cells are not all present in one section).
border between the two ganglia. These cells were 25-30μ in diameter. No cells containing blue fluorescence were found in these ganglia. The arrangement of the fluorescent cells on the surface of the ganglia is shown diagrammatically in figure 2.15. Figure 2.9, photograph B shows some small and large fluorescent cells in the right parietal and visceral ganglia.

There appeared to be a certain degree of symmetry in the arrangement of the fluorescent and some of the non-fluorescent cells around the common border. On each side of the border there were 3 large fluorescent cells with 1 large non-fluorescent cell below them. Above the large cells on each side was a group of small intensely fluorescent yellow cells. 1-3 medium sized yellow fluorescent cells were found where the intestinal and the right pallial nerves entered the ganglia.

The neuropile in both these ganglia contained axons with both types of fluorescence. The fluorescent cells present in the ganglia of Helix aspersa have been summarised in table 2.3.
Table 2.3 Summary of the Fluorescent Cells in the Central Ganglia of Helix Aspersa

<table>
<thead>
<tr>
<th>GANGLION</th>
<th>YELLOW CELLS</th>
<th>BLUE CELLS</th>
<th>NEUROPILE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Left and right pedal ganglia</td>
<td>+ large</td>
<td>+ small</td>
<td>+ yellow</td>
</tr>
<tr>
<td></td>
<td>++ medium</td>
<td>+ small</td>
<td>+ blue</td>
</tr>
<tr>
<td>Left and right Pleural ganglia</td>
<td>-</td>
<td>-</td>
<td>+ yellow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ blue</td>
</tr>
<tr>
<td>Left Parietal ganglion</td>
<td>-</td>
<td>-</td>
<td>+ yellow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ blue</td>
</tr>
<tr>
<td>Right Parietal ganglion</td>
<td>+ large</td>
<td>-</td>
<td>+ yellow</td>
</tr>
<tr>
<td></td>
<td>+ small</td>
<td></td>
<td>+ blue</td>
</tr>
<tr>
<td>Visceral ganglion</td>
<td>+ large</td>
<td>-</td>
<td>+ yellow</td>
</tr>
<tr>
<td></td>
<td>+ small</td>
<td></td>
<td>+ blue</td>
</tr>
<tr>
<td>Right Cerebro-pedal Commissures</td>
<td>++ medium</td>
<td>-</td>
<td>+ yellow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ blue</td>
</tr>
<tr>
<td>Other Commissures</td>
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<td>-</td>
<td>+ yellow</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>+ blue</td>
</tr>
<tr>
<td>Cerebral ganglia</td>
<td>+ large</td>
<td>+ small</td>
<td>+ yellow</td>
</tr>
<tr>
<td></td>
<td>+ small</td>
<td></td>
<td>+ blue</td>
</tr>
</tbody>
</table>

+ Indicates the presence of fluorescent cells or fibres.
++ Indicates that a large proportion of the cells were fluorescent.
2.40 Summary

1. Model systems of the fluorescence produced by formaldehyde and monoamines have been prepared to characterize the properties of the fluorophores.

2. The activation/emission spectra of the 5-HT and DA model systems were measured in isopropanol using a spectrophotofluorimeter. The activation/emission maxima for DA were 415/480 nm. The activation/emission maxima for 5-HT were 415/520 nm.

3. Using filter system 'A' (BG 38 and BG 12 plus barrier filters 47 and 50) both the fluorophores of DA and 5-HT model systems appeared yellow. Their spectra were not readily distinguishable. The specific monoamine fluorescence in the tissue sections of *Helix aspersa* all appeared yellow.

4. A different filter combination, system 'B' (BG 38 and UG 5 plus barrier filter 47 or 44) was devised. Using filter system 'B' the 5-HT fluorescence appeared yellow but the DA fluorescence appeared blue. The spectra of the fluorophores were easily distinguishable, DA had a peak at 480 nm and 5-HT had a peak at 525 nm.

5. Sections of formaldehyde-treated *Helix* ganglia showed some blue and some yellow monoamine fluorescence when examined using filter system 'B'.

6. A comparison of the properties of the models and the tissue fluorescence indicated that the blue fluorescence was due to the presence of DA and the yellow fluorescence was due to the presence of 5-HT.

7. The results of the localisation of the fluorescent cells in *Helix* ganglia have been summarised in table 2.3.
Discussion

The detection of compounds by fluorescence microscopy is limited by the resolution of the microscope. In mammalian systems this presents a problem where fine terminals appear as a diffuse fluorescence rather than discrete fibres. There is no such diffuse fluorescence in the molluscan nervous system that would suggest the presence of numerous fine terminals.

The results of the distribution of neurones containing 5-HT and catecholamines presented here confirm the reports of Seddon et al (1968) and Marsden (1970) on the distribution of monoamine containing neurones in the ganglia of *Helix aspersa* as detected by fluorescence microscopy. These workers have suggested that the catecholamine fluorescence found in *Helix aspersa* ganglia was due to the presence of DA because DA was thought to be the only catecholamine present in the ganglia. Recently, however, Osborne and Cottrell (1970) and A.G. Ramage (1972, personal communication) have demonstrated the presence of NA (80 ng/gm wet weight of tissue) in *Helix* ganglia. In view of this evidence it is necessary to postulate that some of the catecholamine fluorescence could be due to the presence of NA or a mixture of NA and DA. The concentration of DA present in *Helix* ganglia is much greater than that of NA. Levels of DA that have been reported include 5.01 μg/gm tissue (this thesis), 3.84 μg/gm tissue (Marsden, 1970) and 5.16 μg/gm tissue (Jurio and Killick, 1972). Thus, the concentration of DA present is about 60 times as high as the concentration of NA. The majority of the catecholamine fluorescence will probably be due to the presence of DA rather than NA.

The filter systems that were used for studying the distribution of the different types of fluorescence in the present work caused the catecholamine fluorescence to appear blue. This is in contrast to previous workers who have used filter systems where the catecholamine fluorescence appeared green (e.g. Marsden, 1970; Clark, 1966). The 5-HT
fluorescence appeared yellow under both filter systems. It was found easier to distinguish between blue fluorescence and the yellow 5-HT than it was to distinguish between a green fluorescence and the yellow fluorescence. This was particularly true for high concentrations of DA where the fluorescence tended to appear yellow when it was intense (Caspersson, 1966).

The wavelengths of the 5-HT and the catecholamine fluorophores recorded in the microspectrometric studies agreed well with those measured by Caspersson (1966) and Marsden (1970). Only one type of fluorescence was identified in each fluorescent cell both by observation and by microspectrometry. After Dopa pretreatment, however, some of the large yellow neurones appeared yellow-green indicating that they contained DA as well as 5-HT. Pretreatment with 5-HTP did not appear to affect the fluorescence of the DA cells but the fluorescence of these cells was more intense than the 5-HT cells and could possibly obscure any 5-HT fluorescence also present.

The rapid fading of the 5-HT fluorophore compared with the catecholamine fluorophore that was noted has also been reported by other workers (e.g. Corrodi and Jonsson, 1967).

The purpose of the studies carried out in this section had been to locate the positions of large easily accessible neurones that would be suitable for making intracellular recordings and this was achieved.
3.1 Plan of Experiments

The results from the previous section indicated that the only fluorescent cells large enough and accessible enough to use for making intracellular recordings were those containing 5-HT. The most accessible of these cells were found on the dorsal surfaces of the right parietal and visceral ganglia.

The aims of the experiments described in this section were as follows:

1. To make intracellular recordings of the electrical activity in cells of the right parietal and visceral ganglia in the area of the 5-HT containing cells.
2. To determine the responses of these cells to certain drugs added to the experimental bath.
3. To record the responses of the cells to stimulation of the four main nerve trunks.
4. To compare these results with those of other workers on the cells in this area.
5. To subject the ganglia to formaldehyde treatment and to identify the fluorescent cells with respect to the cells recorded from.
6. To look for any similarity in the properties of the cells that contained 5-HT.

3.2 Introduction

Other workers have mapped some of the neurones on the dorsal surfaces of the parietal and visceral ganglia using electrophysiological techniques (Walker et al., 1970; Lambert, 1973; R.J. Gayton, 1971 personal communication).
Criteria that were used for identifying the cells included:—

1. The size, position and appearance of the neurone.
2. The pharmacology of the neurone.
3. The responses of the neurone to peripheral nerve trunk stimulation.
4. Action potential size, shape and frequency.
5. Synaptic activity.
6. Marking the cell with dyes to indicate the path of its axons across
   the ganglia and into nerves.

Once the approximate location of the fluorescent cells (that is
those cells containing either a catecholamine or 5-HT) was found it was
decided to try and establish their physiological and pharmacological
properties. To positively identify a cell it was not sufficient to rely
on size and position alone. It was necessary to test the cell for the
characteristics noted above and then to determine whether this experimental
cell contained either 5-HT or DA. This meant combining the electro-
physiological study with a later histochemical (Fluorescence) study on
the same brain. The following methods of identification were tried with
this purpose in mind:

   a. Whole mounts of Helix brain.
   b. Isolated neurones from Helix brain.
   c. Cryostat sections of the frozen brain.
   d. Procion marking of the cells adjacent to the experimental cell.

The methods and results of a, b, and c will be discussed below.
Procion marking of cells (d) will be discussed seperately.

3.3 Whole Mounts of Helix Brain

a. Method

The preparation of whole mounts has been described in paragraphs
2.13 and 2.20. To improve the results removal of the connective tissue
after or before the histochemical treatment was tried. The whole mounts
were examined under the fluorescence microscope using epi-illumination.

b. Results

In *Helix aspersa* whole mounts proved to be completely unsuccessful. The difficulty seemed to be due to the thick outer connective tissue and every method used to remove it increased the background fluorescence to a high level and so prevented the identification of the fluorescent cells. Marsden (1970) used a solution of pronases (10 mg/ml) to digest away the connective tissue in *Planorbis corneus* but found it unsuccessful in *Helix*.

3.4 Isolated Neurones from Helix Brain

a. Methods

Cells were initially identified by their relative positions and sizes. The cell that was required was removed from the brain using one of the following techniques:

(i) The outer connective tissue was removed to enable the cells to be seen clearly. A slit was then made in the inner connective tissue which allowed the required cell to 'pop out'. A micro-hook had been constructed out of a small piece of tungsten wire set into a glass rod. This hook was placed around the axon hillock and the cell was gently pulled away. The axons were remarkably tough and generally had to be cut in order to remove the cell completely. The cell was then placed onto a glass slide.

(ii) The outer connective tissue was removed and the inner connective slit in the same way as before. The 'popped up' cell was sucked into a 'Microcap' and then expelled onto a glass slide as before.

Different methods were then tried for studying the fluorescence of the isolated nerve cells:

(i) Initially the cells were frozen by placing the slide onto dry-ice chips and then dried by leaving in a desiccator containing dry-ice
chips and phosphorus pentoxide. The dessicator was continuously evacuated until the dry-ice had evaporated. The cells were then exposed to formaldehyde at 70% relative humidity for 1-3 hours at +30°C. They were examined under the fluorescence microscope.

(ii) Cells were dissected in a solution of polyvinylpyrrolidone (PVP 10%) or dimethyl sulphoxide. These compounds act as cryoprotective agents. The cells were frozen and dried as in (i).

(iii) Cells were squashed with a coverslip before they were frozen, dried and exposed to formaldehyde vapour.

(iv) Controls were prepared in the same way as the experimental cells but they were heated in the absence of formaldehyde.

b. Results

(i) Cells that were treated in the manner described in (i) had an intense yellow autofluorescence. This autofluorescence was partly quenched by mounting the cells in xylene or a non-fluorescent mounting medium. The fluorescence that remained in control cells after this treatment was still bright and of a similar colour to the specific fluorescence. Any specific fluorescence present would have been obscured.

(ii) The purpose of the cryoprotective agents was to prevent damage during freezing that could have been the cause of the autofluorescence. There was, however, no significant improvement in the autofluorescence after this treatment.

(iii) This was the most successful method of examining the isolated cells. The autofluorescence was reduced to a level that occurs in the sections. It was concentrated in granules around the axon hillock and coincided with the location of granular brown pigment noted before the cells were removed. Formaldehyde treatment of these cells produced a monoamine fluorescence infrequently and only in small cells. These cells were probably the small intensely fluorescent cells found on the
Figure 3.1

A. An isolated Helix neurone photographed under UV light. Note the intense fluorescence on the folds in the membrane.

B. The same cell photographed using Nomarski optics. The ridges in the membrane can be clearly seen.
common border between the visceral and the right parietal ganglia.

The reasons for the failure of the formaldehyde technique in the isolated cells is not clear. It is probable that the process of isolation damages the cells. Figure 3.1 shows photographs of a control cell that was removed as was described in (i). (A) shows the cell under UV light and (B) was taken using Nomarski optics on the Zeiss photomicroscope. Part of the autofluorescence is caused by reflection of light from the folds in the cell wall. It is this fluorescence that the 'squashing' technique removed.

3.5 Cryostat Sections

a. Method

Bamberger and Norberg (1964) located catecholamine containing cells in frozen sections using the fluorescence technique. The use of cryostat sections and rapid processing could have proved useful in the localisation of the fluorescent cells.

Helix ganglia were frozen onto a block and sections cut using a freezing microtome. These sections were placed onto glass slides and the slides placed in a dessicator containing dry-ice chips. The dessicator was continuously evacuated until the dry-ice had evaporated and the dessicator had come to room temperature. The sections were then exposed to paraformaldehyde at +80°C for 1-3 hours. They were examined under the fluorescence microscope.

b. Results

No specific fluorescence was found in these sections.

The results of these methods indicated that they would not be suitable for use in the identification of fluorescent cells. The fourth method used for the identification of the cells, Procion marking, is described in the following sections.
PROCION DYE MARKING

3.6 Introduction

The technique of dye injection was originally devised by J. Alvarez and E.J. Furshpan (Unpublished). Stretton and Kravitz (1968) looked for a dye with fluorescent properties and a reactive group that would bind to macromolecules in the cell. Procion Yellow M4RS, made by I.C.I Ltd., was found which had the advantages of not diffusing out of the cell and being relatively unaffected by histological treatments. Covalent bonding probably takes place between the dye and cell proteins and carbohydrates.

Stretton and Kravitz (1968) used the dye to study the geometry of neurones in the abdominal ganglia of the lobster abdominal ganglia. The dye can be introduced into a cell body or large axon. For filling cells in the lobster abdominal ganglion Stretton and Kravitz used a 4% dye solution in microelectrodes. Hyperpolarising current pulses of $1 \times 10^{-8}$ to $5 \times 10^{-8}$ Amps and 0.5 seconds duration were applied to the electrode at a rate of one per second for about 30 minutes. The dye is activated at 460 nm and has an emission peak at 550 nm. It is also possible to use pressure injection to fill a cell with the dye (Selverston and Donald, 1969). For both techniques time was usually allowed for diffusion of the dye throughout the neurone. The histochemical staining properties of Procion Yellow M4RS have been discussed by Payton (1970).

METHODS

3.7 The Preparation

The circumoesophageal ganglionic mass was removed from the animal and the right and left pallial, the anal and the intestinal nerve trunks were all kept as long and as undamaged as possible. The isolated brain was placed onto a plasticine-covered slide. The pedal nerves were held under a rubber band and the anterior end of the brain was secured with
entomological pins. The preparation mounted in this way was placed into a Petri dish containing Snail Ringer. Snail Ringer had the following composition:

NaCl  80 mM  
KCl  4 mM  
CaCl$_2$  7 mM  
MgCl$_2$  5 mM  
Tris  5 mM

the solution was adjusted to pH 7.8 with HCl.

The connective tissue over the surface of the ganglia was gently removed by pulling it away with fine pointed forceps. This operation was performed under a binocular microscope. The inner connective tissue was left intact. The slide was then transferred to the experimental bath.

When the outer connective tissue had been removed from the parietal and visceral ganglia the cell bodies could be seen beneath the inner connective tissue. The majority of the cells were colourless, a few had a milky appearance and some contained brown granules. The sizes of the cells ranged from 20-200$\mu$m in diameter but the majority of them fell within the range 50-100$\mu$m. Very young snails were used to try and eliminate the brown pigmentation but the amount of pigmentation did not appear to depend on the age or size of the snail.

3.8 Pretreatment with 5-HTP

To increase the intensity of the specific fluorescence of the 5-HT containing neurones the snails were injected with 100 $\mu$g of 5-HTP ½-1 hour before dissection. 5-HTP is the immediate precursor to 5-HT and is converted to 5-HT in the brain. This increases the level of 5-HT
Figure 3.2

A. Diagram of the experimental bath showing a preparation with the nerves introduced into the suction electrodes. The agar bridge connecting the main bath to the reference bath is also shown.

B. Observation of the preparation using a binocular microscope. Light was focussed onto the preparation using a planar-convex lens.
in the specific neurones.

5-HTP pretreatment did increase the intensity of the 5-HT fluorescence but was not used routinely since on most preparations it was possible to detect the location of the 5-HT neurones.

3.9 The Experimental Bath

A diagram of the bath is given in figure 3.2. The bath was constructed of Perspex with a glass front to allow observation of the brain. The bath was partly filled with paraffin wax which was moulded to support the slide at an angle of 60°. Channels were made in the wax to allow Ringer to enter at the bottom of the bath and to leave at the top of the bath. The volume of the bath was 30 ml.

Four suction electrodes were arranged at the front of the bath. These were made from electrode glass drawn out to a fine tip. They were each connected to a 10 ml syringe by a length of Portex tubing.

3.10 Observation of the Ganglia

The preparation was illuminated with a Beck lamp, focussed onto the preparation by a convex lens. An Olympus binocular microscope (SZ 111) was mounted horizontally in front of the preparation and a magnification of approximately X 50 was used for observation. Figure 3.2 (B) shows the position of the microscope in relation to the bath.

3.11 Microelectrodes

Microelectrodes were made from Pyrex tubing, internal diameter 2 mm and external diameter 4 mm. 7.5 cm lengths were washed in distilled water and dried. The lengths of tubing were pulled on a 'Narishige' vertical electrode puller. Each length gave two electrodes. The electrodes were initially filled with methanol by boiling under reduced pressure
Figure 3.3

Diagram of the experimental set-up for nerve stimulation, cellular recordings and dye injection.

There is a two-way switch to the microelectrode that allows either recordings to be made from the cell or a hyperpolarising current to be passed into the electrode to pulse dye into the cell. The apparatus is further described in the text.
in a flask. The electrodes were then immersed in distilled water for 15 minutes and then in fresh distilled water for a further 30 minutes. During this time the methanol was diluted by the distilled water.

The water was removed from the shank of the electrode and replaced with either 1 M potassium acetate solution or a 4% solution of Procion Yellow dye M4RS in distilled water (w/v). The tips of the electrodes were dipped in distilled water and left (usually overnight) to allow the KAc or the Procion dye to diffuse to the tips.

KAc electrodes with a resistance of 10-20 MΩ were selected for use. Procion electrodes had a high resistance, >100 MΩ. The tips of these electrodes were gently broken down to give a resistance of between 20 and 50 MΩ.

The microelectrode was held in a crocodile clip mounted on a Prior micromanipulator. Using the micromanipulator the electrode could be positioned over the cell and gently lowered onto and finally into the cell. The most usual way of penetrating the cell was to lower the electrode until it just 'dimpled' the surface and then to tap the edge of the micromanipulator. The small but sharp vibrations usually caused the electrode to enter the cell.

3.12 Recording and Display of Cell Activity

A diagrammatic layout of the apparatus used to record the electrical activity of the cell, to stimulate the nerves and to inject Procion dye into the cell is shown in figure 3.3. For recording the electrical activity of the cell the two-way switch (S) was set to make contact with the probe. The potential changes recorded in the cell were relative to the bath potential. Potential changes occurring in the cell were picked up by the microelectrode and fed into a high impedance cathode follower via a silver/silver chloride wire placed in the shank
Figure 3.4
Diagram of the bridge circuit, showing its connections with the other apparatus.
of the electrode. The amplified signal was fed into one input of the differential amplifier of a Tektronix 502 A oscilloscope. The reference electrode was another silver/silver chloride wire and this was placed in a small reference bath containing Ringer solution. This reference bath was in electrical contact with the experimental bath via an agar jelly- KCl bridge. This separate bath prevented electrical interference caused by fluctuations in the Ringer level around the reference electrode. This was most troublesome during washing.

Permanent records were made using a Watanabe 211 pen recorder.

A signal was also led to an audio amplifier and loudspeaker. This system was used to record the spontaneous activity of the cell and the responses of the cell to specific stimuli.

3.13 Modifying the Membrane Potential

The membrane potential could be hyperpolarised or depolarised through the microelectrode using the bridge circuit. Diagrams of the bridge circuit and its connections with other apparatus are shown in figures 3.3 and 3.4. In figure 3.4 the positions of the 3-way switch were 'off', 'hyperpolarise' and 'depolarise'. The stimulation current was controlled by a ten-turn potentiometer.

To measure the change in the membrane potential caused by the bridge current it was necessary to balance the bridge. This was done in the following manner:

1. With the microelectrode in the Ringer and the bridge switch in the 'off' position, the oscilloscope beam was set to a reference position.
2. The 3-way switch was then set to 'hyperpolarise' or 'depolarise' and a large stimulating current was applied. If the bridge was unbalanced this caused a shift in the position of the oscilloscope beam because two different potentials were being applied to the differential inputs of
the oscilloscope (A and B in figure 3.4).

3. The oscilloscope beam was returned to the original reference position by means of the 'Balance' potentiometer. This operation brought the bridge into balance such that:

\[
\frac{R_1}{R_2} = \frac{1 \text{ G} \Omega \text{ resistor in the probe}}{\text{Electrode resistance}}
\]

For this case the potentials applied to the 2 differential inputs of the oscilloscope were equal and no beam deflection occurs.

When a cell was impaled with the bridge in balance then, provided the electrode resistance remained unaltered, any deflection of the oscilloscope beam caused by altering the bridge current was a true representation of the change in membrane potential of the cell.

3.14 Nerve Stimulation

The left pallial, right pallial, anal and intestinal nerve trunks were sucked gently up into the stimulating suction electrodes. Using a Grass S44 stimulator, a Grass SIU 5 and a 4-way switch it was possible to apply a stimulating pulse to each nerve trunk see figure 3.3. The voltage and duration of the pulse were variable. The magnitude of the threshold voltage for each nerve was partly dependent on the fit of the suction electrode around the nerve trunk. In this study the voltages used were 2-5 volts with a duration of 3 ms. Responses to nerve stimulation were displayed on the oscilloscope and on the pen recorder.

3.15 Pharmacology

The selected drugs were made up in Ringer solution in a range of concentrations, e.g. \(10^{-3} - 10^{-6}\) g/ml. Drugs were applied to the preparation from a pipette. Doses of drugs are given in nMoles of drug
Figure 3.5

Data recorded from cell 5 in figure 3.7. In the general map of the right parietal ganglion of Helix this cell is Cell no 1.

**Drugs**  The addition of 5-HT to the bath caused a depolarisation, the addition of ACh also caused a depolarisation. The addition of DA caused a hype polarisation.

**Inputs**  Stimulation of the left and right pallial and anal nerves evoked an IID response in the cell. Stimulation of the intestinal nerve caused a double IPSP and an antidromic AP in this cell. The cell was pulsed with Procion dye for 10 minutes, after which the cell still gave APs.
added to the bath which had a volume of 30 ml. Three different drugs were tested on the neurones of *Helix aspersa*. These were ACh, 5-HT and DA which are putative transmitters within the molluscan nervous system.

3.16 Data recorded

Large cells in the areas of the visceral and right parietal ganglia known to contain fluorescent cells were chosen for recordings. Figure 2.15 shows the areas concerned. When a cell had been impaled with a microelectrode its spontaneous firing pattern and membrane potential were recorded. The responses of the cell to stimulation of the four main nerve trunks were recorded. The responses of the cell to the addition of the drugs ACh, 5-HT and DA were also recorded. See fig. 3.5.

3.17 Cell Marking with Procion Dye

When the relevant data had been recorded from the cell the electrode was removed from the experimental cell and placed in a neighbouring cell. Dye was then ejected from the tip of the electrode by applying hyperpolarising current pulses to the electrode. A separate circuit was used to supply this current and was brought into use by the two-way switch in figure 3.3. The circuit used for pulsing dye is shown in figure 3.6.

Procion dye filled the cell body after a few minutes pulsing. Pulsing was continued for 10-20 minutes by which time the cell was well coloured. For some cells the pharmacology and the nerve inputs of the cell were recorded before dye injection. Injection of Procion dye did not appear to poison the cells. Cells showing spontaneous electrical activity continued firing after being filled with Procion dye.

3.18 Measurement of the Pulsing Current

The current used for the dye injection was not measured directly
Figure 3.6
Diagram of the circuit used for pulsing Procion dye into cells.
The dye is contained in the microelectrode. The total resistance of the circuit is: $R_1 + R_2 + R_3 + R_4 + R_5$. 
but was calculated approximately using Ohm's Law:

\[ V = IR \]

Where: \( V \) = the voltage applied

\( R \) = the total resistance of the circuit = \( R_1 + R_2 + R_3 + R_4 + R_5 \)

\( I \) = the current flowing

\( R_1 \), \( R_2 \), \( R_3 \), \( R_4 \) and \( R_5 \) are individual resistances in the circuit shown in figure 3.6.

\( R_1 \) = Bridge resistance

\( R_2 \) = \( 1 \times 10^9 \) series resistance

\( R_3 \) = Electrode resistance, approximately \( 50 \) MO

\( R_4 \) = Cell resistance, unknown

\( R_5 \) = Resistance of the Ringer solution, unknown.

Since \( R_1 \), \( R_4 \) and \( R_5 \) are small compared with \( R_2 \) and \( R_3 \) we can ignore them for the purpose of obtaining an approximate value for the current.

\[ V = 30 \text{ volts} \]

therefore:

\[ I = \frac{30}{1050 \times 10^6} \]

\[ I = 28.57 \times 10^{-9} \text{ A} \ (=28.57 \text{ nA}) \]

The value of the current used in these experiments fell within the range of current used by Stretton and Kravitz (1968), \( 1-5 \times 10^{-8} \text{ A} \).

The pulse duration used in the present study was 0.5 second at a rate of 1 per second. Pulsing was continued for 15 minutes.

3.19 Fixation

Glutaraldehyde or another suitable histological fixative was used for studies in neuronal geometry. In this study it was intended to use the Falck-Hillarp technique for localisation of 5-HT and DA. The tissue was thus freeze-dried exposed to formaldehyde, embedded and sectioned.
A. Drawing of part of a Helix brain made before it was removed from the bath. The cells that were impaled are numbered 1-5.

B. A table of the responses and nerve inputs of the tested cells. Cells 1, 2 and 5 were injected with Procion Yellow dye. The results of the fluorescence technique have also been included in the table, cell 3 was found to contain specific monoamine fluorescence but cell 4 was non-fluorescent.
3.20 Treatment of the Marked Ganglia

Before the brain was removed from the bath a drawing was made to indicate the positions of the cells being studied. Figure 3.7 shows a drawing of part of a typical brain of Helix made before it was removed from the bath. The excess Ringer was blotted off the surface of the brain and it was arranged on a small piece of paper to hold the ganglia in position. The brain was then frozen in isopentane cooled in liquid nitrogen, transferred to the freeze-dryer and held frozen under vacuum until dry. It was then exposed to formaldehyde vapour at +80°C in the same way as has been described in section 2.18. After histochemical treatment the tissue was embedded in wax, blocked and cut into serial sections 10μ thick. The sections were mounted on clean glass slides.

3.21 Illumination and Examination of the Sections

Procion Yellow K4RS is activated at 460 nm and has an emission peak at 550 nm. The sections were examined under the fluorescence microscope using a high pressure mercury lamp as the light source with exciter filters BG 38 and BG 12 plus barrier filters 47 and 50. This was the best filter combination for studying Procion Yellow fluorescence. Monoamine fluorescence could be studied at the same time. The sections containing the dye marked cells were located.

With the aid of a camera lucida attachment on the microscope outline drawings were made of the sections on tracing paper. The drawings included the edge of the section, the fluorescent cells, the Procion marked cells and the larger non-fluorescent cells. A series of such drawings is shown in figure 3.10(A). Alternate sections only have been shown in this diagram.

3.22 Reconstructing the Ganglia

To determine the relative positions of the fluorescent cells and
the marked cells in the whole ganglia the drawings of the sections were superimposed on one another. If there were a large number of drawings and lower ones were obscured it was possible to increase the translucency of the paper by soaking the paper in xylene. A tracing was made of the superimposed outline drawings and compared with the drawing made of the brain before it was frozen. Figure 3.10(A) shows a series of drawings of serial sections from a typical brain showing fluorescent cells, non-fluorescent cells and Procion marked cells. (B) shows a reconstruction drawing made by superimposing these tracings.
Figure 3.8
Pen recordings from 5 different Helix cells showing the frequency of cell activity and the types of firing patterns. The horizontal scale is 7.5 seconds.
3.23 Cell Activity

When a cell was penetrated with a microelectrode a potential difference across the membrane was recorded, the inside of the cell being negative with respect to the outside. The size of this resting potential or membrane potential usually fell within the range -35 to -55 mV. Most cells in addition to the resting potential also showed spontaneous changes in electrical activity. This activity often took the form of overshooting action potentials. The pattern of firing (ie. the size and frequency of the APs) could be used as an additional criterion for identification of the cell. Examples of different types of firing pattern are shown in figure 3.8.

Cells that were silent when they were impaled could be made to fire by depolarising the membrane potential until it reached the threshold to fire. Usually, however, experiments were carried out with the cells at their natural resting potentials.

Procion Yellow dye did not appear to be poisonous to the cells in that cells showing a spontaneous activity when they were first impaled continued to fire after they had been filled with Procion dye. Figure 3.5 shows a cell in the right parietal ganglion that has been filled with Procion dye (indicated on the diagram by ----) and still shows spontaneous action potentials of 70 mV.

3.24 Nerve Stimulation

A variety of responses was evoked by stimulation of the four main nerve trunks. These responses included:

1. Excitatory post synaptic potentials (EPSP)
2. Inhibitory post synaptic potentials (IPSP)
3. Inhibition of long duration (ILD)
4. Antidromic action potentials (AD AP)

5. Various biphasic responses.

Abbreviations that have been used in the text and in figures are given in brackets.

It was often possible to observe more than one of these responses in a cell after a single nerve stimulation. There was also sometimes a change in the response when the stimulus strength (voltage) was increased. The voltage used routinely was just above the threshold for the particular nerve and so this phenomenon was not encountered. The value of the threshold stimulus depended on the fit of the suction electrode around the nerve trunk, for a close fit a smaller voltage was required.

Figure 3.5 shows the nerve inputs from cell 5 in figure 3.7. In this case the response of the cell to stimulation of each of the nerve trunks was an ILD with the addition of an antidromic AP through the intestinal nerve.

3.25 Pharmacology

The responses of the cells to each drug tested were classified into two major types:

(i) Hyperpolarisation

(ii) Depolarisation

A hyperpolarisation was usually accompanied by an inhibition of firing and a depolarisation was usually accompanied by an excitation (an increase in the firing rate of the cell)

The type of response shown by a cell was sometimes dependent on the amount of drug that was added to the preparation. Some cells easily became desensitised to a particular drug. It was possible to have a biphasic response to a drug.
Figure 3.9
Photograph of a section of the right parietal (R.P.) and visceral (Vis) ganglia of Helix aspersa. This section was one from a set of serial sections that have been drawn in figure 3.10 A, the section in this photograph was section 12. Note the Procion marked cell in the visceral ganglion near the entry of the intestinal nerve and the second Procion marked cell appearing next to it.
a. ACh

Most cells showed a response to the addition of ACh. Some of the cells were depolarised by ACh, these cells are known as 'D' cells. A few cells were hyperpolarised by ACh, these cells are known as 'H' cells.

b. 5-HT

Cells that were sensitive to 5-HT were depolarised. Cells that are hyperpolarised by 5-HT have been reported by Gerschenfeld (1971) and Lambert (1973) but this response was not found in any cells used in this study.

c. DA

Not all the cells responded to the addition of DA but those that did respond were hyperpolarised and firing was inhibited. Figure 3.5 shows the pharmacology of cell 5 in figure 3.7 to these three drugs. This cell was depolarised by 5-HT and ACh and hyperpolarised by DA.

3.26 Examination of the Sections

a. Procion fluorescence

The cells that contained the Procion dye were easily located in the sections. Procion Yellow had an orange fluorescence under the lighting conditions used in the fluorescence microscope. The dye diffused to some extent into the axons of the cells. To obtain complete diffusion of the dye throughout the neurone it is necessary to pulse the dye into the cell for a longer period of time and then to incubate the ganglia at 4°C for a further 16 hours before fixation and embedding. Long injection times were not used in this study because the main purpose was to localise the fluorescence due to monoamines at their original storage sites and it was important to freeze the ganglia as soon as possible after removal from the animal.

Figure 3.9 shows a section of the right parietal and visceral ganglia of *Helix*, two cells in the visceral ganglion contain Procion dye.
Figure 3.10
Reconstruction of the ganglia of *Helix aspersa* from serial sections.

A. A series of drawings made with the camera lucida attachment on the microscope of serial sections of the right parietal and visceral ganglia containing Procion marked cells. Alternate sections have been presented here.

B. Diagram of the combined section drawings. The numbers given to the cells correspond to the numbers given to the cells in figure 3.7 and not to the numbers given to the cells in the general map of *Helix* brain.
Figure 3.11

A photograph of a section through the right parietal and visceral ganglia of Helix. In one cell Procion dye can be seen in the nucleus and specific, formaldehyde-induced fluorescence due to 5-HT is present in the cytoplasm.
b. Monoamine fluorescence

As far as it was possible to determine the 5-HT fluorescence in the snails that had been pretreated with 5-HTP was located in the same cells as in the control snails. There did not appear to be any increase in the number of fluorescent cells after pretreatment with 5-HTP but the intensity of the fluorescence in the specific cells was increased. In later experiments 5-HTP pretreatment was omitted and the monoamine fluorescence was still clearly visible.

Generally when Procion dye was injected into a cell the fluorescence of the dye spread throughout the cytoplasm and the nucleus and masked the presence of any specific monoamine fluorescence. Occasionally it happened that the Procion dye was only injected into the nucleus of the cell and did not diffuse into the cytoplasm. When this happened in a cell that contained 5-HT in its cytoplasm the fluorescence due to the monoamine was not masked but was still visible. In sections of such cells the nucleus was orange and the surrounding cytoplasm was a fluorescent yellow. Figure 3.11 shows a cell where the Procion marking is confined to the nucleus and the cytoplasm shows specific 5-HT fluorescence.

The spontaneous activity of the cells that were marked in the nucleus was indistinguishable from that in cells which were later found to contain dye in the cytoplasm and the nucleus. It was not possible to predetermine the position of the electrode within the cell when marking with the dye.

3.27 Reconstructing the Ganglia

The marked cells were found at one end of the series of sections because the brain was sectioned longitudinally. It was found necessary to reconstruct the ganglia from the camera lucida drawings because variations in the angle at which the sections were cut had a considerable
effect on the apparent relative positions of the cells. For instance one cell might appear to be next to another when, in fact, it was beneath it.

Figure 3.10 (A) shows a series of drawings of serial sections made with the camera lucida and (B) is a tracing of these drawings superimposed. In (A) and (B) the cells which contain Procion dye and those which contain specific fluorescence have been indicated by shading, non-fluorescent cells have been left open. Figure 3.9 shows a photograph of one section in the series from which the drawings were made. This section was section 12, sections 11 and 13 appear in figure 3.10. The tracing of the reconstructed ganglia was compared with the drawing of the brain that was made before it was removed from the bath, figure 3.7. By using the Procion cells as a guide, the cells whose fluorescence has been determined can be identified with the cells where the pharmacology has been determined. The table of results in figure 3.7 correlates the results for that particular brain.

3.28 Identified Cells in Helix aspersa Brain

Recordings were made from the areas in the right parietal and visceral ganglia where large fluorescent cells had been located, see the results in section 2.39 and also figure 2.15. These large cells were found along the border between the two ganglia and they contained yellow fluorescence.

Some cells were easily identifiable from one preparation to another. The criteria used for the identification of the cells in this study were as follows:

1. The size and appearance of the neurone and its position with respect to various landmarks in the ganglia, such as the nerve trunks, and other large cells.
Figure 3.12

Diagram of the cells in the right parietal and visceral ganglia of *Helix aspersa* that have been used in this study.

The numbers and letters are those referred to in the results sections 3.29 and 3.30.
2. The pharmacology of the neurone. In this study, the response of the cell to the addition of ACh, 5-HT and DA to the bath.

3. The nerve inputs of the neurone. That is the response of the cell to stimulation of the four major nerve trunks, the right and left pallial, the intestinal and the anal nerves.

Cells in the right parietal ganglion were assigned the number given to them by Lambert (1973) in the general map of Helix neurones. The cells in the visceral have been assigned a letter for identification. Figure 3.12 shows the labelling of the cells in these two ganglia that has been used in this study.

The results described below are tabulated in table 3.1 and the positions of the cells are shown in figures 3.12 and 3.20.

3.29 Right Parietal Ganglion

Cell 1

The position of this cell in the right parietal ganglion is shown in figure 3.12. This was the largest cell in the right parietal ganglion. Its size was between 150 and 200\(\mu\). It has been given the name of the 'big D' cell by previous workers. Figure 3.5 shows the typical responses of this cell to stimulation of the nerve trunks and to the addition of drugs to the bath. ACh and 5-HT both caused a depolarisation of the membrane potential which was accompanied by an excitation. DA caused a hyperpolarisation of the membrane potential. The nerve inputs for this cell were very characteristic. Stimulation of the right pallial, anal and left pallial nerves produced a long IPSP or an ILD response. Stimulation of the intestinal nerve trunk produced an antidromic AP followed by a double IPSP. The first phase of this IPSP was fast and the second was a slow phase. Figure 3.13 of the responses of the 'big D' cell to stimulation of the nerves clearly shows
Figure 3.13

The responses of cell 1 in the right parietal ganglion of *Helix aspersa* to stimulation of the four main nerve trunks (3.5 V, 2 ms).

A. Stimulation of the right pallial nerve evoked an ILD in this cell. Stimulation of the intestinal nerve trunk evoked an antidromic AP and a double IPSP. The first part of this double IPSP is a fast phase and the second is a slow phase. This response was characteristic of cell 1 (big 'D' cell) in the right parietal ganglion of *Helix*.

B. Stimulation of the anal and the left pallial nerve trunks evoked an ILD response in each case.
this double IPSP after stimulation of the intestinal nerve trunk.

The results of the fluorescence studies showed that cell 1 was always non-fluorescent and did not, therefore contain a significant store of either 5-HT or DA.

Cell 2

The position of this cell in the right parietal ganglion is shown in figure 3.12. One of the characteristics of this cell was the fast firing rate (3 AP/second). Application of ACh to this cell can either cause a hyperpolarisation or a depolarisation. The nerve inputs for this cell are shown in figure 3.14. Trace A shows an antidromic AP and an EPSP following stimulation of the right pallial nerve trunk. Trace B shows EPSPs in response to stimulation of the anal and left pallial nerves. Stimulation of the intestinal nerve trunk produced a short inhibition of firing but this did not appear to be accompanied by either an IPSP or an EPSP.

Cell 2 did not contain any specific monoamine fluorescence after exposure to formaldehyde vapour.

Cell 3

This was a large cell (100-150μ) found at the bottom of the right parietal ganglion, its position is shown in figure 3.12. The responses of this cell to stimulation of the nerve trunks are shown in figure 3.15. Stimulation of each of the nerve trunks evoked an EPSP in the cell body. In addition stimulation of the right pallial nerve evoked an antidromic AP in the cell. This cell was hyperpolarised by the addition of ACh to the bath (figure 3.15). This cell was the only consistently 'H' cell that was found in the area of the right parietal ganglion that was studied.

Fluorescence studies showed that there were no biogenic amines present in this cell.
Figure 3.14

The responses of cell 2 in the right parietal ganglion of Helix aspersa to stimulation of the four main nerve trunks, (3V).

A. Stimulation of the right pallial nerve produced an antidromic AP and an EPSP in this cell. The response to stimulation of the intestinal was not identified.

B. Stimulation of the anal and the left pallial nerves produced an EPSP in this cell.
Figure 3.16

The responses of cell 3 in the right parietal ganglion of *Helix aspersa* to nerve stimulation (3V, 3ms) and to the addition of ACh.

A. Stimulation of the left pallial and anal nerves produced an EPSP in this cell.

B. Stimulation of the intestinal and right pallial nerves also produced an EPSP in this cell.

C. The response of this cell to ACh was a hyperpolarisation.
The responses of cell 4 in the right parietal ganglion of Helix aspersa to stimulation of the four main nerve trunks (3V, 2ms).

A. ILD responses to stimulation of the right pallial and intestinal nerves.

B. Long ILD response to stimulation of the anal nerve.

C. ILD response to stimulation of the left pallial nerve.

Figure 3.16
Cell 4

Cell 4 was a large cell approximately 100µ in diameter that was found above cell 3 in the right parietal ganglion. Figure 3.12 shows the position of this cell. The application of ACh to this cell caused a depolarisation in the membrane potential and an increase in the firing rate of the cell. The membrane potential and the firing rate returned to normal on washing. The addition of 5-HT to the preparation depolarised this cell and caused an increase in the firing rate. The effect was reversed on washing. DA added to the bath hyperpolarised the cell and caused an inhibition of firing. The normal resting potential and firing rate returned on washing. The responses of the cell to stimulation of the four nerve trunks are shown in figure 3.16. Trace A shows an ILD response evoked in the cell after stimulation through the right pallial and the intestinal nerves. In trace B stimulation of the anal nerve produced a long ILD response in the cell. Stimulation of left pallial nerve also produced an ILD response in this cell (trace C).

After formaldehyde treatment the cytoplasm of this cell contained a yellow fluorescence. This fluorescence had the same characteristics as the fluorescence in the 5-HT model systems.

Cell 5

This cell was positioned anterior to cell 4 in the right parietal ganglion of Helix, see figure 3.12. The properties of this cell were very similar to those of cell 4. The pharmacology of the cell is shown in figure 3.17. Trace A shows the depolarisation and excitation in response to the addition of ACh to the bath. Trace B shows the effect of 5-HT added to the bath, there was a depolarisation and excitation. The effects of ACh and of 5-HT were removed by washing. The addition of DA to the preparation caused a hyperpolarisation and an inhibition of firing, (trace C). The effect of DA was removed by flushing the bath with Ringer solution. The nerve inputs of this cell were similar to
Figure 3.17
The responses of cell 5 in the right parietal ganglion of *Helix aspersa* to drugs added to the bath.

A. This cell was depolarised by the addition of ACh.

B. 5-HT caused a depolarisation in the cell.

C. The addition of DA hyperpolarised the cell.
those of cell 4. Stimulation of the right pallial nerve produced an
ILD response in the cell, stimulation of the anal and the left pallial
nerves also produced an ILD response in the cell.

Fluorescence histochemistry on this cell showed that there was
a yellow fluorescence present in the cytoplasm of the cell that had the
same characteristics as the 5-HT fluorescence in the model systems.

Cell 6

This cell in the right parietal ganglion of Helix was anterior
to cell 5, its position is shown in figure 3.12. The cell was large
(100 μ) and its pharmacology and nerve inputs were similar to those of
cells 4 and 5. The addition of ACh to the bath caused a depolarisation
in this cell and the addition of 5-HT to the preparation also caused a
depolarisation. This cell was hyperpolarised by DA. The response of the
cell to stimulation of the four main nerve trunks was an ILD response
in each case.

After exposure to formaldehyde the cytoplasm of this cell contained
a specific monoamine fluorescence that was characteristic of the presence
of 5-HT.

Small Yellow Cells

Anterior to cells 4, 5 and 6 there was a group of 15-25 small,
intensely fluorescent yellow cells. Their position is indicated in
figure 2.15. These cells were too small to record from. Some medium
sized fluorescent cells were also shown in the diagram of the locations
of the fluorescent cells. The pharmacology and nerve inputs of these
cells has not been established.
Figure 3.18

Recordings from cell A of the visceral ganglion of *Helix aspersa*.

A. The responses of the cell to stimulation of the intestinal and left pallial nerves were EPSPs.

B. The cell was depolarised sufficiently to cause it to fire, the addition of ACh to the depolarised cell caused a hyperpolarisation and an inhibition in the firing. The addition of ACh to the cell at its normal resting potential caused a hyperpolarisation.
3.30 Visceral Ganglion

Cell A

This large cell (100-150 μ) was found at the bottom right hand corner of the ganglion. It often appeared to 'hang off' the edge of the ganglion. The position of this cell is shown in figure 3.12. When the cell was first penetrated with a microelectrode there was often a burst of action potentials. After this initial activity the cell usually became silent. This initial burst of APs was probably due to damage or shock of some kind. Stimulation of the four major nerve trunks evoked EPSPs. Figure 3.18 shows EPSPs evoked after stimulation of the intestinal and left pallial nerves, trace A. In trace B the cell was depolarised sufficiently using the bridge circuit to cause it to fire. The addition of ACh to the cell caused a hyperpolarisation. The cell was then returned to its natural resting potential and became silent. The addition of ACh to the cell when it was silent also caused a hyperpolarisation. This cell was depolarised by the application of 5-HT.

Fluorescence histochemistry on this 'H' cell showed that there was no 5-HT or DA present in the cytoplasm.

Cell B

This cell was usually found close to the 'H' cell, cell A. The response of this cell to specific stimulation is shown in figure 3.19. Trace A shows EPSPs evoked by stimulation of the right pallial nerve, trace B shows an EPSP evoked by stimulation of the intestinal nerve. Trace C shows stimulation of the anal nerve which produces an antidromic AP and an EPSP in this cell. In trace D repeated stimulation of the anal nerve is followed one to one by the antidromic spike, until the somatic part of the AP, the 'S' spike fatigued and only the axonic or 'A' spike remained. This is more clearly seen on the oscilloscope screen. Stimulation of the left pallial nerve also produced an EPSP.
Figure 3.19

Responses of cell B in the visceral ganglion of Helix aspersa to stimulation of the four main nerve trunks (A-E), and the responses of this cell to the addition of ACh and DA to the bath.

A. Stimulation of the right pallial nerve evoked EFSPs in this cell.
B. Stimulation of the intestinal nerve evoked an EPSP in this cell.
C. Shows an antidromic AP and an EPSP after stimulation of the anal nerve.
D. Repetative stimuli through the anal nerve demonstrates that the antidromic spike follows one to one. The somal spike then fatigues and leaves only the axonal spike.
E. Stimulation of the left pallial nerve evoked an EPSP in the cell.
F. The addition of ACh to this cell caused a depolarisation in this cell.
G. The addition of DA to this cell caused a hyperpolarisation. The firing pattern in this cell was of a bursting type.
in this cell. The addition of ACh onto this cell caused a depolarisation of the membrane potential and an excitation, trace F. Trace G shows a hyperpolarisation and an inhibition after the addition of DA to the cell. Addition of 5-HT to cell B caused a depolarisation.

After formaldehyde treatment this cell was found to contain a specific monoamine fluorescence in the cytoplasm which had the characteristics of 5-HT fluorescence.

Cell C

Cell C was a large cell (100 μ) found next to cell A, the 'H' cell, and cell B (see figure 3.12). The application of ACh or 5-HT to this cell caused a depolarisation and an excitation. The addition of DA to the cell caused a hyperpolarisation. Stimulation of the right pallial, left pallial, the intestinal or the anal nerve evoked an EPSP in this cell.

Histochemical treatment of this cell with formaldehyde indicated that it contained 5-HT in its cytoplasm.

Cell D

This cell was smaller (80 μ) than those already described and its position is shown in figure 3.12. Preliminary investigations have indicated that the cytoplasm of the cell was fluorescent and that the fluorescence was due to 5-HT. The response of the cell to the addition of ACh or 5-HT was a depolarisation. The response of the cell to the addition of DA was a hyperpolarisation. Stimulation of the left pallial or the anal nerve produced an EPSP in the cell.

Cell E

This cell was smaller (80 μ) than cells A-C and was found in the position indicated in figure 3.12. Application of ACh or 5-HT to the cell caused a depolarisation in the membrane potential. The application of DA to this cell caused a hyperpolarisation. A slow IPSP was evoked in the cell after stimulation of each of the four major
nerve trunks.

The cytoplasm of this cell was found to contain yellow fluorescence after exposure to formaldehyde. This fluorescence was characteristic of 5-HT fluorescence.

Cell F

The position of this cell, half-way up the visceral ganglion on its border with the right parietal is shown in figure 3.12. This cell was depolarised by the addition of ACh or 5-HT and hyperpolarised by the addition of DA to the bath. The responses to the stimulation of the right pallial and the intestinal was biphasic, consisting of an EPSP followed by a slow IPSP. Stimulation of the anal or the left pallial nerves produced an EPSP in the cell.

Fluorescence studies on this cell indicated that the cytoplasm contained 5-HT.
Figure 3.20

Diagram of the brain of *Helix aspersa* to show the positions of the cells that have been identified. Their pharmacology and fluorescence characteristics have been listed in table 3.1.
Table 3.1 The Pharmacology and Fluorescence Properties of the Identified Cells in Helix aspersa

**Right Parietal Ganglion**

<table>
<thead>
<tr>
<th>Cell</th>
<th>Fluorescence</th>
<th>ACh</th>
<th>DA</th>
<th>5-HT</th>
<th>R.Pallial</th>
<th>Anal</th>
<th>Intestinal</th>
<th>L.Pallial</th>
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<td>D</td>
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<td>H</td>
<td>D</td>
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</table>

**Visceral Ganglion**

<table>
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<th>DA</th>
<th>5-HT</th>
<th>R.Pallial</th>
<th>Anal</th>
<th>Intestinal</th>
<th>L.Pallial</th>
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<td>H</td>
<td>-</td>
<td>D</td>
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<td>EPSP</td>
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<td>D</td>
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<td>AD+</td>
<td>EPSP</td>
<td>EPSP</td>
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<td>EPSP</td>
<td>Biphasic</td>
<td>EPSP</td>
</tr>
</tbody>
</table>
3.31 Summary

1. Six large cells in the right parietal ganglion and six large cells in the visceral ganglion of *Helix aspersa* have been identified using electrophysiological techniques.

2. Fluorescence studies on these cells have shown that in the right parietal ganglion 3 out of the 6 cells contained 5-HT in their cytoplasm. In the visceral ganglion 5 out of the 6 cells contained 5-HT in their cytoplasm.

3. The 5-HT containing cells in both ganglia had a similar pharmacology. These cells were depolarised by ACh and 5-HT and hyperpolarised by DA.

4. The nerve inputs of the cells in the right parietal ganglion were all IID type responses. In contrast most of the responses to nerve stimulation in the visceral cells were of an EPSP type.
Discussion

The reason for the failure to demonstrate 5-HT in the cryostat sections using the formaldehyde technique could be that the amine diffused away from the storage site at some stage of the processing. Hamberger and Norberg (1964) demonstrated the presence of catecholamines in frozen sections but not 5-HT. 5-HT tends to diffuse to a greater extent than the catecholamines. This could be because the catecholamines are bound to a greater extent than 5-HT within their storage sites.

Some progress had been made in the technique using single isolated cells. The autofluorescence had been reduced to a tolerable level and a few small cells had been found that contained some specific fluorescence. This technique could probably be improved to demonstrate reliably the presence of monoamines within cells.

Procion dye had been used successfully on Helix neurones (Kerkut, French and Walker, 1970) but the brain was fixed in glutaraldehyde after the cells had been filled with the dye. In the present study it was found that the fluorescent properties of the Procion dye were not destroyed by freeze-drying or exposure to formaldehyde. This meant that it was suitable for combined use with the histochemical fluorescence technique for localising monoamines. The fluorescent properties of the dye enabled simultaneous examination of the monoamine fluorescence and dye fluorescence in the sections.

The proportions of types of responses to specific stimulation that were found are not necessarily equal to the proportions found for the ganglia taken as a whole because only a small group of cells was used in this study. For example most cells in this study were depolarised by the addition of ACh but for a larger area of the right parietal ganglion the ratio of 'D' cells : 'H' cells is 10:71 (Lambert, 1973).

Wilgenburg and Leewen (1971) have characterised some neurones in
the visceral and parietal ganglia of *Helix pomatia* by their pharmacology. They have shown 3 'D' cells and 1 'H' cell in both the visceral and right parietal ganglia in an almost identical arrangement to that found for cells 3, 4, 5 and 6 in the right parietal and cells A, B, C and E in the visceral ganglia of *Helix aspersa* found in the present study.

The pharmacology of the 5-HT containing cells in the right parietal and visceral ganglia was identical for the drugs tested in this study. All the 5-HT containing cells were depolarised by the addition of ACh or 5-HT and they were all hyperpolarised by the addition of DA to the bath. In this study no cells were found that were depolarised by DA (only one or two cells have been reported in *Helix* that show this response). No cells were found that were hyperpolarised by 5-HT although certain cells in other parts of the ganglia do show this response. Two or three cells that were hyperpolarised by ACh were present in the area of the fluorescent cells but they were always non-fluorescent after formaldehyde treatment. Cell 1 (the big 'D' cell) had a similar pharmacology to the 5-HT containing cells but this cell was always non-fluorescent after the histochemical treatment.

The nerve inputs of the 5-HT containing cells in the right parietal ganglion were similar. Stimulation of the four main nerve trunks produced an ILD response in the majority of cases. The similarity of the responses to specific stimulation in cells 4, 5 and 6 of the right parietal ganglion, which all contain 5-HT, suggests that these cells have a similar function.

In the visceral ganglion the nerve inputs of the 5-HT containing cells were different from those in the right parietal ganglion. Four of the five cells showed EPSP's after stimulation of all or some of the nerve trunks. One of the cells showed a slow IPSP after stimulation of the major nerve trunks. It is possible that some of these cells have similar functions.
The pathways of the 5-HT containing cells have not been traced using Procion dye. Only one antidromic AP was identified for the 5-HT cells. Stimulation of the anal nerve produced an antidromic AP in cell B. Elucidation of the axonal pathways of the 5-HT cells would be of value in determining the function of the cells. Procion yellow could be employed for this purpose.

A new technique has been developed for tracing the paths of axons and their branches which should prove useful for the 5-HT cells. The cell is filled with CoCl₂ from a microelectrode and this is allowed to spread throughout the neurone and its branches. The ganglionic mass is then immersed in \((\text{NH}_4)_2\text{S}\) solution and a black precipitate of cobalt sulphide is deposited throughout the neurone. The whole ganglionic mass is cleared using creosote and the treated neurone can be examined as a whole mount preparation.

Other cells known to contain 5-HT where the pharmacology has been determined are the Retzius cells of the Leech ganglia. These cells are hyperpolarised by the addition of 5-HT and depolarised by the addition of ACh (Kerkut and Walker, 1967; Kerkut, Sedden and Walker, 1967b). The giant cells in the cerebral ganglia of Helix aspersa which have been shown to contain 5-HT are depolarised by iontophoretic application of ACh (Kandel and Tauc, 1966).
Chapter 4

THE LOCALISATION OF THE DOPAMINE AND 5-HYDROXYTRYPTAMINE CONTAINING NEURONES IN PLANORBIS CORNEUS

4.1 Plan of Experiments

From the results of the previous sections it can be seen that in Helix aspersa brain only 5-HT containing neurones and neurones with no biogenic amines present in their cytoplasm were large enough and accessible enough for making intracellular recordings. Other work (Marsden and Kerkut, 1970) had shown that the water snail Planorbis corneus had a very similar arrangement of fluorescent neurones to that found in Helix with the exception of a very large DA containing cell in one of the pedal ganglia.

The purpose of the experiments in this section was to study the arrangement of the fluorescent neurones in the ganglia of Planorbis corneus in order to become familiar with their positions and in particular to locate the large DA containing neurone.

4.2 The Preparation

The circumoesophageal ganglia of the water snail Planorbis corneus was used for these experiments. In particular a large cell on the ventral surface of the left pedal ganglion was studied. Snails were obtained from a dealer and kept in an aquarium at 10°C or at room temperature until required. The animals kept at room temperature remained active but no difference was found in the results of the fluorescence.
Figure 4.1

Photograph of the ventral surface of the circum-oesophageal ganglionic mass of Planorbis corneus. The two cerebral ganglia are out of focus below the suboesophageal ganglia. See figure 4.3 for a diagram of the ganglia.
4.3 The Dissection

The dissection of Planorbis was essentially similar to the isolation of Helix ganglia described in section 2.12. The shell was removed and the foot pinned onto a wax block. The dorsal skin was cut and pinned back, exposing the gut and the reproductive apparatus. This and any other tissue was removed to expose the ganglia. The circum-oesophageal ganglia of Planorbis was smaller than that of Helix (about half the size). The outer connective tissue covering the ganglia was transparent and using the binocular microscope the orange-coloured cell bodies could be seen beneath. The connective tissue was removed for whole mount studies. The isolated ganglia are shown in figure 4.1.

4.4 Freezing the Tissue

a. For sections

The Planorbis tissue was frozen in the same way as described for Helix (paragraph 2.13) by immersing in precooled isopentane.

b. For whole mounts

Planorbis ganglia prepared for study as whole mounts were orientated on a small piece of paper before freezing in cooled isopentane.

4.5 Freeze-drying

During freeze-drying the same temperature and pressure were used for Planorbis as for Helix, that is -40°C and 0.01 torr but because the thickness of the tissue was less the drying time could be reduced. Overnight drying was used routinely.

4.6 Exposure of the Tissue to Formaldehyde Vapour

The freeze-dried tissue was exposed to formaldehyde vapour at +80°C for 1-3 hours. The relative humidity of the paraformaldehyde used was 60-70%. Control tissues were heated in the absence of formaldehyde.
for the same length of time.

4.7 Preparation and Examination of the Tissue

a. Sections

The freeze-dried, formaldehyde treated tissue was impregnated with wax (M.P. 56°C) in the manner described for Helix (paragraph 2.19). The tissue was then embedded, blocked and sectioned at 10μm. The sections were placed onto cleaned glass slides and heat flattened before examination under the fluorescence microscope. Transmitted light was used for the sections with filter system 'B' (paragraph 2.29) that is exciter filters BG 38 and UG 5 plus barrier filters 47 and/or 44.

b. Whole mounts

Whole mounts were cleared in light grade liquid paraffin under vacuum on the freeze-drier for 10-20 minutes. They were then examined under the Zeiss, large fluorescence microscope using epi-illumination. Filter system 'B' was used. The specimen was placed in a shallow dish containing liquid paraffin. This rested on a slide on the stage of the microscope. Light of the selected wavelength passed down the objective and onto the specimen. Reflected and emitted light passed back up the objective and to the eyepieces. The barrier filters were in the usual position and filtered out excess UV light.

4.8 Photography

The photography of sections of Planorbis brain was carried out on the photomicroscope and has been described in section 2.22. For whole mounts a single lens reflex camera was fitted onto the upright tube of the triocular head of the microscope. Test strips of various exposure times were taken to ensure correct exposure. The films used were the same as for Helix. For black and white photography Kodak Tri X Pan film
(ASA 400) was used. The film was developed in D.76 and fixed in F.5. Prints were made on Kodak bromide paper and developed in D.163 diluted to 25% with water and fixed in F.5. For colour transparencies Kodak High Speed Ektachrome film was used and colour prints were made from transparencies by Boots Ltd.

4.9 Differentiation Between Specific and Autofluorescence

It was possible to distinguish between the specific and the non-specific fluorescence by omitting the formaldehnyde during the histochemical treatment. The specific fluorescence was no longer present under these conditions.

4.10 Differentiation Between Monoamines

The main criteria used for the differentiation of catecholamine fluorescence and the 5-HT fluorescence was the colour difference. Using the filter system 'B' (UG 5 and BG 38 + 47 or 44) described in section 2.29, the catecholamine fluorescence appeared blue while that of 5-HT was yellow.

4.11 Differentiation Between the DA and NA Fluorophores

As in the case of Helix the fluorescence of these two catecholamines was not distinguished. Marsden (1970) has reported that Planorbis ganglia contained 9.53 μg DA/g wet weight tissue whilst noradrenaline was undetectable. In the light of these results it seems more likely that the catecholamine fluorescence was due to DA than to NA.

4.12 The Structure of Planorbis Brain

The brain of Planorbis was made up of nine ganglia. The connections between the ganglia were the same as for Helix although the cerebral ganglia were not separated by long commissures. A diagram of the
Dorsal view of the brain of *P. corneus* to show the arrangement of the ganglia. The cerebral ganglia have been separated (---) to display the suboesophageal ganglia and are seen on the ventral side.
Ventral view of the ganglia of *Planorbis corneus*. The positions of the statocysts in the pedal ganglia is shown.
arrangement is given in figure 4.2 and figure 4.3.

a. Cerebral Ganglia

This pair of ganglia lay dorsal to the other seven ganglia. A connective joined the left and right cerebral ganglia. Leaving each ganglia were two additional connectives. One of these joined to the corresponding pedal ganglion and the other to the corresponding pleural ganglion. These short connectives would correspond to the long commissures in Helix.

b. Suboesophageal ganglia

The remaining seven ganglia were arranged in a circle. The two pedal ganglia lay anteriorly. These were large ganglia. The right pedal ganglion had a connection with the right pleural ganglion lying next to it and this in turn was connected to the right parietal ganglion. Both these ganglia were small. The right parietal was separated from the left parietal ganglion by the visceral ganglion. The visceral and the left parietal ganglia were as large as the pedal ganglia. The left pleural ganglion was a small ganglion lying between the left parietal and left pedal ganglion. It made connections with both. The ganglia of Planorbis had a similar nerve supply to the Helix ganglia.
4.13 The Location of the Fluorescent Cells in Planorbis corneus

From the whole mount and section studies it was found that the cerebral, pedal, visceral and left parietal ganglia of Planorbis contained fluorescent cells. Most of the fluorescent cells were yellow but there was one large cell in the left pedal ganglion that contained a blue fluorescence. This cell was very large (>200 µ). From the studies using sections small blue fluorescent cells were found that were not seen in the whole mounts because they were below the surface of the ganglia. The neuropile in all the ganglia was found to contain fluorescent fibres as well as non-fluorescent ones. The fluorescence was either blue or yellow but the blue was predominant.

The distribution of the fluorescent neurones over the dorsal surface of the ganglia of Planorbis is shown in figure 4.4 and the distribution of the fluorescent cells over the ventral surface is shown in figure 4.5.

4.14 Identification of the Fluorescence

Two types of specific fluorescence were found in the ganglia of Planorbis corneus. The first type appeared to be yellow when examined under filter system 'B' (BG 38 and UG 5 + 47 and/or 44). The characteristics of this fluorescence corresponded with the characteristics of the fluorescence in the model 5-HT spots. The second type of fluorescence appeared to be blue under filter system 'B'. The characteristics of this fluorescence corresponded with those of the DA model spots. It is possible that some of the blue catecholamine fluorescence was due to the presence of NA. Chemical estimations (Marsden, 1970) indicate that the concentration of DA in the ganglia is high while NA was
Figure 4.4

Diagram of the dorsal surface of the circumoesophageal ganglia of Planorbis corneus showing the position of the cells that contain specific fluorescence after formaldehyde treatment.
Diagram of the ventral surface of the circumoesophageal ganglia of *P. corneus* showing the position of the cells that contain specific fluorescence after formaldehyde treatment.
A small amount of autofluorescence was also present in the sections but this was easily distinguished from the specific fluorescence by omitting the formaldehyde from the histochemical treatment. There was also a colour difference between the specific and the autofluorescence.

4.15 Cerebral Ganglia

This pair of ganglia contained a similar range of fluorescent cells to those found in the cerebral ganglia of Helix. There were groups of small blue and yellow cells (10-15μ). Groups of larger cells and a few isolated large cells were also present. It is possible that one of the large yellow cells corresponds to the giant yellow cells in Helix cerebral ganglia. Figure 4.6 (A) is a photograph of the cerebral ganglia in Planorbis showing a group of yellow fluorescent cells (15-40μ) in each ganglia. The neuropile contained fibres and varicosities of both types of specific fluorescence but the blue fluorescence predominated.

4.16 Pedal Ganglia

In each pedal ganglia there was a statocyst situated posteriorly. The large blue cell was located above the statocyst on the ventral surface of the left pedal ganglion. There was no such large blue cell in the right pedal ganglion. Also on the ventral surfaces of both pedal ganglia there were several medium to large yellow fluorescent cells (75-200μ). The dorsal surface also has a few yellow fluorescent cells. Figure 4.6 (B) shows a photograph of the ventral surface of the pedal, the large blue cell has been indicated. The remaining fluorescent cells were yellow.

In Helix the distribution of the yellow cells was similar to that in Planorbis but no large blue cell was found.
A. Photograph of the cerebral ganglia of *Planorbis corneus* showing a group of fluorescent cells in each ganglion. The fluorescence was yellow. C, is the connective between the two ganglia and N, is a nerve leaving the ganglion.

B. Photograph of a whole mount of *Planorbis* brain showing the ventral surface of the pedal ganglia and the presence of fluorescent cells. Bl, is a large cell in the left pedal ganglion containing blue fluorescence. The other fluorescent cells were all yellow. N, is a nerve leaving the ganglion.
4.17 Pleural Ganglia

These ganglia were very small and contained no fluorescent cells. The neuropile contained blue and yellow fluorescent fibres in addition to non-fluorescent fibres.

4.18 Right Parietal Ganglion

This ganglion was also very small and did not contain any fluorescent cells but like the other ganglia the neuropile contained fluorescent fibres of both types.

4.19 Left Parietal and Visceral Ganglia

These were two large ganglia lying posterior to the pedal ganglia. Yellow fluorescent cells were found in both these ganglia, mostly lying on the dorsal surface but a few were found on the ventral surface. Figure 4.7 (A) shows a photograph of the fluorescent cells on the dorsal surface of the visceral (V) and left parietal (LP) ganglia in a whole mount preparation of Planorbis brain.
A. Photograph of the dorsal surface of the visceral (V) and left parietal (LP) ganglia of Planorbis corneus showing clusters of fluorescent cells. The fluorescence was yellow.

B. Photograph of a nerve trunk containing fluorescent fibres in Planorbis corneus.
SUMMARY

1. The central ganglia of Planorbis corneus have a similar structure to the central ganglia of Helix aspersa.

2. The distribution of fluorescent cells after treatment with formaldehyde was similar in both pulmonates with the exception of a large blue cell present in Planorbis but not in Helix.

3. In Planorbis cells containing yellow fluorescence were found in the pedal, cerebral, left parietal and visceral ganglia. This yellow fluorescence had similar characteristics to the fluorescence of 5-HT model spots.

4. Small cells containing a blue fluorescence were found in the cerebral and pedal ganglia. A large blue cell (200μ) was found on the ventral surface of the left pedal ganglion. The blue fluorescence present in these cells corresponded to the fluorescence in the CA model spots. Planorbis ganglia contains a high concentration of DA with no detectable NA so the catecholamine fluorescence is most likely to be due to the presence of DA.
Discussion

The results presented on the distribution of monoamine containing cells in *Planorbis corneus* confirm the results of Marsden and Kerkut (1970) and Marsden (1970) with a discrepancy in the labelling of the right and left sides of the brain. In this study the ganglia of *Planorbis* have been described by the same convention used for *Helix* ganglia.

The fluorescent cells in *Planorbis* have a similar distribution to those in *Helix* with one or two notable differences. The most striking difference is the presence of a large blue fluorescent cell in the left pedal ganglion of *Planorbis*, no such cell was found in *Helix*. In *Planorbis* the connectives joining the cerebral ganglia with the pedal and pleural ganglia were much shorter than those in *Helix*. No fluorescent cells were found in the cerebro-pedal connectives of *Planorbis* to correspond with those present in *Helix*.

The blue fluorescence in the large pedal cell had the characteristics of catecholamine fluorescence with a peak in the emission wavelength at 480nm. Marsden (1970) reported a concentration of 9.53 μg DA/g tissue in *Planorbis* ganglia but was unable to detect any NA present. It is thus most likely that the blue fluorescence is due to the presence of DA in the cell.

The rest of the DA containing cells in *Planorbis* were small and were found below the surface of the ganglia as they were in *Helix*. The cells containing yellow, 5-HT fluorescence were mostly larger (75-150μ) and were found on the surface of the ganglia in the same way as those in *Helix*. The neuropile of *Planorbis* contained fibres and varicosities with both types of fluorescence but the blue fluorescence was predominant, this is similar to the case found in *Helix*. 
Chapter 5

THE IDENTIFICATION OF NEURONES IN PLANORBIS CORNEUS GANGLIA

5.1 Introduction

Various criteria could be used to identify neurones in Planorbis ganglia. For example:

1. The position of the cell in the ganglia with reference to the entry of nerves, other cells and structures (e.g. the statocysts).
2. The size and appearance of the cell.
3. The response of the cell to possible chemical transmitters, e.g. ACh, 5-HT, catecholamines and various amino acids. This is referred to as the pharmacology of the cell.
4. The pattern of any spontaneous electrical activity and action potential shape.
5. Nerve inputs onto the cell.
6. The path of axons leaving the cell identified by dye injection or other staining techniques.

For this study the first 5 criteria were used to identify the nerve cells. Nerve stimulation was not used for Planorbis. The methods used for determining the pharmacology of the Planorbis cells, and in particular the large blue cell, were the same as those used for Helix except that the brain was examined as a whole mount after the histochemical treatment instead of as sections.

5.2 Pretreatment with Dopa

Some of the snails were injected with 100 µg Dopa in 0.1 ml saline 1/2 - 2 hours prior to dissection. The purpose of this treatment was to increase the fluorescence of the blue cell by increasing the levels of DA present in the cell. In later experiments the fluorescence was
5.3 The Preparation

The circumoesophageal ganglionic mass of Planorbis was removed from the animal and secured, ventral side uppermost to a slide covered in plasticine by means of entomological pins. It was placed in a Petri dish containing a Ringer solution made up as follows:

- NaCl: 50.0 mM
- KCl: 1.6 mM
- CaCl$_2$: 4.0 mM
- MgCl$_2$: 8.0 mM
- Tris: 5.0 mM

adjusted to pH 7.5 with HCl.

The cell bodies could be seen through the outer connective tissue because this was virtually transparent and the cells themselves had a natural orange colour. The connective tissue was gently removed from the ventral surfaces of the pedal ganglia using fine forceps. The brain was then placed in the experimental bath described for Helix (paragraph 3.9). The bath contained 30 ml of the Ringer solution.

5.4 Microelectrodes

Microelectrodes were prepared in the same way as described for Helix (paragraph 3.11) and were filled with a 4% solution of Procion Yellow dye, M4RS in distilled water. A cell was selected by its position and size and the microelectrode was moved into position above the cell by means of a micromanipulator. The electrode was then lowered until it just dimpled the cell and the edge of the micromanipulator was gently tapped until the electrode entered the cell.
Potential changes in the cell relative to the bath potential were measured and recorded in the same way as for Helix (paragraph 3.12).

5.5 Pharmacology

Four drugs were used in this study. They were ACh, 5-HT, DA and glutamate. A series of suitable dilutions was made up for each drug in the Ringer solution. Tartrate was added to the DA solutions to prevent oxidation of the DA. The drugs were added to the bath in a volume of 0.2 ml from a pipette. The doses of drugs quoted in the figures are nMoles of drug added to the bath. Most of the cells showed a response to all the four drugs tested.

5.6 Data Recorded

The height and rate of firing of APs in the cell and the response of the cell to the addition of each of the drugs in turn was recorded. It was possible for the cells to desensitise to subthreshold doses of glutamate and hence not to show a response. To overcome this problem a large initial dose of glutamate was used. For the other drugs the dose was increased until a definite response was obtained. The membrane potential of the cell was usually recorded when the electrode was removed from the cell.

5.7 Dye marking

Neighbouring cells of the experimental cell were pulsed with Procion dye by applying negative current pulses to the electrode. Pulsing was usually continued for 15 minutes using pulses of 0.5 seconds duration at a rate of 1 per second. The dye could not be seen in the cells while it was being pulsed because it was masked by the natural orange colour of the cells. After freeze-drying, the orange colour of
the cells disappeared and the Procion dye was easily visible. The marked cells were used as a reference for locating the cell that had been identified. A drawing was made of the brain showing the relative positions of the impaled cells and any large nearby cells, statocysts etc.

5.8 Histochemical Treatment of the Ganglia

The brain was removed from the bath, blotted, orientated on a small piece of paper and quenched in isopentane which had been pre-cooled in liquid nitrogen. The frozen brain was then transferred to the freeze-dryer and left overnight at -40°C and 0.01 torr. The freeze-dried tissue was then exposed to formaldehyde vapour at 70% relative humidity and +80°C for 1-3 hours, cleared in liquid paraffin under vacuum and examined under the fluorescence microscope, using epi-illumination, for the experimental cell and the Procion marked cells. Filter system B (BG 38 and UG 5 + 47 and/or 44) was used which allowed the differentiation between 5-HT and catecholamine fluorescence to be made by a colour difference. It was also possible to distinguish between the specific and the autofluorescence by control tissue that had been heated in the absence of formaldehyde and by a difference in the colour of the two types of fluorescence. A drawing of the whole mount showing the positions of the fluorescent cells and the Procion cells was made and directly compared with the drawing made of the brain before histochemical treatment.
Figure 5.1
Diagram showing the positions of the identified cells on the ventral surface of the pedal ganglia of *Planorbis corneus*.
5.9 Electrical Activity in the Cells

The membrane potentials recorded in Planorbis cells fell within the range 35-65 mV. Most of the cells showed spontaneous overshooting action potentials. The size of the APs was 50-70 mV.

5.10 Pharmacology of the Cells in the Pedal Ganglia

The pharmacology of seven cells in the pedal ganglia has been determined. Figure 5.1 is a diagram of the ventral surface of the ganglia of Planorbis showing the positions of the identified cells within the pedal ganglia. The identified cells have been numbered 1-7.

**Cell 1**

Cell 1 was the largest cell in the left pedal ganglion (150-250μ). It was found on the ventral surface quite close to the statocyst in this ganglion. After treatment with formaldehyde this cell showed a blue fluorescence within the cytoplasm, the nucleus was non-fluorescent. The wavelength of the fluorescence had a maximum at 480 nm and showed the same characteristics as the fluorescence in the DA model spots. This cell usually showed spontaneous APs (60-70 mV) when impaled with a microelectrode. Some cells hyperpolarised and fell silent after a time.

Figure 5.2 shows a photograph of the pedal ganglia of Planorbis and the presence of the large blue fluorescent cell has been indicated.

The responses of cell 1 to the addition of drugs is shown in figures 5.3 and 5.4. The addition of ACh to the preparation caused an initial depolarisation followed by an inhibition, figure 5.3, and sometimes a hyperpolarisation, figure 5.4. This response is described as an inhibitory depolarisation and is discussed in greater detail in paragraph 5.11. The addition of 5-HT caused an increase in the firing
Figure 5.2
Photograph of the ventral surface of the pedal ganglia of Planorbis to show the cells containing monoamine fluorescence. B is the large cell containing blue, catecholamine fluorescence in the left pedal ganglion. The other fluorescent cells contained yellow, 5-HT fluorescence.
rate which lasted for a long time despite washing the preparation (see figure 5.4, trace B and figure 5.3, trace 4). 5-HT was often slower to show an effect than ACh. A large dose of glutamate was used to avoid subthreshold desensitisation of the response, the addition of 110 nMoles glutamate to cell 1 caused a hyperpolarisation, see figure 5.4 trace C.

The addition of DA to cell 1 of a snail that had not been pretreated with Dopa was a hyperpolarisation, see figure 5.3, trace 5 and figure 5.4, trace D. In snails that had been pretreated with Dopa the response of cell 1 to DA was no longer a hyperpolarisation but was a depolarisation. In certain cases this response was similar to the inhibitory depolarisation caused by ACh on this cell. In figure 5.5 trace A shows the response of cell 1 in a non-pretreated brain to the addition of DA to the bath. Trace B shows the response of cell 1 in a Dopa pretreated brain to the addition of DA to the bath.

Cell 2

Cell 2 was located to the right hand side (looking at the ventral surface) and slightly anterior to cell 1. It was smaller than cell 1 (100-150µ) and gave spontaneous APs of about 60 mV when impaled. Figure 5.6 shows the responses of cell 2 to the addition of drugs to the bath. Trace A shows an inhibitory depolarisation caused by the addition of ACh. This cell was very sensitive to 5-HT. Trace B shows a long-lasting depolarisation caused by the addition of 0.5 nMoles of 5-HT to the bath. There was a short delay between the addition of the 5-HT and the onset of the response. The addition of DA to the preparation caused an increase in the rate of firing in this cell. The response was more rapid than that of 5-HT but not as long-lasting, see trace C in figure 5.6.

The response to glutamate in this cell was not consistent. Half of the cells tested did not show a response to glutamate. Of the
The responses of cell 1 in the left pedal ganglion of Planorbis to drugs added to the bath. The recordings were continuous except where indicated.

A. Shows an inhibitory depolarisation to ACh and a hyperpolarisation to DA. B. 5-HT caused a slight depolarisation. C. Glutamate produced a hyperpolarisation. D. A larger dose of 5-HT produced a long-depolarisation. E. DA caused a depolarisation.
Figure 5.4

Responses of cell 1 in the left pedal ganglion of P. corneus to drugs added to the bath. The preparation was washed 20 seconds after the had been added. The recordings are continuous.

A. 1 nMole ACh caused a depolarisation followed by a hyperpolarisation.

B. 5-HT caused a long-lasting depolarisation. C. Glutamate caused a hyperpolarisation. D. DA caused a long lasting hyperpolarisation.
Figure 5.5

The effect of Dopa pretreatment on the DA response of Cell 1 in Planorbis corneus.

A. DA added to a preparation from an untreated snail caused a hyperpolarisation in this cell.

B. DA added to a preparation from a snail that had been pretreated with Dopa caused an inhibitory depolarisation in this cell.
remaining four cells two gave a depolarisation and excitation response, for example trace D in figure 5.6. The other two cells showed a definite hyperpolarising response. A possible explanation of this is that there are two cells occurring in an almost identical position with similar responses to the addition of ACh, 5-HT and DA. A second possibility is that the cells that were depolarised by glutamate were actually cell 3 in a different position.

After formaldehyde treatment cell 2 did not contain any specific fluorescence.

Cell 3

This cell was located on the right hand side and slightly posterior to cell 1, see figure 5.1. It was about the same size as cell 2 (100 μ). The APs in this cell were about 50 mV. The pharmacology of this cell is shown in figure 5.7. In trace A the addition of ACh gave an inhibitory depolarisation. In trace C the addition of 5-HT caused a slight depolarisation and a long lasting excitation. Glutamate caused a depolarisation and an excitation, see trace D. DA also caused a depolarisation and a long lasting excitation in this cell, see trace E.

There was no specific fluorescence present in this cell following formaldehyde treatment.

Cell 4

This cell was anterior and slightly to the left of cell 1 in the left pedal ganglion of Planorbis. It was about the same size as the surrounding cells (100μ). The APs in this cell were about 60 mV. The pharmacology of the cell is shown in figure 5.8. The addition of DA to the bath caused an excitatory response in this cell. The addition of 5-HT to the bath caused a long lasting depolarisation and increase in the firing rate. Trace A shows both these responses, it can be seen that after the response to DA the firing rate returned rapidly to normal
Figure 5.6

The response of cell 2 in the left pedal ganglion of *P. corneus* to drugs added to the bath.

A. 1 nMole ACh caused an inhibitory depolarisation in this cell.
B. 5-HT produced a long-lasting depolarisation.
C. DA caused a depolarisation.
D. Glutamate caused a depolarisation in this cell.
but the response to 5-HT was prolonged. In trace B it is seen that glutamate causes a hyperpolarisation and ACh causes a normal depolarisation accompanied by an increase in the firing rate.

The cytoplasm of this cell showed a yellow fluorescence after treatment with formaldehyde. This fluorescence had a peak at 525 nm and had the same characteristics as the fluorescence in the 5-HT model systems. The nucleus of the cell was always non-fluorescent.

Cell 5

Cell 5 was a large cell (100-150μ) at the top of the left pedal ganglion of Planorbis. The cell showed spontaneous APs when impaled with a microelectrode. Figure 5.9 shows the responses of the cell to drugs added to the bath. In trace A the addition of ACh caused an excitatory depolarisation. In trace B the addition of 5-HT caused a depolarisation and an increase in the firing rate that was more long lasting than the ACh response. The addition of glutamate to the cell caused a brief depolarisation and increase in firing rate followed by a short inhibition, this 'bursting' pattern continued for some time. In trace C the addition of DA caused a biphasic response. The initial phase was a small hyper-polarisation followed by a depolarisation. The cell in figure 5.9 required a larger dose of DA to show a response than other cells of this type.

Formaldehyde treatment produced a yellow fluorescence in the cytoplasm of this cell which had the characteristics of 5-HT fluorescence.

Cell 6

Cell 6 was located in the right pedal ganglion of Planorbis in a similar position to that which the large DA cell (cell 1) occupied in the left pedal ganglion. It was about the same size as the large-DA containing cell (150-200μ). The position of this cell is shown in figure 5.1 and the photograph in figure 5.2 also shows this cell.

Spontaneous APs from this cell were about 50-70 mV. Figure 5.10
Figure 3.7

The responses of cell 3 in the left pedal ganglion of Planorbis to drugs added to the bath.


E. DA caused a depolarisation.
The responses of cell 4 in the left pedal ganglion of Planorbis to drugs added to the bath. When the cell had responded to each drug the brain was washed.

A. The cell was depolarised by both DA and 5-HT. The 5-HT response persisted despite washing.

B. Glutamate hyperpolarised the cell and ACh depolarised the cell.
The responses of cell 5 in the left pedal ganglion of Planorbis to drugs added to the bath.

A. ACh caused a depolarisation and excitation.

B. 5-HT caused a depolarisation. Glutamate caused a brief increase in the firing rate followed by an inhibition. The cell continued to give bursts of APs followed by an inhibition.

C. DA caused a small hyperpolarisation followed by a depolarisation.
Figure 5.10

The responses of cell 6 to drugs added to the bath. This cell was found in the right pedal ganglion of Planorbis corneus.

A. ACh and 5-HT both caused a depolarisation.

B. DA caused a hyperpolarisation in this cell.

C. Glutamate also caused a hyperpolarisation.
shows the pharmacology of this cell. In trace A the addition of ACh to
the bath caused a depolarisation of the membrane potential and the
addition of 5-HT to the bath caused a similar depolarisation to that of
ACh but usually at a lower dose than the ACh response. Trace B shows
a large hyperpolarisation caused by the addition of DA to the bath, this
hyperpolarisation was approximately 15 mV. The response to glutamate
in trace C was biphasic. There was a short depolarisation with a burst
of 3 or 4 APs followed by a longer period of hyperpolarisation accompanied
by an inhibition of firing.

After formaldehyde treatment the cytoplasm of cell 6 contained
a yellow fluorescence that appeared to be due to the presence of 5-HT.
The nucleus of the cell was non-fluorescent.

Cell 7

The pharmacology of this cell has not been well tested and its
formaldehyde fluorescence has not been established. Preliminary results
indicate that ACh causes a depolarisation. 5-HT and DA also cause a
depolarisation and glutamate causes a hyperpolarisation.

The results of the fluorescence of the identified Planorbis cells
is shown in table 5.1 and the pharmacology of the cells is shown in
table 5.2.

5.11 The Responses of Planorbis Cells to ACh

Some Planorbis cells gave a 'normal' depolarisation response
when ACh was added to the bath. This depolarisation was accompanied by
an increase in the rate of firing of APs. In certain cells, however,
it was noticed that there was a depolarisation which was accompanied by
a decrease in the size of the APs and an inhibition in the firing rate.
Cell 1 was one of the cells to give this inhibitory depolarisation to
Table 5.1

The fluorescence characteristics of the identified neurones in *Planorbis corneus* and the amine that is most likely to be responsible for the fluorescence.

<table>
<thead>
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<th>CELL No.</th>
<th>FLUORESCENCE</th>
<th>AMINES</th>
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<tbody>
<tr>
<td>1</td>
<td>BLUE</td>
<td>Dopamine</td>
</tr>
<tr>
<td>2</td>
<td>NONE</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>NONE</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>YELLOW</td>
<td>5-HT</td>
</tr>
<tr>
<td>5</td>
<td>YELLOW</td>
<td>5-HT</td>
</tr>
<tr>
<td>6</td>
<td>YELLOW</td>
<td>5-HT</td>
</tr>
<tr>
<td>7</td>
<td>?</td>
<td>?</td>
</tr>
<tr>
<td>CELL No.</td>
<td>ACh</td>
<td>5-HT</td>
</tr>
<tr>
<td>---------</td>
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<td>------</td>
</tr>
<tr>
<td>1</td>
<td>D inhib</td>
<td>D</td>
</tr>
<tr>
<td>2</td>
<td>D inhib</td>
<td>D</td>
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<tr>
<td>3</td>
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<tr>
<td>7</td>
<td>D</td>
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</table>

**Key**

- **D** ______ Depolarisation
- **H** ______ Hyperpolarisation
- **D inhib** ______ Inhibitory Depolarisation
- **ILD** ______ Inhibition

*Table 5.2*

The pharmacology of the identified neurones in the pedal ganglia of *Planorbis corneus.*
Responses of different Planorbis cells to ACh added to the bath.

A. A 'classical' depolarisation where the depolarisation is accompanied by an increase in the size of the APs and their rate of firing.

B. A depolarisation accompanied by a reduction in AP size.

C. A depolarisation with a complete inhibition of firing.

D. A large depolarisation of the membrane potential in a silent cell. There is an initial short burst of APs followed by inhibition.
ACh. Very few cells gave a normal hyperpolarising response to ACh. The size of the inhibitory response was often found to increase with time. Figure 5.11 shows the different types of ACh responses found in Planorbis neurones. Trace A shows a 'classical' depolarisation response where the depolarisation is accompanied by an increase in the frequency of the APs. Trace B shows an inhibitory depolarisation where there is a decrease in the size of the APs but an increase in the frequency. Trace C shows a depolarisation of the membrane potential with a complete inhibition of firing. Trace D shows a large depolarisation in a silent cell which is accompanied by a few APs.

A similar phenomenon can be seen when a KCl electrode is used to record from a Cl\(^-\) 'H' cell (ie. a cell where the ACh 'H' response is mediated by Cl\(^-\) ions). Initially the response to ACh is a hyperpolarisation. As Cl\(^-\) ions from the electrode leak into the cell the internal Cl\(^-\) ion concentration, \([\text{Cl}^-]_i\), is increased, the difference between internal and external Cl\(^-\) ion concentration is decreased and the equilibrium potential for Cl\(^-\) ions, \(E_{\text{Cl}}\), moves towards 0 mV. \([\text{Cl}^-]_i\) varies from cell to cell and hence \(E_{\text{Cl}}\) also varies. If \(E_{\text{Cl}}\) moves to a value less negative than the resting potential then the addition of ACh will cause a depolarisation. This depolarisation is often seen to be inhibitory and cause a block in the formation of APs.

5.12 Test For the Presence of Chloride Ions in the Procion Yellow Solution

The electrodes used for all the Planorbis cell recordings were filled with a 4% solution of Procion Yellow dye in distilled water. The dye was supplied by I.C.I. Ltd. A solution of the dye was mixed with a silver nitrate solution to test for the presence of chloride ions. No white precipitate was formed and the result was negative. It has been shown (Kerkut and Thomas, 1964) that a number of ions can substitute
The effect of lowering the chloride concentration of the Ringer solution, $[\text{Cl}^-]_o$, on the size of the ACh response in cell 1 of Planorbis.

A. The response of the cell to the addition of 1 nMole ACh.

B and C. ACh (10 nMole) caused an inhibitory depolarisation (9mV in C).

D and E. The ACh response increased to 15mV when $[\text{Cl}^-]_o$ was reduced to 7mM.

F. The response decreased when normal $[\text{Cl}^-]_o$ was restored.
Figure 5.13

The effect of Cl⁻ free Ringer solution on an inhibitory depolarisation caused by Ach on a Planorbis neurone.

A and B. The response of the cell to 1 and 10 nMole Ach, respectively. Subsequent doses were 10 nMole.

C and D. The Ach response increased in the presence of Cl⁻ free Ringer.

E. The response was reduced again on restoring Cl⁻ to the bathing medium.
for chloride in causing a reversal of a hyperpolarisation. It was possible that the solution of the dye contained a different ion which had a similar effect.

A second possible explanation for this observation is given by Frazier et al (1967) for a similar response in Aplysia neurones. In these neurones the membrane potential is greater than the equilibrium potential for the ACh response. Despite the direction of the response to ACh these cells are inhibited by ACh and are 'H' cells.

5.13 Effect of Reducing the Chloride Ion Concentration in the Ringer

Figure 5.12 shows the effect of replacing some of the chloride ions in the Ringer solution with acetate ions on the inhibitory depolarisation response to ACh. Traces A and B show the response of the cell to the addition of 1 nMole and 10 nMoles respectively, a dose of 10 nMoles was chosen for subsequent use. Trace C shows a depolarisation of 9 mV after the addition of ACh. The experimental bath was then flushed with Ringer solution where the chloride concentration had been reduced from 75 mM to 7mM. In trace D the depolarisation caused by 10 nMoles of ACh was 13 mV. In trace E the depolarisation caused by the same dose of ACh had increased to 15 mV. The chloride concentration of the Ringer solution was restored to its normal value and the size of the depolarisation fell to 11 mV in trace F.

The effect of a chloride free Ringer solution on the ACh inhibitory depolarisation is shown in figure 5.13. Traces A and B show the response of the cell to the addition of 1 and 10 nMoles ACh respectively. A dose of 10 nMoles was chosen for the rest of the experiment. The Ringer solution in the bath was replaced with a Ringer solution where the chloride had been replaced with acetate. The addition of ACh to the cell in the presence of Cl⁻ free Ringer solution showed an increase in the size of the response from 6.6 mV to 20 mV, traces C and D. The
Figure 5.14
The reversal of the ACh response in cell 1 of Planorbis with time.
The arrows indicate the addition of 11 nMole ACh.
A. ACh added 1m after the cell was impaled caused a hyperpolarisation.
B. The addition of ACh 1m 40s after impaling caused a depolarisation.
C and D. The depolarisation to ACh was increased at 2m 30s and 7m 50s.
response was reduced to 8.5 mV when the normal Ringer was restored to the bath, trace F.

These results indicate that the response to ACh in these cells was mediated, at least in part, by Cl- ions. The value of $E_{Cl}$ for Planorbis cells was not known but in many Helix neurones it is known to be around -60 mV. The resting potentials of the Planorbis neurones varied but were usually about -40 mV. Although $E_{Cl}$ was not known for these cells it did not seem likely that their resting potentials were negative enough for this to be the explanation. The most likely explanation seemed to be that an ion was leaking into the cell from the electrode. An enquiry was made to I.C.I. Ltd. as to whether a solution of Procion dye would contain Cl- ions, Dr. G.V. Davy (I.C.I. Ltd.) has kindly confirmed this.

5.14 Effect of Time on the ACh Response

Additional evidence that these cells are 'H' cells is provided by the observation that the response to ACh that was added immediately after the cell was impaled was sometimes a hyperpolarisation. After a short time the response of the cell to ACh became a depolarisation accompanied by an inhibition. In figure 5.14 trace A shows a slight hyperpolarisation caused by the addition of ACh immediately after the cell had been impaled with an electrode containing Procion dye. Subsequent additions of ACh caused a depolarisation that increased in size, traces B-D.

5.15 Conclusions

In the light of the information obtained on the ACh response of these cells it seems that they are 'H' cells where the [Cl] has been altered. It will be necessary to confirm that the inhibitory 'D'
response cells show an 'H' response when an electrode that does not contain Cl ions is used for recording, for example an electrode containing potassium acetate would be suitable.
SUMMARY

1. Seven large neurones in the pedal ganglion of *Planorbis corneus* have been identified using electrical activity and drug responses as the criteria for identification.

2. Fluorescence studies have been carried out on 6 of these neurones. 3 of the 6 cells contained a yellow fluorescence, 1 of the 6 contained a blue fluorescence and the remaining 2 contained no specific fluorescence.

3. The yellow fluorescence had the characteristics of 5-HT fluorescence. The blue fluorescence corresponded to catecholamine fluorescence and was most likely to be due to the presence of DA.

4. The 5-HT containing cells were depolarised by both ACh and 5-HT. Their responses to DA were not similar. Their responses to glutamate were in two cases a hyperpolarisation and the third cell showed a biphasic response where the second phase was inhibitory.

   The 5-HT containing cells in *Helix* showed similar responses to ACh and 5-HT.

5. The results indicate that the DA cell was an 'H' cell (i.e. a cell that is hyperpolarised by ACh). 5-HT caused a depolarisation in this cell. DA and glutamate both caused a hyperpolarisation.

6. The results indicated that the non-fluorescent cells were also 'H' cells. In these cells 5-HT and DA both caused a depolarisation and the glutamate response was a depolarisation in one cell and a hyperpolarisation in the other.

7. The ACh response in the DA cell and the two non-fluorescent cells was mediated at least in part by Cl⁻ ions.
Discussion

In this chapter the results of the pharmacology and electrical activity of some cells in the pedal ganglia of *Planorbus corneus* have been presented. The result of exposing these cells to formaldehyde has also been reported.

Only neurones in the pedal ganglia of *Planorbus* were used for intracellular recordings. These neurones showed a spontaneous electrical activity that took the form of overshooting APs and was similar to the activity recorded in the *Helix* right parietal and visceral neurones. Attempts at recording from neurones in the pedal ganglia of *Helix* had been unsuccessful because removing the connective tissue covering the ganglia damaged the cells beneath. The identification of the cells after formaldehyde treatment was easier in *Planorbus* than in *Helix* because whole mounts could be used instead of serial sections.

All the tested 5-HT neurones in *Planorbus* were depolarised by ACh and 5-HT. The responses of the *Helix* 5-HT neurones to ACh and 5-HT were also depolarisations. The DA response in the *Planorbus* 5-HT neurones was not consistent. In one case it was a hyperpolarisation similar to the *Helix* neurones that contained 5-HT. In another cell it was a depolarisation and in the third cell the response was biphasic. The glutamate response for the *Planorbus* cells was a hyperpolarisation in two cases and a biphasic response in the third cell. Glutamate was not tested in *Helix*.

The large DA containing cell in *Planorbus*, cell 1, was an 'H' cell and was inhibited by ACh. The effect of changing [Cl\textsubscript{i}] on this response has been discussed in section 5.11. The 5-HT response of cell 1 was a depolarisation. (All the cells tested were depolarised by 5-HT). Glutamate on cell 1 caused a hyperpolarisation of the membrane potential. The addition of DA to cell 1 in untreated snails caused a hyperpolarisation. Where snails had been pretreated with Dopa the DA response of cell 1
was often a depolarisation. It was interesting that treatment with Dopa which probably increases the level of DA present in the cell appears to affect the response of this cell to the addition of DA. Aghajanian (1972) has reported that the injection of compounds affecting the metabolism of 5-HT or compounds that were structurally similar to 5-HT had an effect on the firing rate of the cells of the Raphe nuclei in mammalian brain. These neurones are known to contain 5-HT. The pretreatment of snails with Dopa could possibly affect the responses of the other cells.

The non-fluorescent cells that were identified in Planorbis were 'H' cells, that is they were inhibited by ACh. These cells were depolarised by 5-HT and DA and either depolarised or hyperpolarised by glutamate.

It was interesting to find that the responses of the 5-HT containing cells of Planorbis to ACh and 5-HT were the same as the responses of the Helix 5-HT neurones to these drugs. The outstanding difference between the pharmacology of the DA cell of Planorbis and the 5-HT cells of both pulmonates was that the DA containing cell was inhibited by ACh. The type of response to ACh seen in cell 1 was also found in the non-fluorescent cells. The main difference in pharmacology between the DA cell and the non-fluorescent cells was that the DA cell was hyperpolarised by DA but the non-fluorescent cells were depolarised by DA.

The pharmacology of cells that have been shown to contain DA or 5-HT has not previously been reported for Planorbis.
GENERAL DISCUSSION

The monoamines are of particular interest because there is evidence to suggest that they have a transmitter function in the CNS of vertebrates and invertebrates. The criteria that need to be fulfilled before a substance can be regarded as a transmitter have been discussed in section 1.1. Werman (1966) and Kerkut (1967) have suggested that there is no need for a substance to satisfy all the criteria for a transmitter that have been suggested in the past.

DA and 5-HT have been suggested as transmitters in *Helix, Planorbis* and other molluscan nervous systems. The evidence in support of a transmitter role for these amines is outlined below:

1. **The presence of the compounds in the nervous system**

   DA and 5-HT are present in the ganglia of *Helix* and *Planorbis* (Kerkut, Seddon and Walker, 1966; Marsden, 1970). DA is the major catecholamine present in the nervous system of these pulmonates. Both amines have been localised within the neurones and terminal varicosities using fluorescence microscopy (Dahl et al, 1966; Sedden et al, 1968; Marsden, 1970; this thesis). The distribution of the monoamine fluorescence in molluscan neurones is similar to the distribution in vertebrate neurones, i.e. the fluorescence in the soma is medium-strong, it is less strong in the axon and intense at the terminal varicosities. Gerschenfeld (1963) has described the presence of presynaptic dense-core vesicles in *Helix pomatia* that could be the storage sites for catecholamines.

2. **The presence of precursors**

   Dopa, the immediate precursor to DA, has been found in the blood of *Helix aspersa* (Kerkut et al, 1966). It is thought that the hydroxylation of tyrosine to Dopa takes place outside the ganglia.

3. **The presence of synthesising enzymes**

   Dopa can be converted to DA within the ganglia of *Helix aspersa* (Kerkut et al, 1966) and the decarboxylation of 5-HP to 5-HT by an
homogenate of *Helix* ganglia has been shown (Kerkut and Cottrell, 1963). It has been suggested that Dopa decarboxylase and 5-HTP decarboxylase are the same enzyme, amino acid decarboxylase (Cardot, 1963). There is a blood brain barrier to the entry of DA and 5-HT into neurones whereas Dopa and 5-HTP are able to enter the neurones freely. The decarboxylase enzyme may be located within the cytoplasm of the neurone or the cell membrane. No Dopa was detected in the ganglia of normal snails (Sedden, 1967) which suggests that the decarboxylation of Dopa either takes place freely or it is possible that the decarboxylase enzyme forms part of a specific uptake mechanism in the cell membrane.

4. The presence of a specific release mechanism

Gerschenfeld and Stefani (1968) have reported an increase in release of 5-HT by the central ganglia of *Cryptomphallus aspersa* in response to electrical stimulation. S-Rozsa and Perenyi (1968) have demonstrated a release of 5-HT from *Helix pomatia* heart following stimulation of the extracardial nerve. Reserpine causes a release of 5-HT in *Helix aspersa* and *Planorbis corneus* (Kerkut et al, 1966; Marsden, 1970).

5. The presence of an inactivation system

A chemical transmitter must be inactivated shortly after its release to account for its short time course. Two inactivation mechanisms have been established in nervous systems:

a. Enzymic. For example, cholinesterase in the cholinergic nervous system.

b. Re-uptake by the terminals. For example, the re-uptake of catecholamines by the presynaptic terminals in the vertebrate sympathetic system.

In vertebrate systems 5-HT is mainly metabolised by MAO (Sjoerdsm et al, 1955) to 5-HIAA. In most molluscan nervous systems MAO is thought to be absent or present in only small quantities (Blascho et al, 1937; Blascho and Hope, 1957; Kerkut and Cottrell, 1963; Kerkut et al, 1966).
Marsden and Kerkut (1970) have reported an increase in the yellow fluorescence in *Planorbis* after treatment with a MAO inhibitor and Marsden (1972) has demonstrated the presence of 5-HIAA in the ganglia of *Planorbis corneus*. He found no unidentified compounds present and has suggested that no other enzyme mechanisms were of major importance in 5-HT metabolism. Jurio and Killick (1972) have shown that metabolites of monoamines requiring the presence of MAO were absent or present in low concentrations in *Helix* ganglia. No evidence has been found for the presence of COMT activity in *Helix aspersa*. Diffusion away from the receptor site has been suggested by Gerschenfeld and Stefani (1968) as a possible inactivation mechanism for 5-HT in molluscs.

If 5-HT functions as a transmitter in the molluscan nervous system then the mechanism by which it is inactivated has still to be conclusively decided.

Possible Transmitter Functions of 5-Hydroxytryptamine

There is good evidence to support the idea that 5-HT is the excitatory transmitter onto *Venus* heart (see section 1.38) and 5-HT is probably an excitatory transmitter in other invertebrate hearts. S-Rozsa (1966) has demonstrated a release of 5-HT from *Helix* heart after stimulation of the cardiac nerve and Chase et al (1968) have shown a similar release from *Aplysia* heart. This release was reduced when Ca\(^{++}\) was removed from the bathing medium.

It is thought that 5-HT neurones have a motor function in some invertebrate nervous systems (Rude, 1966; Clark, 1966). The pedal ganglia of *Helix* contain many 5-HT neurones and nerves from these ganglia innervate the muscles of the foot. The cardiac nerve which supplies the heart is a branch of the intestinal nerve which originates in the visceral ganglion. It is possible that some of the 5-HT containing cells in the visceral or right parietal ganglia may send axons down the intestinal nerve trunk and innervate the heart, where 5-HT functions as
the excitatory transmitter. A branch of the 'big D' cell, cell 1, has been traced down the intestinal nerve using Procion dye (Kerkut, French and Walker, 1970). Cell 1 did not contain 5-HT. Chemical estimations have shown 5-HT to be present in the heart of Helix (Jurio and Killick, 1972). There was a considerable seasonal variation and in the winter 5-HT was undetectable.

There is evidence that 5-HT could function as a neurotransmitter in the leech, Hirudo medicinalis. 5-HT has been shown to be contained in the Retzius cells and in their axons (Kerkut et al, 1967a; Marsden, 1970). Axons from these cells pass down the lateral nerve and to the muscles of the body wall. Yellow varicosities found in the body wall after formaldehyde treatment are thought to be the terminals of the axons. Contractions of the body wall induced by ACh are inhibited by 5-HT. Walker, Woodruff and Kerkut (1968) have found that 5-HT inhibited excitatory potentials recorded in leech muscle fibres. This evidence suggests that 5-HT functions as an inhibitory transmitter in the muscles of the body wall in the leech.

5-HT is not entirely associated with motor functions as cells within the central nervous systems of invertebrates respond to it. For instance Cottrell (1970b) has presented evidence for 5-HT being the transmitter between the Giant Serotonin Cell (GSC) and a non-serotonin containing cell in the buccal ganglion with which it makes contact, see section 1.38.

5-HT has an excitatory effect on many Helix neurones and an inhibitory action on a few (Kerkut and Walker, 1962; Lambert, 1973; this thesis). 5-HT has an excitatory effect on some neurones in Planorbis (this thesis). Gerschenfeld and Stefani (1968) have presented evidence to suggest that 5-HT is the chemical transmitter responsible for the slow EPSP of the CIIDA neurones in Cryptonphallus aspersa (see section 1.38). They have suggested diffusion away from the receptor
sites as a possible inactivation mechanism. Gerschenfeld and Stefani (1966; 1968) demonstrated that the 5-HT receptors were located on the axon hillock and the first part of the axon but not on the soma. Gerschenfeld (1963) has reported the presence of axo-axonic synapses in molluscs and was unable to find any evidence for axo-somatic synapses. Tors'ka et al (1968) have reported axo-somatic and axo-axonal connections in the ganglionic layer of Planorbis and axo-dendritic and dendrodendritic connections in the neuropile.

5-HT has been suggested to be the transmitter involved in an EPSP response in a certain 'H' cell in the visceral ganglia of Helix aspersa (Kerkut, Ralph, Walker, Woodruff and Woods, 1971).

Small 5-HT containing cells within the nervous system of invertebrates may function as interneurones. There is no evidence for 5-HT containing sensory cells in the peripheral system of invertebrates.

Possible Transmitter Functions of Dopamine

DA has been shown to have an inhibitory effect on neurones in Helix aspersa (Kerkut and Walker, 1962; this thesis). Kerkut et al, (1969) have postulated that DA is the transmitter involved in the ILD response of the 'big D' cell, cell 1. They put forward the following evidence to support this idea:

a. Both the ILD and the DA hyperpolarisation were shown to be due to an increase in K⁺ permeability.

b. The equilibrium potential for the DA response is in the same range as that for the ILD response.

c. Both responses were selectively blocked by the α-blocker ergometrine. Walker, Ralph, Woodruff and Kerkut (1971) have presented evidence for a DA IPSP in a cell in the visceral ganglion of Helix.

Catecholamine containing cells have commonly been ascribed a sensory function e.g. in Nephtys (Clark, 1966), in a bivalve (Sweeney, 1968) and a slug (Osborne and Cottrell, 1971). Jurio and Killick (1972)
found DA present in the foot-muscle of Helix and have suggested that it may be associated with sub-epidermal sensory cells.

Only small DA neurones were found in Helix ganglia and the morphology and distribution of these cells suggests an interneurone function. There were large numbers of DA containing fibres and nerve terminals present in the neuropiles of all the ganglia. These fibres could originate from sensory cells in the periphery and from the small DA cells that are thought to be interneurones.

The case for Planorbis is similar with respect to the small DA cells and the fluorescent fibres in the neuropile. The large DA cell in the pedal ganglia is interesting because there was no corresponding cell in Helix and there have been no reports of a similar cell in other invertebrates. It would be of interest to trace the path of the axons from this cell in order to help elucidate its function.

There is thus considerable evidence to suggest that DA and 5-HT have a transmitter function in the ganglia of gastropods and other invertebrates.

Other Studies on Identifiable Monoamine Containing Cells

The giant Retzius cells in the ganglion of the leech Hirudo medicinalis have been shown to contain 5-HT (Kerkut, Sedden and Walker, 1967a). The cells are 60-80μ in diameter and are easily recognisable. Electrophysiological studies on these neurones (Kerkut, Sedden and Walker, 1967b) have shown that they have a resting potential between 40 and 50 mV and spontaneous APs of 60-80 mV. The pharmacology of the cells has been investigated and it has been shown that they are depolarised by the addition of ACh. The addition of 5-HT to these cells causes a hyperpolarisation. DA on these cells causes a hyperpolarisation.

The Giant Serotonin Cell of the metacerebral ganglia in Helix has been studied in considerable detail and this cell is depolarised by the application of ACh (Kandell and Tauc, 1966).
The results of the pharmacology of identified 5-HT containing cells are summarised in the table below. These cells are all 'D' cells, that is they are depolarised by the addition of ACh. The pulmonate 5-HT cells were also depolarised by 5-HT. The leech Retzius cells were hyperpolarised by 5-HT.

Pharmacology of the 5-HT Containing Neurones

<table>
<thead>
<tr>
<th></th>
<th>ACh</th>
<th>5-HT</th>
<th>DA</th>
<th>Glutamate</th>
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</thead>
<tbody>
<tr>
<td><strong>Helix aspersa</strong></td>
<td>D</td>
<td>D</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td><strong>Planorbus corneus</strong></td>
<td>D</td>
<td>D</td>
<td>D</td>
<td>H</td>
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<tr>
<td></td>
<td>D</td>
<td>D</td>
<td>H-D</td>
<td>Bursts of Activity</td>
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<td></td>
<td>D</td>
<td>D</td>
<td>H</td>
<td></td>
</tr>
<tr>
<td><strong>Hirudo medicinalis</strong></td>
<td>D</td>
<td>H</td>
<td>H</td>
<td></td>
</tr>
</tbody>
</table>

Glaizer (1967) described a neurone in the suboesophageal ganglia of Helix aspersa that responded to NA but not to DA and he proposed that different receptors exist for DA and for NA. The identification of NA within Helix ganglia in addition to DA (Osborne and Cottrell, 1969) supports the possibility that NA may have a transmitter function within the molluscan nervous system.
SUGGESTIONS FOR FURTHER EXPERIMENTS

In addition to extending the work described here for other cells in the two pulmonates, several points have arisen during this study which it would be interesting to follow up more closely.

1. Pretreatment with Dopa appeared to affect the responses of certain cells in Planorbis to the addition of dopamine. It would be interesting to investigate the effect of Dopa more thoroughly and also to investigate the effect of 5-HTP. In addition the effect of drugs which specifically lower the monoamine content of the cells (e.g. reserpine) could be investigated in this respect.

2. The technique described for tracing the paths of axons using cobalt chloride should prove useful in determining the areas innervated by the cells that have been identified. This would be helpful in determining their function.
APPENDIX

Acetylcholine.

\[
\text{CH}_3\text{C}=\text{O}\text{CH}_2\text{CH}_2\text{N}^+\text{CH}_3\text{CH}_3
\]

Dopamine (3-hydroxytyramine).

\[
\text{HO-}\text{C-H}_2\text{C-H}_2\text{NH}_2
\]

5-hydroxytryptamine.

\[
\text{HO-}\text{C-H}_2\text{C-H}_2\text{NH}_2
\]

Glutamate (used as the monosodium salt).

\[
\text{HOOC-C-H}_2\text{C-H}_2\text{COOH}
\]
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Addendum

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