A QUANTITATIVE STUDY OF RAPHE NEURONES
OF THE RAT

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The experiments described in the following pages were the work of the author of this thesis.

The investigations were supervised by Professor W. E. Watson, and were performed in the Department of Physiology, Faculty of Medicine, Edinburgh University.
SUMMARY

Rats were given drugs, many of which affect 5-hydroxytryptamine (5HT) metabolism in brain. Interference microscopy was used to measure the nucleolar dry mass of isolated nuclei from various interneurones. In all experiments the nucleolar dry mass of the median raphe neurones in the brainstem was measured: these neurones synthesize 5HT. The isolated nuclei of various hypothalamic neurones were also examined: the nucleolar dry mass of the suprachiasmatic neurones (which are postsynaptic for 5HT-containing terminals) and of the arcuate neurones was measured in most experiments. In a few experiments, the nucleolar dry mass of ventromedial, dorsomedial, preoptic and posterior neurones was also measured.

Following the daily administration of the monoamine-depleting drug, reserpine, the nucleolar dry mass of raphe neurones increased significantly from day 5 onwards, to an extent dependent on the dosage. Four days after withdrawing the drug, the dry mass value was again normal. The nucleolar dry mass of the suprachiasmatic neurones increased in one experiment, but not in another when larger doses of reserpine were used. The nucleolar dry mass of other hypothalamic neurones (apart from the ventromedial neurones) was not affected by reserpine. The drug oxypertine, which depletes only noradrenaline in brain, was given daily for 8 days; the nucleolar dry mass of raphe neurones did not change.

The daily administration of monoamine oxidase inhibitors alone for up to 10 days, or together with reserpine for 7 days, did not alter the
nucleolar dry mass of the neurones studied. The stresses of dehydration for 5 days or the daily injection of aqueous and oily solutions for 9 days did not affect the nucleolar dry mass of the raphe neurones.

Tricyclic antidepressant drugs (which inhibit the uptake of 5HT by neurones) were given daily for up to 9 days. The nucleolar dry mass of raphe and suprachiasmatic neurones decreased. For raphe neurones, the larger the drug dosage and the more potent the drug, the less pronounced was the decrease in dry mass. With suprachiasmatic neurones the higher drug dosages were the more effective.

The administration of lithium, which accelerates the uptake of 5HT by neurones, increased the nucleolar dry mass of raphe neurones, but not of suprachiasmatic and arcuate neurones.

Various monoamines, their precursors and their analogues were given by intraventricular infusion or by intraperitoneal injection for 7 days or more. The nucleolar dry mass of raphe neurones decreased only after 5HT, melatonin and tryptophan.

When diamorphine was given daily, the nucleolar dry mass of raphe and suprachiasmatic neurones was maximal at days 11-12 and days 15-18 respectively. The nucleolar dry mass of other neurones did not change.

In rats receiving barbitone orally for up to 37 days, the nucleolar dry mass of raphe, arcuate and suprachiasmatic neurones was unchanged at day 5; but a significant decrement was seen by day 14. This nucleolar change was most pronounced in the raphe neurones (where it was maintained for 37 days).
and least pronounced in the suprachiasmatic neurones. Six days after withdrawing barbitone, the nucleolar dry mass of these neurones reverted to normal.

The effect on neuronal nucleolar dry mass of altering the levels of various steroid hormones was investigated. With daily injections of testosterone for 6–14 days, the nucleolar dry mass of raphe neurones increased, while that of suprachiasmatic neurones decreased. The injection of cortisone for 14 days had no effect on the raphe neuronal parameter. After adrenalectomy, alone or combined with cortisone injections, no nucleolar changes were seen in the raphe, arcuate or suprachiasmatic neurones.

The changes in the nucleolar dry mass of various neurones were discussed in relation to the altered metabolism of brain 5HT reported to follow these experimental procedures.
ACKNOWLEDGEMENTS

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'I have often admired your consummate wisdom, O Cato. It is shown in many ways, but in none more perfectly than in the singular ease and cheerfulness with which you bear the weight of years' (Cicero: 'De Senectute'). Thus I pay tribute to the brio and sagacity of my youthful, octogenarian father. While this thesis was being prepared, this Celtic Cato frequently gave enthusiastic encouragement and supported morale. He also lent that calming sense of perspective which was celebrated by the scientist, Omar, son of Ibrahim the Tentmaker:

The Wordly Hopes men set their Hearts upon
Turns Ashes - or it prospers; and anon,
Like Snow upon the Desert's dusty Face
Lighting a little Hour or two - is gone.

How long, how long, in infinite Pursuit
Of this and That endeavour and dispute?
Better be merry with the fruitful Grape
Than sadden after none, or bitter, Fruit.

But leave the wise to wrangle, and with me
The Quarrel of the Universe let be:
And, in some corner of the Hubbub coucht,
Make Game of that which makes as much of Thee.
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<tr>
<td>AAAT</td>
<td>aromatic-L-amino acid transferase</td>
</tr>
<tr>
<td>ACTH</td>
<td>adrenocorticotropic hormone</td>
</tr>
<tr>
<td>CSF</td>
<td>cerebrospinal fluid</td>
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<tr>
<td>5HT</td>
<td>5-hydroxytryptamine</td>
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<td>5HTP</td>
<td>5-hydroxytryptophan</td>
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<td>5HIAA</td>
<td>5-hydroxyindole acetic acid</td>
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<tr>
<td>MAO</td>
<td>monoamine oxidase</td>
</tr>
<tr>
<td>N.S.</td>
<td>not significant</td>
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<tr>
<td>OPD</td>
<td>optical path difference</td>
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<tr>
<td>6-OHDA</td>
<td>6-hydroxydopamine</td>
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<tr>
<td>PCPA</td>
<td>p-chlorophenylalanine</td>
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<tr>
<td>RNA</td>
<td>ribonucleic acid</td>
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<tr>
<td>S.E.</td>
<td>standard error</td>
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<td>S.C.</td>
<td>suprachiasmatic</td>
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SECTION 1. INTRODUCTION
1.1 The metabolic role of neuronal nucleoli

The nucleolus is essential for the synthesis and assembly of cellular ribosomes (1-3). For example, anucleolar mutants of *Xenopus* cannot synthesize ribosomal ribonucleic acid (4); they lack the ribonucleoprotein granules which are components of normal nucleoli (5) and which, it is thought, are the precursors of ribosomes (6). Using both autoradiographic and sedimentation techniques, the incorporation of 3H-cytidine into ribonucleic acid by fibroblasts has been studied in the presence and absence of actinomycin D: ribosomal ribonucleic acid was found to originate from nucleolar ribonucleic acid (7). This finding has been supported by the results of studies on nucleic acid hybridization (8), and also by the demonstration by microchemical methods that the base compositions of both nucleolar and ribosomal ribonucleic acid are similar (9).

In neurones, nucleolar changes accompany prolonged alterations of cellular synthetic activity. For example, nucleolar volume is correlated with the cytoplasmic content of ribonucleic acid (10), which in turn is linked to the rate of protein synthesis in the neurone (11). During the sustained osmotic stimulation which accompanies salt loading, the volume and ribonucleic acid content of nucleoli in supraoptic neurones increase (12). The relationship between nucleolar changes and the rates of ribosome and protein synthesis in various neurones has been confirmed in other studies (13-15).

Techniques of ultraviolet absorption microspectrography and interference microscopy have been used to monitor changes in the nucleic acid
content and dry mass respectively of neuronal nucleoli (16, 17). In the response of hypoglossal neurones to injury, the nucleic acid content of these neurones' nucleoli rises initially, to be followed by a similar change in their cell bodies (18). During dehydration, the supraoptic neurones of rats yield increasing values for nucleolar nucleic acid and dry mass, and also for cell body nucleic acid (19, 20). In lactating rats, a similar quantitative relationship has been demonstrated between nucleolar nucleic acid and dry mass of the paraventricular neurones and the functional demand for hormone production by these neurones (21, 22).

The hypoglossal, supraoptic and paraventricular cells have distinctive motoneurone or neurosecretory functions. The aim of the present investigation was to detect changes in the nucleolar dry mass of various interneurones in the brainstem and hypothalamus.

1.2 The raphe neurones

1.2.1 Introduction

The raphe nuclei are situated in the brainstem of the central nervous system; many neurones in these nuclei contain 5-hydroxytryptamine (5HT) (23, 24). By using techniques such as histofluorescence (25, 26), biochemical analysis and lesion-induced degeneration, axons from the 5HT-containing raphe neurones have been demonstrated in tracts which terminate in various regions of the central nervous system, - particularly in the forebrain, hypothalamus, and spinal cord (27-36).
Fig. 1.2.2. SOME PATHWAYS FOR 5HT METABOLISM
1.2.2 The biochemistry of 5HT in raphe neurones

The 5HT within the raphe neurones is synthesized there. The principal and other suggested pathways for brain 5HT metabolism are summarised in Fig. 1.2.2.

1.2.3 5HT synthesis in raphe neurones

Tryptophan is actively transported (63) across the membrane of all parts of raphe neurones (64); the isolated neuronal terminals retain the capacity for accumulating tryptophan (65, 66). The speed of this process and the resulting concentration of tryptophan in raphe neurones may influence their rate of 5HT synthesis, since the rate-limiting enzymic step for increases in 5HT production is the hydroxylation of tryptophan (67-74). However there is much evidence to support the view that major changes in the rate of 5HT synthesis depend on the availability of tryptophan hydroxylase, rather than of its substrate. For example, the saturation of tryptophan hydroxylase by tryptophan in brain tissue is almost complete (75-77). Excess tryptophan can inhibit tryptophan hydroxylase, at least in vitro (77). When more tryptophan is made available in brain tissue, there is not a consistently parallel increase in the concentration or turnover of 5HT (78-81). In regions of the adult or developing brain, 5HT concentration correlates well with tryptophan hydroxylase activity (82-84) but not with the concentration of tryptophan (85). Lastly, when tryptophan hydroxylase is inhibited, its declining activity runs parallel to the fall in 5HT concentration (37).

Tryptophan hydroxylase is specific (86-88). In brain, its presence exclusively in the raphe neurones ensures that only in these neurones is 5HT
produced from tryptophan (89). Tryptophan hydroxylase is synthesized mainly in the cell bodies of the raphe neurones, and is then transported distally to their terminals (36, 90-92). However the synthesis of some enzyme within the terminals themselves cannot be excluded in view of the evidence that ribonucleoprotein is present in neuronal axons and terminals (93-98) and that protein synthesis takes place there (99-106). For example, the production of the biosynthetic enzymes for noradrenaline in noradrenergic terminals may be controlled by "informational molecules" synthesized in the cell bodies of the neurones, and subsequently transported to the terminals (107-108).

Although the enzyme aromatic-L-amino-acid transferase (AAAT), like tryptophan hydroxylase, is probably synthesized in the cell bodies of the raphe neurones and then transported to their terminals (109, 110), AAAT differs from tryptophan hydroxylase in significant respects. In brain tissues other than the pineal gland, AAAT is not saturated (78, 86, 111). AAAT is also non-specific (112-115), closely-related enzymes being present in other monoaminergic neurones (116) as well as in neuroglia and capillary endothelium (113).

Whereas 5HT-synthesizing enzymes are formed mainly in the cell bodies of the raphe neurones, there is evidence that 5HT itself is produced mostly within the terminals of these neurones. For example 5HT is synthesized autonomously by the terminals in brain slices (117) and synaptosomes (118); adult values for 5HT concentration in the maturing brain are
approached only as the nerve endings develop (118); in the circadian rhythm described for brain 5 HT concentration (120-123), the amplitude of change is maximal in regions rich in 5HT-containing terminals, but minimal in those regions containing the raphe neuronal cell bodies (124). Nevertheless, some of the 5HT in raphe neuronal terminals may be synthesized in their cell bodies and then conveyed along their axons to the terminals.

Various findings support this contention. Firstly, when the molluscan brain is perfused with solution containing tritiated tryptophan or 5-hydroxytryptophan (5HTP), the isotope label is detectable over the cell bodies of the 5HT-containing neurones, - and in particular over the Golgi apparatus -, whereas little or no label is found over the terminals (125). Secondly, when monoamine oxidase (MAO) in mammalian brain is inhibited, 5HT fluorescence is seen forming firstly as a perinuclear ring in the raphe neurones and then permeating their cell body cytoplasm before eventually spreading to their axons and terminals (126); in noradrenaline-containing neurones a similar sequence has been demonstrated after MAO inhibition, and also for the return of noradrenaline histofluorescence after reserpine (126-128). Thirdly, small amounts of 5HT accumulate above constricted or damaged segments of the 5HT-containing axons in molluscan and mammalian central nervous systems (129, 130), in a manner resembling the accumulation of noradrenaline in ligated axons of sympathetic neurones (131-134).

1.2.4 5HT breakdown in raphe neurones

The breakdown of 5HT into 5-hydroxy-indole acetic acid (5HIAA) is catalyzed by MAO (135-137), a family of relatively nonspecific enzymes
MAO plays an important role in regulating the amount of "free" 5HT in the cytoplasm of raphe neurones (146, 147).

The cellular site of MAO synthesis in raphe neurones is unclear. However, the inference of work on other monoaminergic neurones is that neuronal MAO is synthesized in the cell bodies (148, 149) and terminals (103) of neurones, but possibly also in adjacent non-neural cells (149, 150). Indeed, MAO-containing cells which are post-synaptic to monoaminergic neurones, or which have no apparent synaptic communication with them, may be partly responsible for inactivating the monoamines released from those neurones (145, 151-155).

1.2.5 5HT storage in raphe neurones

Many vesicles of diameter ranging from 50nm to 170nm are present at the raphe neuronal terminals close to the synaptic clefts (156, 157). In various monoamine-containing cells, comparable organelles act as monoamine stores (158-164); this is also their function in raphe neuronal terminals (165, 166). Indeed, most of the 5HT in raphe neurones is bound within these vesicles (126, 167-171) which afford protection from cytoplasmic MAO (172-175). When the 5HT content of raphe neurones is suddenly increased, either by tryptophan-loading (81, 169, 176) or by the administration of 5HT (137, 177), the intraneuronal concentration of 5HT rises only initially; the concurrently-elevated concentration of 5HIAA results from free cytoplasmic 5HT being degraded by MAO, once the vesicular stores for 5HT have been filled. When these vesicles are disrupted by reserpine, the concentration
of 5HT in brain falls precipitously, while that of 5HIAA increases.

Because of their anatomical and biochemical complexity, it was long assumed that the vesicles in monoaminergic terminals are transported there from their biosynthetic sites in the neuronal cell bodies (178, 179). This view has been sustained by subsequent findings. For example, vesicles are reported in the perinuclear zone of raphe neurones, often close to the Golgi apparatus (24, 74, 180). In other neurones, this cellular region is also implicated in the synthesis of vesicles (181,182); for instance, workers have demonstrated the reserpine-sensitivity of the perinuclear ring of noradrenaline fluorescence, which is seen in noradrenergic neurones after reserpinnzation (126) (see Introduction 1.2.3).

The subsequent transport of vesicles to neuronal terminals has been shown clearly in noradrenergic neurones and in 5HT-containing snail neurones: after axonal ligation (129, 179), electrocoagulation (35), or colchicine application (183), vesicles accumulate proximal to the affected axonal segments. After the disruption of the vesicles in noradrenergic terminals by reserpine (184), recovery of function in these terminals depends on the arrival by axonal transport of vesicles synthesized in the cell bodies (29, 185-189).

Therefore, from both direct and indirect evidence, it appears that the synthesis of the storage vesicles of raphe neurones is a metabolic function of their cell bodies.
1.2.6 The release and recapture of 5HT by raphe neurones

There is considerable evidence that raphe neurones release 5HT from their terminals: (a) endogenous and exogenous 5HT is released from brain slices when these tissues are stimulated electrically or by exposure to a high-potassium medium; the release of tritiated water and 14C-urea is unchanged under these circumstances (190-192); (b) small amounts of 5HT are released continuously into cerebrospinal fluid from brain tissue under basal conditions: when the cell bodies of the raphe neurones are stimulated, more 5HT is released (193-195), and the concentration of 5HIAA in brain and cerebrospinal fluid rises (196-199); (c) in the distal segment of a transected spinal cord, the failure of impulse transmission is associated with the conservation of 5HT and the decline of 5HIAA formation (200).

When noradrenergic neurones are stimulated, only the vesicular noradrenaline is released from their terminals. As yet there is no direct evidence that vesicular 5HT is of similar importance for the function of raphe neuronal terminals (201).

Extraneuronal 5HT, whether of endogenous or exogenous origin, is actively accumulated by the raphe neurones. The activity of the membrane pump responsible for this function has been demonstrated in raphe and other neuronal terminals by autoradiography (177, 202-206), electron microscopy (125) and histofluorescence (209). But the evidence for a comparable uptake efficiency in the cell bodies of 5HT-containing neurones is conflicting (125, 165, 209-211).
The re-uptake of released 5HT by the raphe neuronal terminals has two consequences. Firstly, the partial replenishment of the presynaptic 5HT stores by this mechanism may minimize the need for 5HT synthesis during neuronal activity: in sympathetic neurones, for example, 50-95% of the noradrenaline released from the terminals is recoverable by membrane transport (147, 212). Secondly, the actions of the released 5HT on the postsynaptic cell membrane are terminated (see Discussion 3.3.4.3).

1.2.7 The effects of 5HT on the membranes of postsynaptic cells

The iontophoretic administration of 5HT provokes excitation or depression of cerebellar (213) and cortical neurones (214–216); with brainstem neurones, excitation, inhibition or mixed responses have been elicited (217–219); with spinal motoneurones, 5HT is purely inhibitory (220). In the suprachiasmatic nucleus, which contains large numbers of raphe neuronal terminals (29, 32, 156, 210, 213, 221–223), neuronal firing is inhibited by iontophoretically-applied 5HT and by the electrical stimulation of the raphe neurone bodies in the midbrain (213).

However there is evidence that tachyphylaxis occurs for 5HT in peripheral tissues (153, 224–228) and also in the 5HT synapses of the molluscan (227) and mammalian (216, 219, 226, 229) central nervous system.

1.2.8 Measurement of brain 5HT turnover

Raphe neuronal 5HT is the major source of the 5HT in brain (23, 29, 230, 231). Therefore if 5HT turnover in brain is measured, a major aspect of raphe neuronal metabolism can be monitored (155).
The methods by which brain 5HT turnover is measured fall into two categories (155, 232). In the first category comes the use of various specific metabolic inhibitors. For example, 5HT turnover can be measured indirectly, (a) from the initial rate of increase in brain 5HIAA concentration after the active expulsion of this 5HT metabolite has been blocked by probenecid or dinitrophenol (233, 234); and (b) from the initial rate of 5HIAA decline or 5HT rise after using a MAO inhibitor (235). In the second category of methods, isotopically-labelled substances are used (236): for example, (a) after MAO inhibition, the rate at which 14C-labelled tryptophan and 5HTP are converted into 5HT can be measured (61, 237); and (b) after tritiated 5HT has been taken up by brain tissue either in vitro or in vivo (after its intraventricular injection), the rate of disappearance of isotopic activity also illustrates turnover (155, 238).

However the theoretical bases of these techniques for measuring 5HT turnover are suspect (232). Firstly, the inhibition of reactions by the relevant drugs is probably never specific and absolute (239-241). Secondly, other metabolic pathways for 5HT (231) (see Introduction 1.2.2) provide alternative routes whereby the pharmacological blockade of individual steps of the principal 5HT metabolic pathway can be circumvented by 5HT or its precursors (242, 243). The reliability of turnover studies which are interpreted on the assumption that 5HT metabolism is completely and specifically blocked at one particular stage is therefore questionable. Thirdly, the uptake, storage, and destruction of exogenous, isotopically-labelled substances are assumed to exemplify the intracellular fate of endogenous substances (232,
while this assumption may be tenable for isotopically-labelled tryptophan (64), for exogenous 5HT this assumption is questionable (177, 202, 203, 246-248). The genesis of this controversy over the fate of exogenous 5HT may lie in discrepancies in experimental procedures, - for example in the types of anaesthesia (202, 203), homogenization and centrifugation used (249, 250).

Although such technical factors make it difficult to measure small changes of turnover of brain 5HT convincingly, several conclusions concerning 5HT metabolism in brain can be accepted.

Firstly, 5HT turnover is faster in brain than in other tissues (61, 251). Secondly, the regional turnover of 5HT in brain cannot be predicted from the local 5HT concentration (252, 253). Thirdly, the rate of 5HT metabolism varies in different regions of the brain (238, 253-255), and can be altered by various procedures.

1.2.9 Alterations in the rate of 5HT synthesis in raphe neurones

There have been many reports of an accelerated synthesis of 5HT in brain following the electrical stimulation of the raphe nuclei (197, 256-260) or of regions rich in 5HT terminals (261). In the raphe neuronal terminals of the transected spinal cord, the amount of spontaneous electrical activity in the cord segments may determine the rate of 5HT synthesis (24, 262, 263). Various drugs alter the rate of 5HT metabolism: for example, the turnover decreases after the administration of lysergic acid (264, 265) and imipramine-like drugs (222, 266-268); turnover increases after reserpine and lithium (235, 269, 270).
In monoaminergic neurones, transient changes in the rate of monoamine production may result from the altered activity of a constant amount of synthesizing enzyme already present in the nerve terminals (271-273). The biochemical mechanisms responsible for these changes may include variations in the precursor uptake (see Introduction 1.2.3), intracellular electrolyte concentration (274-277), cofactor and coenzyme availability (40, 87, 278, 279), or end-product inhibition (237, 280-282). But in noradrenergic neurones, prolonged increases in the rate of noradrenaline synthesis result from greater amounts of synthetic enzymes being produced by induction in the cell bodies of these neurones (283-288); this process can be aborted by inhibiting the synthesis of ribonucleic acid and protein (108, 289-291). A similar biosynthetic mechanism may operate in 5HT-synthesizing cells (269, 292, 293).

1.3 Conclusions

Certain conclusions can be drawn from the published work on raphe neurones. 5HT is probably a neurotransmitter (190, 294-296). The enzymes necessary for 5HT synthesis and breakdown, and for the synthesis of storage vesicles are produced in the cell bodies of the raphe neurones. Changes in the rate of 5HT formation have been detected and measured using various techniques. Long-term alterations in 5HT-synthesis-rates may be mediated by the protein-biosynthetic mechanisms in the cell bodies of these neurones.

When 5HT is released from the terminals of raphe neurones, the electrical activity of most postsynaptic neurones - such as those in the suprapineal and pineal nucleus - is inhibited.
1.4 Aims of thesis

In the present study, the technique of interference microscopy was used with the following intentions:

a) to confirm that, in interneurones such as the median raphe neurones, changes in nucleolar dry mass could be induced which were comparable to those reported in motor neurones and neurosecretory neurones (see Introduction 1.1);

b) to see if such changes in the nucleolar dry mass of raphe neurones were followed by changes in the nucleolar dry mass of neurones postsynaptic to raphe neurones, for example the suprachiasmatic neurones in the hypothalamus (see Introduction 1.2.7);

c) to ascertain whether or not drug-induced changes in the nucleolar dry mass of the median raphe and suprachiasmatic neurones could be related to the concurrent alterations in 5HT metabolism reported by other investigators using other techniques.
SECTION 2. GENERAL METHODS
2.1 Preparation of animals

Unless otherwise stated, male albino rats, aged 3-3½ months and weighing 250-300 gm were used in the experiments. They were grouped 4-6 per cage, and allowed free access to water and rat pellets. After arrival in the departmental animal house, the rats were allowed at least 10 days to adapt before any experimental procedure was started. Drugs and control solutions were administered as described for each experiment. Daily intraperitoneal injections were given at 09.00 hours; in some experiments, a second injection was given at 16.00 hours. In most cases, animals were killed between 11.00 hours and 13.00 hours.

The animals were killed by exsanguination under light ether anaesthesia.

2.2 Preparation of specimens for microscopy

A longitudinal midline incision was made in the scalp. The brain was exposed using bone nibblers to remove the vault of the skull. Two transverse cuts were made in the brainstem, vertical to the base of the skull, above and below the superior corpora quadrigemina. A thin slice of brainstem tissue, about 1 mm thick, was then freed from underlying tags of tissue, lifted out, and placed in a watch-glass containing cold homogenization medium. With a dissecting microscope, the median raphe region was identified by its relationship to obvious adjacent structures (Fig. 2.2.a.)(297). A small block of median raphe neurone tissue (1 x 0.5 x 0.25 mm) was excised from this region (Figs. 2.2.b.-d.). No other raphe nuclei were examined.
Fig. 2.2.a.: Diagram of brainstem, transverse section. Abbreviations represent cerebral aqueduct (c.a.), dorsal raphe nucleus (d.r.n.), superior cerebellar peduncle (s.c.p.), tectospinal tract (t.t.), median raphe nucleus (m.r.n.), trigeminal nerve, sensory root (t.), and raphe (r.).

Fig. 2.2.b.: Transverse section of brainstem, stained by H & E, showing median raphe region partially excised; magnification x 18.
Fig. 2.2.c.: Transverse section of median raphe region, partially excised, and stained by H & E; magnification x 250.

Fig. 2.2.d.: Section of median raphe region, totally excised, prepared as in Fig. 2.2.c.
Fig. 2.2.e.: Diagram of medial aspect of bisected hypothalamus; abbreviations represent thalamus (t.), anterior commissure (a.c.), preoptic nucleus (pr.n.), optic chiasm (o.c.), suprachiasmatic nucleus (s.n.), ventromedial nucleus (v.n.), arcuate nucleus (a.n.), dorsomedial nucleus (d.n.), posterior nucleus (po.n.), and mamillary nucleus (m.b.).

Fig. 2.2.f.: Preparation of medial hypothalamus, stained by H & E, magnification x 25.
To obtain tissue containing hypothalamic nuclei, the rest of the brain was removed from the skull, placed in a watch-glass containing homogenization fluid, and then viewed down a dissection microscope (x 12 magnification). The base of the brain was bisected by means of a midline incision which ran posteriorly through the hypothalamus from a point directly anterior to the optic chiasm. The underlying cavity of the third ventricle was seen. The two brain halves were gently cut apart, leaving the medial (ventricular) surface of each hypothalamus exposed.

The various hypothalamic nuclei were identified by means of adjacent landmarks (Figs. 2.2.e.-f.)(297). Small blocks of tissue (0.5mm cubed) were excised as indicated later in the text. These tissues contained the supra-chiasmatic, arcuate, preoptic, ventromedial, dorsomedial and posterior nuclei. Usually the corresponding tissues from both halves of the hypothalamus were pooled.

Each block of tissue was transferred to a small homogenizer (Fig. 2.2.g.) containing approximately 2.5μl of homogenization fluid. The composition of this fluid was: 0.34 M sucrose, 0.002 M MgCl₂, 0.001 M KCl, and 0.005 M Tris, adjusted to pH 6.5 with 1N HCl (298). After gentle mechanical disaggregation of the tissue, the resulting suspension of isolated neuronal nuclei was transferred by Pasteur pipette to a glass slide (specimen slide) and covered by a glass coverslip. A similar droplet containing only the homogenization fluid was pipetted on to a matching slide and covered by a matching coverslip to act as a reference slide.
Fig. 2.2.g.: Small glass homogeniser with plunger; each small division on the scale represents 1 mm.

Fig. 2.3.a.: Photomicrograph of intraneuronal nucleus with nucleolus.
Fig. 2.3.b.: Photomicrograph of intraglial nucleus.
2.3 Identification and selection of nucleoli for measurement

The isolated nuclei of neurones were readily distinguished from those nuclei within neurones or neuroglia (Figs. 2.3.a. and b., Fig. 2.4.3.). Compared to glial nuclei, the nuclei of neurones were larger, their nucleoplasm was less granular, and their nucleoli were more prominent.

To avoid any tendency to select for photography those neuronal nuclei with large nucleoli, all isolated neuronal nuclei within each microscopic field were photographed. Another field was then examined in a different part of the slide according to a predetermined pattern, so that examples of neuronal nucleoli from all parts of the slide could be randomly selected and their dry mass measured.

2.4 Measurement of nucleolar dry mass of isolated neuronal nuclei

2.4.1 Analysis of measurements

Isolated neuronal nuclei were identified and their individual values for nucleolar dry mass were calculated. To obtain each pooled mean value for the nucleolar dry mass of raphe neurones, usually between 150 and 350 nucleoli from 4–6 rats were observed. For the various hypothalamic neurones, pooled mean values were based on the dry mass measurements of 50 to 100 neuronal nucleoli from the same rats. In the experiments described as preliminary, fewer observations were made. The results for pooled mean nucleolar dry mass of all neurones were evaluated statistically using Student's test. Experimental groups were compared with control
groups in order to obtain "p" values; "N. S." represents "no significant change".

2.4.2 Interference microscopy - theory

The interference microscope provides a method for interpreting the retardation imposed on a beam of light by a translucent body or organelle in its path in terms of its dry mass: dry mass is the mass of substance other than water. Two beams of light are of equal frequency and originally in phase. One beam (the object beam) traverses the organelle being examined, while the other (reference beam) provides a comparison beam. When these beams are recombined and allowed to interfere, a fringe or banded pattern is produced by the alternating zones of constructive and destructive interference of the two beams. If the organelle is denser than the surrounding medium - say water - and has the higher refractive index, the beam traversing the organelle will be retarded. The image of the organelle is seen by interference contrast; the optical path difference between the two beams is shown by the lateral shift of those interference bands passing through the organelle. The magnitude of this optical path difference (OPD) depends partly on the dry mass and refractive index of the translucent organelle. The mathematical calculations and formulae, from which the equation for the dry mass of neuronal nucleoli is derived, can be found in Appendix I.

The use of interference microscopy to measure the dry mass of cells, nuclei and nucleoli is justified by the close agreement between the
Fig. 2.4.3.: Photomicrograph of an isolated raphe neuronal nucleus, with nucleolus; illumination firstly with fringe pattern of bands, secondly with uniform background.

Fig. 2.4.4.: Microdensitometric traces, - one scan (passing through the centre of the nucleolus in an isolated neuronal nucleus) is superimposed upon a second scan (portraying the fringe pattern of interference bands); the abbreviations are explained in text, section 2.4.4.
values for specific refractive increment and dry mass obtained by this technique and by biochemical analysis (299, 300), X-ray microradiography (301-304), ultraviolet microabsorption (303, 305) and refractometry (306).

2.4.3 **Interference microscopy - technique**

In these investigations the Leitz double-beam interference microscope was used, with objectives X 50/0.85 and condensers NA 0.5. The procedure was similar to that described elsewhere (16, 20, 21). The specimen and reference slides were placed on the appropriate stages of the microscope. Having obtained the fringe pattern of alternating dark and light bands (Fig. 2.4.3.), white light was used to identify the "zero order" band, which is a white band flanked symmetrically first by black bands and then by a sequence of coloured bands. Along the zero order band the optical path differences (OPD) between light of all wavelengths is zero; with this band in the centre of the field, the contrast of the fringe pattern is maximal. After the illumination had been switched to monochromatic light (546 nm wavelength), an isolated neuronal nucleus was selected for photography and brought into focus. The bands were separated until the field was uniformly illuminated; at this juncture, there was no variation in OPD over the field. The optimal conditions for subsequent measurements were obtained when the centre of the nucleolus and the background were of equal brightness; under these circumstances, the relationship between OPD and light intensity was linear (16). Photomicrographs were taken firstly of the nucleolus with uniform background illumination; and secondly
of the adjacent clear field, with the original fringe pattern (Fig. 2.4.3.).

Photomicrographs were taken on Kodak Plus X 35 mm film; they were developed using Kodak 19b developer solution. Having been washed and dried, the films were scanned by microdensitometry. Objects lying on the object plane of the interference microscope were thus portrayed in density profile on the microdensitometer trace at a magnification of $4.2 \times 10^3$.

2.4.4 Microdensitometry - technique

Photographic film containing the photomicrographs of the neuronal nuclei was placed on the specimen table of the Joyce-Loebl Automatic Recording Microdensitometer Mk IIIc. The photomicrographs were scanned in the long axis of the film.

The instrumental settings were:

- grey wedge range: 0-2D
- objective magnification: x 22
- scanning slit aperture (width x height): 140 x 300 nm
- arm ratio: 20 to 1

In the first scan, a tracing of the nucleolus within its nucleus was obtained. The nucleolar profile selected was that describing the maximal nucleolar width in the axis of scan. The edges of the nucleoli were easily seen on the scan, and the outermost rims of each nucleolus were marked on the density tracing paper. The distance between these marks was the nucleolar diameter, $d$ cm.
Table 2.4.5.

<table>
<thead>
<tr>
<th>NUCLEUS</th>
<th>Dm/d</th>
<th>Standard error</th>
<th>No</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raphe</td>
<td>0.0667</td>
<td>0.0029</td>
<td>1.415</td>
</tr>
<tr>
<td>Suprachiasmatic</td>
<td>0.050</td>
<td>0.0023</td>
<td>1.399</td>
</tr>
<tr>
<td>Arcuate</td>
<td>0.053</td>
<td>0.0017</td>
<td>1.402</td>
</tr>
<tr>
<td>Dorso-medial</td>
<td>0.047</td>
<td>0.0015</td>
<td>1.396</td>
</tr>
<tr>
<td>Ventro-medial</td>
<td>0.050</td>
<td>0.0018</td>
<td>1.396</td>
</tr>
<tr>
<td>Posterior</td>
<td>0.052</td>
<td>0.002</td>
<td>1.401</td>
</tr>
<tr>
<td>Preoptic</td>
<td>0.047</td>
<td>0.002</td>
<td>1.396</td>
</tr>
</tbody>
</table>

Dm : nucleolar OPD

d : nucleolar diameter

No : nucleolar refractive index (corrected)
Superimposed on this first tracing was a second trace, obtained by scanning the photomicrograph of the banded field adjacent to the isolated nucleus.

Using these traces (Fig. 2.4.4.) the following measurements were made for each nucleolus:

\[ d = \text{nucleolar diameter} \]

\[ \text{La} = 1 \text{ wavelength of the light used (546 nm), or the OPD between equivalent points of intensity on adjacent bands of fringe pattern.} \]

\[ a = \text{measure of optical retardation imposed by nucleolus} \]

\[ a/\text{La} = \text{peak nucleolar OPD}. \]

2.4.5 Sources of error in procedures

There is negligible loss of substance from the nucleoli in isolated nuclei (18). In the present investigation, when slides of isolated neuronal nuclei were examined directly and up to six hours after their preparation, the values obtained for mean nucleolar dry mass were comparable.

The measurement of nucleolar dry mass by interference microscopy is subject to variables. Of these, the corrected values for nucleolar refractive index (No) were estimated from samples of 50 nucleoli randomly selected from each group of neurones studied (Table 2.4.5.). Of the sources of error due to the optics of the microscope, light scattering and glare were unlikely to be significant under the conditions of these experiments (20, 21, 307).
During microdensitometry, the potential observer error inherent in determining accurately the nucleolar rims - and hence in calculating the value for nucleolar diameter - was minimized by the author performing all the measurements. When nucleoli were scanned twice in these circumstances, the values for diameter were accurately reproduced.

Hence the effects of the potential variables and sources of error were negligible. Nevertheless their existence favours the interpretation of the results in this thesis as describing trends in nucleolar dry mass values, rather than the absolute values.

2.5 Drugs used in investigations

1) Reserpine (1mg/ml) in stabilized aqueous solution: Halewood Chemicals Ltd., Staines, Middlesex, England.

2) Oxypertine ('Integrin'): Winthrop Labs., Surbiton-upon-Thames, U.K.; as 1-(2-(5,6-dimethoxy-2-methylindole-3-yl)ethyl)-4-phenylpiperazine.

3) Nialamide ('Niamid'): Chas. Pfizer & Co., New York; as nialamide (1-(2-(benzylcarbamyl)ethyl)-2-isonicotinoyl-hydrazine) monochloride.

4) Tranylcypromine ('Parnate'): Smith, Kline & French Labs. Ltd., Welwyn Garden City, Herts.

5) Imipramine ('Tofranil'): Geigy (U.K.) Ltd., Macclesfield, Cheshire; as the 5-(3-dimethylaminopropyl)-10,11-dihydrodibenz (b,f)azepine hydrochloride; 25mg per 2ml vial.
6) Desipramine ('Pertofran'): Geigy (U.K.,) Ltd.; as 10,11-dihydro-5-(3-methylaminopropyl)dibenz(b,f)azepine hydrochloride; 25mg per 2ml vial.


9) 6-hydroxydopamine HBr: Sigma Ltd.; as 2,4,5-trihydroxyphenethylamine.

10) Noradrenaline acid tartrate: Winthrop Labs.

11) Histamine acid phosphate: BDH Chemicals.

12) Tryptophan (L-tryptophan): BDH Chemicals.

13) Phenylalanine (L-β-phenylalanine): BDH Chemicals.


15) Diamorphine (1mg per 1ml ampoule) in aqueous solution: Dept. of Pharmacy, Royal Infirmary, Edinburgh.


18) Testosterone propionate, 50 mg/ml in ethyloleate 20%, arachis oil 80%: Evans Medical Ltd., Speke, Liverpool.

19) Almond Oil B.P.: Mackenzie & Co.
SECTION 3. CHANGES IN NUCLEOLAR DRY MASS OF VARIOUS INTERNEURONES IN THE CENTRAL NERVOUS SYSTEM OF THE RAT
3.1 The nucleolar dry mass response of raphe neurones and of various neurones in the hypothalamus to the drug-induced depletion of brain 5HT

3.1.1 Introduction

The aims of this experiment were firstly to produce and measure changes in the nucleolar dry mass of those interneurones principally concerned in brain 5HT metabolism, i.e. the raphe and suprachiasmatic neurones; secondly to test the specificity of those changes by examining concurrently the nucleoli of various other interneurones which are not involved in brain 5HT metabolism.

Reserpine combines with (184, 308, 309) and disrupts (174, 310) the vesicles which contain most of the 5HT in raphe neurones (see Introduction 1.2.5)(182, 231). The 5HT thereby liberated (166, 167) is inactivated by cytoplasmic MAO (126), and the brain concentration of 5HT falls while that of its metabolite 5HIAA rises (74, 311, 312); 5HT histo-fluorescence in neurones disappears (126, 135). Subsequently the rate of 5HT synthesis increases (235). Hence it was of interest to investigate the involvement of raphe neuronal nucleoli in the restoration of brain 5HT content which follows the reserpine-induced depletion.

Whereas reserpine also depletes neuronal catecholamines by a similar mechanism (126, 132, 147), oxypertine is an indole derivative which rapidly decreases the catecholamine content of brain without altering brain 5HT metabolism (313, 314-316). By observing the
nucleolar dry mass of raphe neurones after the administration of oxypertine. It was hoped to verify the specific nature of the raphe neuronal response to reserpine.

3.1.2 Methods

Two experiments with reserpine were carried out. In the first experiment, rats were given reserpine 1mg daily for up to 9 days ("high-dose" reserpine) by intraperitoneal injection. In the second experiment, rats were given intraperitoneal injections of reserpine, 0.5mg, daily for up to 10 days ("low-dose" reserpine); after this time the drug was withdrawn, and the rats were kept for up to 10 days more. Control animals in both groups were given intraperitoneal injections of appropriate volumes of Ringer lactate solution.

During these experiments, rats were killed in batches at various stages (see Results 3.1.3). The dry mass response of nucleoli from raphe, suprachiasmatic, posterior, preoptic and ventromedial neurones was observed using the interference microscope.

In the oxypertine experiment, rats received a suspension of oxypertine (2mg in 1ml Ringer lactate) by intraperitoneal injection daily for 7-8 days. Control animals received similar injections of Ringer lactate alone. At the end of the experiment, only the raphe neuronal nucleoli were examined.
Fig. 3.1.A. Raphe and suprachiasmatic neurones: columns indicate pooled mean nucleolar dry mass, with standard errors; rats received reserpine (high dose) for up to 9 days.

Fig. 3.1.B. Raphe and suprachiasmatic neurones: data illustrated as in Fig. 3.1.A., but with reference to the administration and subsequent withdrawal of low-dose reserpine.
Fig. 3.1.C. Various other neurones: data as in Fig. 3.1.B.

Fig. 3.1.D. Raphe neurones: data presented as in Fig. 3.1.A., but experimental animals received oxypertine for 7-8 days.
<table>
<thead>
<tr>
<th>NUCLEUS</th>
<th>DRUG GROUP (with number of animals)</th>
<th>POOLED MEAN NUCLEOLAR DRY MASS (g x 10^-12)</th>
<th>STANDARD ERROR OF MEAN (S.E.)</th>
<th>NUMBER OF NUCLEOLI OBSERVED</th>
<th>p VALUE</th>
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<tbody>
<tr>
<td>Raphe</td>
<td>Control, (3)</td>
<td>8.31</td>
<td>0.38</td>
<td>152</td>
<td>-</td>
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<td></td>
<td>Reserpine d 4-5, (5)</td>
<td>9.36</td>
<td>0.36</td>
<td>248</td>
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<td>Reserpine d 6-9, (4)</td>
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<td>244</td>
<td>0.005-0.001</td>
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<td>Supra-</td>
<td>Control, (3)</td>
<td>6.43</td>
<td>0.31</td>
<td>68</td>
<td>-</td>
</tr>
<tr>
<td>chiasmatic</td>
<td>Reserpine d 5-6, (2)</td>
<td>6.95</td>
<td>0.38</td>
<td>40</td>
<td>N.S.</td>
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<tr>
<td>(S.C.)</td>
<td>d 8-9, (3)</td>
<td>6.86</td>
<td>0.27</td>
<td>57</td>
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<td>-</td>
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<td>0.39</td>
<td>95</td>
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</tr>
<tr>
<td>E C</td>
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<td>0.21</td>
<td>90</td>
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<td>0.36</td>
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</table>

Posterior/
<table>
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<tr>
<th>NUCLEUS</th>
<th>DRUG GROUP (with number of animals)</th>
<th>POOLED MEAN NUCLEOLAR DRY MASS (g x 10^{-12})</th>
<th>STANDARD ERROR OF MEAN (S.E.)</th>
<th>NUMBER OF NUCLEOLI OBSERVED</th>
<th>p VALUE</th>
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<tr>
<td>Posterior</td>
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<td>0.27</td>
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<td>Pre-optic</td>
<td>Control, (5)</td>
<td>6.22</td>
<td>0.21</td>
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<td>0.34</td>
<td>59</td>
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<tr>
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<td>Withdraw d 2, (3)</td>
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<td>0.29</td>
<td>64</td>
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<td>d 4-6, (5)</td>
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</tr>
<tr>
<td>Ventro-medial</td>
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<td>0.23</td>
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<td></td>
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<td>0.19</td>
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<tr>
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<td>Withdraw d 2, (3)</td>
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<td>0.05-0.025</td>
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<tr>
<td></td>
<td>d 4-6, (5)</td>
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<td>0.22</td>
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</tr>
<tr>
<td></td>
<td>d 9, (2)</td>
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<td>0.35</td>
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</tr>
<tr>
<td>Raphe</td>
<td>Control, (4)</td>
<td>8.78</td>
<td>0.29</td>
<td>293</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Oxypertine, (5)</td>
<td>9.09</td>
<td>0.38</td>
<td>386</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
3.1.3 Results

In these experiments (Figs. 3.1.A. & B., Table 3.1) reserpine affected a dose-related increase in the nucleolar dry mass of the raphe neurones.

In the low-dose reserpine experiment, the increased nucleolar dry mass of the raphe neurones returned to normal values after reserpine administration had ceased; but up to the ninth day of withdrawal, no "overshoot" was apparent (Fig. 3.1.B.).

With low-dose reserpine, the nucleolar responses of the raphe neurones preceded similar changes in the nucleolar dry mass of the suprachiasmatic neurones (Fig. 3.1.B.). However no significant changes were seen in the suprachiasmatic neurones after high-dose reserpine (Fig. 3.1.A.).

The changes seen in the ventromedial neurones after low-dose reserpine bore no relationship in time or direction to the responses of the raphe and suprachiasmatic neurones. No significant changes were seen in the posterior or preoptic neuronal nucleoli during this experiment (Fig. 3.1.C.).

The administration of oxypertine to rats for 7-8 days did not alter the nucleolar dry mass of their raphe neurones (Fig. 3.1.D.).

In the rats receiving high-dose reserpine, sedation was marked. Most rats lost weight and suffered from diarrhoea; some also developed crusting around the nose and eyes. The mortality rate was maximal between days 4 and 6; during the experiment, about one rat in four died.
The rats receiving low-dose reserpine did not show these florid signs. However they lost weight: their initial average weight of about 300 gm each declined to around 220 gm at day 4 of reserpine, rose again to about 250 gm at days 8-10, and was about 270 gm at the end of the experiment. Water intake also declined: normal intake averaged about 25ml/rat/day; by day 2 intake was reduced by 50%, and it was only 25% of normal at day 5; but 2 days after reserpine had been discontinued, water intake was again normal. Food intake was not measured, but was probably reduced while the animals were receiving reserpine.

Signs of drug toxicity were not apparent during the oxypertine experiment.
3.1.4 Discussion

These experiments demonstrated that the raphe interneurones concerned in 5HT synthesis showed changes in their nucleolar dry mass after the administration of a 5HT-depleting drug. The time-course of these nucleolar changes was similar to that reported for the nucleolar responses to prolonged stimulation in motoneurones and neurosecretory cells (see Introduction 1.1.).

The increased dry mass of raphe neuronal nucleoli after reserpine can be interpreted as a sign of increased ribosome and protein synthesis (see Introduction 1.1). In the morphologically-similar noradrenergic neurones (317), reserpine also promotes the destruction of noradrenaline by damaging the storage vesicles (255, 318). During the subsequent restoration of the amine content of these neurones (25), a slow increase in the amount of noradrenaline-synthesizing enzymes is found in their cell bodies (109, 319-321) (see Introduction 1.2.9); the restoration of noradrenaline content is delayed when inhibitors of protein and RNA synthesis are administered (108, 289, 321). In raphe neurones a similar, dose-dependent increase in the production of 5HT-synthesizing enzymes (269, 293, 322) probably contributes to the accelerated formation of 5HT after reserpine (235). This view is supported by the nucleolar changes observed in the present study, since the 5HT-synthesizing enzymes are formed mainly if not entirely within the cell bodies of the raphe neurones (see Introduction 1.2.3).
The damage caused by reserpine to the storage vesicles is probably irreversible (189). Therefore for the restoration of brain monoamine levels, protein components for new vesicles must be synthesized in the nerve cell bodies, and then transported to the nerve terminals (see Introduction 1.2.5). These processes, which take place some 2-5 days after giving reserpine, may also be linked with the increased nucleolar dry mass of the raphe neurones (317).

Reserpine induces anorexia (323). The decline in brain 5HT concentration resulting from a low-tryptophan diet is more pronounced when reserpine is also given (324). In the high-dose reserpine experiment in this investigation, food intake was severely depressed: it is possible that the concurrent dramatic increase in the nucleolar dry mass of the raphe neurones was a response to the low brain 5HT concentration (see Concluding Discussion 4.1), which in turn resulted from both a decreased synthesis and an enhanced destruction of 5HT.

According to the evidence of histofluorescence and other anatomical studies (29, 32, 213), the suprachiasmatic nucleus includes many 5HT-containing nerve endings, but none containing noradrenaline; around 5% of the boutons in the nucleus may contain 5HT (156). During the administration of reserpine, the amount of 5HT in cerebral ventricular perfusates from the experimental animals does not increase as the brain 5HT concentration plummets (325); this indicates that 5HT from the disrupted vesicles of raphe neurones is metabolized by MAO intraneuronally, and is
not released into the synaptic spaces (147). Hence one probable effect of reserpine is to diminish the amount of 5HT released from the raphe neuronal terminals on to the suprachiasmatic neurones (326). When 5HT is released on to suprachiasmatic neurones, either directly by iontophoresis or indirectly by stimulating electrically the raphe neurones, the spontaneous firing of the suprachiasmatic neurones is inhibited (see Introduction 1.2.7). Therefore during the administration of low-dose reserpine, the increased metabolic activity of suprachiasmatic neuronal nucleoli may be a consequence of attenuating the normal degree of 5HT inhibition of these neurones (327).

Moreover, with the cessation of reserpine administration and the consequent replenishment of 5HT in raphe neuronal terminals, the normal degree of trans-synaptic inhibition of the suprachiasmatic neurones could be restored. This sequence of events is compatible with the restoration of the normal nucleolar dry mass values for suprachiasmatic neurones after reserpine withdrawal.

The absence of any significant response by suprachiasmatic neurones to high-dose reserpine is difficult to explain. Perhaps this dosage provoked an exceptionally vigorous biosynthetic response by the raphe neurones - an accelerated production of vesicles, synthesizing enzymes and ultimately of 5HT itself may have sustained the release of 5HT from a small, reserpine-resistant pool of 5HT (203, 328) in the raphe terminals in the suprachiasmatic nucleus; in noradrenergic neurones, functional recovery after reserpine is
associated with the presence of a low concentration of noradrenaline - the small, functional pool in the terminals (134, 188, 329, 330). Alternatively at this dosage of reserpine, hormones with effects antagonistic to those of 5HT may have been released from other terminals in the suprachiasmatic nucleus. This may have happened as a specific response to reserpine, as a consequence of the anorexic and sedative effects of reserpine (323, 331), or as a response to the general stress associated with the repeated injections and dehydration; for besides its effects on 5HT and noradrenaline in neurones, reserpine alters the metabolism of acetylcholine in brain (332-334). Reserpine also enhances the secretion of ACTH from the anterior pituitary (335): ACTH and corticosteroids increase 5HT synthesis in brain (336).

In the other hypothalamic nuclei studied, 5HT-containing terminals are scanty while noradrenergic terminals are more abundant (29). Only the neurones of the ventromedial nucleus showed nucleolar changes during the administration of reserpine; these changes differed qualitatively from the responses of the raphe and suprachiasmatic neurones. Alterations in the nucleolar dry mass of the neurones in the ventromedial nucleus develop rapidly after adrenalectomy and after surgical stress (21). But because the physiological functions of these neurones are not known, the significance of their nucleolar changes cannot be evaluated.
Like reserpine, oxypertine has sedative properties (243, 337). In various rodents, oxypertine rapidly decreases the catecholamine content of brain; this decrease is prevented by MAO inhibition. Hence oxypertine is considered to exert a reserpine-like action on catecholamine-containing vesicles but not on 5HT-containing vesicles, since 5HT metabolism is not altered (313-316, 338). The unchanged nucleolar dry mass of the raphe neurones in the present study supports both this view and the contention that the nucleolar changes in the raphe neurones after reserpine were not the consequence of noradrenaline depletion in the brain.

3.1.5 Conclusions

Increments in the nucleolar dry mass of the raphe and suprachiasmatic interneurones were produced by the administration of reserpine. Interpretations of these changes were compatible with the known alterations of 5HT turnover and of enzyme synthesis after reserpine. Changes in the nucleoli of the ventromedial neurones could not be explained. No other neurones showed nucleolar changes. Oxypertine did not alter the nucleolar dry mass of raphe neurones.
3.2 Changes in the nucleolar dry mass of raphe neurones in response to drugs which inhibit neuronal MAO, and to various stresses

3.2.1 Introduction

The amount of 5HT in brain is increased when MAO inhibitors are used to block the main metabolic pathway for 5HT degradation (25, 31, 202). The nucleolar responses of raphe and other neurones to the administration of these drugs were investigated. The drugs used were nialamide alone (126) and tranylcypromine alone (331, 339). In addition, nialamide was given together with reserpine, in order to observe the effect of increasing the concentration of "free" cytoplasmic 5HT in raphe neurones, while decreasing the amount of 5HT bound in their vesicles (132, 183, 211, 340).

In these and other experiments, drugs were prepared in aqueous and oily solvents, some of which were not available for use as control injections. In the reserpine experiment (Results 3.1.3) the water intake of the rats was reduced. Hence it was decided to ascertain also whether or not physiological responses to the stresses of repeated parenteral injections and of dehydration might influence the nucleolar dry mass of raphe neurones.
3.2.2 Methods

Three experiments were performed using MAO inhibitors. Nialamide, 10mg dissolved in 0.2ml saline, was given to each rat daily for up to 10 days by intraperitoneal injection. Tranylcypromine, 5mg was given similarly for 8 days. Nialamide, 50mg alone on day 1 but 25mg when combined with reserpine 0.5mg subsequently, was given in 0.5ml saline daily for up to 7 days. Control animals from each group were given intraperitoneal injections of 0.5ml saline daily for 5-9 days. At the end of these experiments the rats were killed. The nucleolar responses of raphe, suprachiasmatic, dorsomedial and arcuate neurones were scrutinized as described earlier. In the tranylcypromine experiment, only the raphe neurones were examined.

For the experiments on the effects of stresses, rats were given intraperitoneal injections (1ml daily) of almond oil or of Ringer lactate solution. Other rats were handled similarly but received no injections.

Tissue containing median raphe neurones was excised from the brains of rats which had been deprived of drinking water for 5 days; similar tissue was also obtained from the brains of control animals (see Footnote).

In these stressed animals, the nucleolar parameter of only the raphe neurones was measured.

(Footnote: these tissue samples were obtained with the cooperation of Dr R. M. Watt, Dept. of Physiology, Edinburgh University.)
Fig. 3.2.A. Raphe neurones: animals received drugs as shown in columns; these represent pooled mean nucleolar dry mass with standard errors.

Fig. 3.2.B. Raphe neurones: data represented as in Fig. 3.2.A., but experimental animals received tranylcypromine for 8 days.
Fig. 3.2.C. Various neurones: data presented as in Fig. 3.2.A.

Fig. 3.2.D. & E. Raphe neurones: columns indicate pooled mean nucleolar dry mass, with standard error, in rats subjected to various stresses.
<table>
<thead>
<tr>
<th>NUCLEUS</th>
<th>DRUG GROUP (with number of animals)</th>
<th>POOLED MEAN NUCLEOLAR DRY MASS (g x 10^-12)</th>
<th>STANDARD ERROR OF MEAN (S.E.)</th>
<th>NUMBER OF NUCLEOLI OBSERVED</th>
<th>p VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raphe</td>
<td>Control, (3)</td>
<td>8.31</td>
<td>0.38</td>
<td>152</td>
<td>-</td>
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<td></td>
<td>Nial. d 2-4, (6)</td>
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<td>Nial.-Res. d 2-4, (4)</td>
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<td>25 mg 0.5 mg d 5-7, (6)</td>
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</tr>
<tr>
<td></td>
<td>Control, (4)</td>
<td>9.72</td>
<td>0.37</td>
<td>128</td>
<td>-</td>
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<tr>
<td></td>
<td>Tranylcypromine, (5)</td>
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<td></td>
<td></td>
<td>5 mg</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.C.</td>
<td>Control, (3)</td>
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<td>68</td>
<td>-</td>
</tr>
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<td>Nial. d 2-4, (6)</td>
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<td>0.29</td>
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<td>N.S.</td>
</tr>
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<td>Arcuate</td>
<td>Control, (2)</td>
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<td>0.36</td>
<td>41</td>
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<td>Nial. d 2-4, (6)</td>
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<td>Dorso-</td>
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<td>POOLED MEAN NUCLEOLAR DRY MASS (g x 10^{-12})</td>
<td>STANDARD ERROR OF MEAN (S.E.)</td>
<td>NUMBER OF NUCLEOLI OBSERVED</td>
<td>p VALUE</td>
</tr>
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<td>-------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-------------------------------</td>
<td>-----------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>Dorso-medial</td>
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<td>0.37</td>
<td>40</td>
<td>-</td>
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<td>Nial.-Res. d 2-4, (4)</td>
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<td>0.27</td>
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</tr>
<tr>
<td></td>
<td>25mg O.5ng d 5-7, (5)</td>
<td>5.93</td>
<td>0.20</td>
<td>103</td>
<td>N.S.</td>
</tr>
<tr>
<td>Raphe</td>
<td>Control, (5)</td>
<td>7.66</td>
<td>0.26</td>
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<td>Dehydration, (4)</td>
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</tr>
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<td>8.71</td>
<td>0.30</td>
<td>180</td>
<td>-</td>
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<tr>
<td></td>
<td>Oil, (3) 1ml</td>
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<td>Ringer lactate, (3) 1ml</td>
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<td>0.37</td>
<td>120</td>
<td>N.S.</td>
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</table>
3.2.3 Results

Tranylcypromine and nialamide produced no significant changes either in the raphe neuronal nucleoli or in the hypothalamic neurones studied. The combination of nialamide with reserpine also proved ineffectual on these neuronal nucleoli (Figs. 3.2.A.-C., Table 3.2.).

No significant changes were seen in the nucleolar dry mass of the raphe neurones after repeated injections of aqueous and oily solutions, or after dehydration (Figs. 3.2.D.-E., Table 3.2.).

During the administration of nialamide, the behaviour of the rats (assessed subjectively) began to alter at day 3: the animals became very reactive to light touch, they escaped from the cages with alacrity during handling, and they frequently adopted aggressive postures towards each other - standing on their hind legs and swaying from side to side. Otherwise their physical condition remained good. The behaviour of the rats receiving tranylcypromine was less remarkable, while the rats receiving nialamide and reserpine combined were more lethargic.

The animals in both stress experiments appeared healthy and behaved normally during the investigations. After 5 days of dehydration, the average weight loss was about 25%.
3.2.4 Discussion

After MAO inhibition (with or without reserpine) the 5HT content and histofluorescence intensity of the raphe nuclei increase (25, 200, 211), although an increased fluorescence in the median raphe nucleus may not be evident (341). The rate of 5HT synthesis probably does not alter (200, 336, 342), or it may decline (237, 280-282) (see General Discussion 4.1). The resting firing rate of raphe neurones is depressed (264, 343, 344). In the present study the unaltered nucleolar dry mass of the raphe neurones indicates that whatever the other changes were in raphe neuronal metabolism, ribosomal synthesis does not alter significantly when MAO inhibitors are given for 7 days (Figs. 3.2.A. & B.). In an earlier experiment, reserpine caused an increment in the nucleolar dry mass of raphe neurones (see Results 3.1.3). In this experiment, reserpine given with nialamide failed to effect a similar change. This finding is of interest, in view of the report that, in noradrenergic neurones, the trans-synaptic induction of tyrosine hydroxylase by reserpine is blocked when MAO is inhibited (321).

There is some controversy as to whether the excess cytoplasmic 5HT in raphe neurones spills out across the neuronal membrane during MAO inhibition. In vivo, the administration of MAO inhibitors alone, or with reserpine, increases the normal release of 5HT from brain tissue into ventricular perfusates (193, 325); after a massive dose of nialamide (500mg/kg body weight intraperitoneally) extracellular 5HT histofluorescence is reported (23, 345). However in the presence of these drugs, there is an
unimpeded accumulation and retention of tritiated 5HT by brain slices (137). Moreover, studies using histofluorescence and biochemical techniques indicate that with the dosages of MAO inhibitors comparable to those used in the present study, the raphe neuronal membrane pump may prevent the accumulating intracellular 5HT from leaking out and increasing the extraneuronal levels of 5HT (25, 340). A similar situation is found in noradrenergic terminals: despite pretreatment with reserpine and nialamide, a sustained noradrenaline fluorescence can still be produced by the intraneuronal accumulation of exogenous noradrenaline (346).

In the present study, the nucleolar dry mass of the suprachiasmatic neurones did not alter after the inhibition of MAO, even with the concurrent administration of reserpine. This evidence of their unchanged metabolic activity is consistent with the view that, when these drugs are used in the dosage described in the present investigation, there is no gross accumulation of 5HT outside the raphe neuronal terminals in the suprachiasmatic nucleus. When reserpine in a similar dose was given alone, the nucleolar dry mass of the suprachiasmatic neurones increased, possibly as a result of attenuating 5HT inhibition (see Results 3.1.3 and Discussion 3.1.4). But raphe neuronal 5HT is not depleted, when reserpine is given after a MAO inhibitor (211); and there may be a continuing release of 5HT adequate to maintain the normal degree of inhibition of the suprachiasmatic neurones. This hypothesis is compatible with the results of the present study.
Behavioural excitation in animals receiving MAO inhibitors has been reported elsewhere (201) and will be discussed later (see Discussion 3.4.4).

Various stresses, including intermittent electroshock (347), horizontal oscillation (348), handling (349), restraint and isolation (350), reportedly alter the metabolism of various brain monoamines including 5HT. However a dissenting opinion has been expressed that stress may not affect significantly central 5HT neurones (351). This opinion is corroborated by the results of the present study.

After 5 days dehydration, the nucleolar dry mass of rat supraoptic neurones was markedly elevated (19); however this stress did not alter significantly the nucleolar metabolism of the raphe neurones. Elsewhere, repeated daily injections of saline for 16 days have been found to increase the activity of midbrain tryptophan hydroxylase by 23% (352); however when the rats in the present study were given daily intraperitoneal injections of Ringer lactate or almond oil, the absence of nucleolar changes in the raphe neurones indicated that these particular nonspecific stresses were inconsequential, insofar as the synthesis of ribosomes and protein by raphe neurones was concerned.

3.2.5 Conclusions

Following MAO inhibition, without or with reserpine, no changes were detected in the nucleolar dry mass of raphe and suprachiasmatic neurones. These findings are compatible firstly with the evidence that the
rate of 5HT synthesis by raphe neurones may not change appreciably when
the concentration of intraneuronal 5HT rises; and secondly with the contention
that there is no accumulation of extraneuronal 5HT sufficient to increase
significantly the 5HT-induced inhibition of the metabolism of the supra-
chiasmatic neurones.

There were no changes in the nucleolar dry mass of raphe neurones
after rats had been stressed by repeated injections of various "control"
solutions or by dehydration.
3.3 The effects on the nucleolar dry mass of raphe neurones of drugs which alter the uptake of 5HT by raphe neurones

3.3.1 Introduction

Tricyclic antidepressant drugs inhibit the active uptake of tryptophan and 5HT by raphe neurones (222, 264, 353, 354); the rate of 5HT synthesis declines after their administration (266, 268). These drugs probably do not affect the uptake or binding of reserpine in rat tissues (355).

Like the tricyclic antidepressant drugs, lithium is used in psychiatric therapy (356, 357); after the administration of lithium, the extraneuronal level of monoamines decreases (357, 358), and 5HT turnover is altered (270, 359).

The effects of two tricyclic antidepressant drugs (imipramine and desipramine), of desipramine together with reserpine, and of lithium on the nucleolar dry mass of the raphe, suprachiasmatic and arcuate neurones were investigated.
3.3.2 Methods

In two experiments (one year apart) the tricyclic antidepressant drugs, imipramine and desipramine, were given to rats; some rats were also given reserpine. All drugs were administered by intraperitoneal injection.

In the first experiment, one group of rats was given low-dose desipramine (2.5mg) daily for 8-9 days, either alone or with reserpine (0.5mg) on the second and subsequent days. A second group of rats received desipramine (10mg on alternate days), but of the five animals in this group only one survived until day 6, when it was killed.

In the second experiment which lasted 8-9 days, some rats received daily injections of imipramine in low (2.5mg) or high (5mg) doses. Other rats were given high-dose desipramine (5mg) daily.

Control rats in both experiments received appropriate aliquots either of Ringer lactate solution or of the solvent for imipramine provided by Geigy Ltd.

In the lithium experiment, each rat was given daily intraperitoneal injections of 30mg lithium carbonate in 1ml Ringer lactate. Control animals received daily injections of Ringer lactate alone. The injections were given for 10-14 days, when the rats were killed.

In all experiments, the raphe, suprachiasmatic and arcuate nuclei were dissected out, and the dry mass of their neuronal nucleoli was measured.
Fig. 3.3.A. Various neurones: columns indicate pooled mean nucleolar dry mass, with standard error; drugs given are shown in the columns.

Fig. 3.3.B. Various neurones: data presented as in Fig. 3.3.A., but with different drug regimes.
Fig. 3.3.C. Various neurones: data presented as in Fig. 3.3.A., but experimental animals received lithium for 10 to 14 days.
<table>
<thead>
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<th>NUCLEUS</th>
<th>DRUG GROUP (with number of animals)</th>
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N.S. (0.2-0.1)
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<th>STANDARD ERROR OF MEAN (S.E.)</th>
<th>NUMBER OF NUCLEOLI OBSERVED</th>
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3.3.3 Results

Apart from the single rat receiving 10mg desipramine on alternate days, the nucleolar dry mass of raphe neurones in all animals receiving tricyclic antidepressant drugs was decreased (Figs. 3.3.A.-B., Table 3.3.). This decrement of the nucleolar dry mass was more significant after low doses of the drugs than after high doses: nucleolar dry mass decreased by 14-15% against 9-10% respectively. In the regime of desipramine 10mg on alternate days, there was no nucleolar response (Fig. 3.3.A.); however because of the high mortality in this experiment, this result was obtained from only one animal; this result, therefore, was recorded in the thesis merely because it indicated the same trend in the changes in nucleolar parameter as was found after the other experiments using tricyclic drugs, namely, that high doses of these drugs had less effect on the nucleolar dry mass of raphe neurones than did low doses. There was no decrement in the nucleolar dry mass of raphe neurones after desipramine and reserpine combined.

In contrast to the raphe neurones, the nucleolar dry mass of the suprachiasmatic neurones was more depressed by the high doses of desipramine and imipramine than by the low doses (Figs. 3.3.A. & B.); after desipramine and reserpine combined, the decrement was not significant.

In the arcuate neurones, the nucleolar parameter tended to decrease after these drugs, but the decrements were not statistically significant (Fig. 3.3.B.).
After lithium, the nucleolar dry mass of the raphe neurones was increased (Fig. 3.3.C.). The changes in the nucleolar parameter of suprachiasmatic and arcuate neurones were not statistically significant.

Apart from the considerable mortality in the high-desipramine series (10mg on alternate days) which has already been described, there was little sign of drug toxicity in these experiments. Some rats receiving tricyclic antidepressant drugs, especially low-dose desipramine, became exceptionally alert and reacted excessively to various auditory and tactile stimuli. The rats receiving lithium became increasingly excitable from day 8 onwards.
3.3.4 Discussion

The effects of tricyclic antidepressant drugs include: diminished electrical and synthetic activity in raphe neurones, extraneuronal accumulation of 5HT resulting from the inhibition of the membrane pump for monoamines, and altered activity of postsynaptic neurones.

3.3.4.1 Decreased raphe neuronal activity: shortly after the administration of imipramine, the spontaneous firing rate of raphe neurones decreases; this inhibition lasts for over 30 minutes (343, 360). The diminished impulse activity in the raphe neurones is accompanied by a decreased breakdown of 5HT in their terminals: for example, the retention of exogenous, isotopically-labelled 5HT in brain is prolonged when tricyclic drugs are given after the intracisternal dose of 5HT (136, 202); there is also a slower diminution of the endogenous 5HT content of raphe terminals in the suprachiasmatic nucleus, if further 5HT synthesis is inhibited (222, 361). The rate of 5HT synthesis also declines (263, 267). When probenecid is used to block the egress of 5HIAA (see Introduction 1.2.8), the slower rate of turnover is evident from the decrease in 5HIAA and 5HT content of brain which follows the administration of tricyclic antidepressants (266, 268, 353). The long-term administration of imipramine (20mg/kg intraperitoneally for 10 days) results in the continuing decline of the levels of 5HT and 5HIAA in almost all areas of the brain (362). However after much smaller doses of imipramine (10mg/kg every 48 hours for 7 days), the tryptophan hydroxylase activity in rat midbrain does not change (322).
The indications that with tricyclic antidepressant drugs the electrical and metabolic activities of raphe neurones are both inhibited, are compatible with the finding of the present investigation that the nucleolar parameter of these neurones is also decreased. The observation that the lower doses of tricyclic antidepressants in these experiments were more effective in causing these changes than the high doses or the reserpine-desipramine combination is discussed later (see Discussion 3.3.4.3 and Concluding Discussion 4.1).

3.3.4.2 Extraneuronal accumulation of 5HT: tricyclic antidepressant drugs inhibit the membrane pump for monoamines of many cells including monoaminergic neurones, pericytes, and endothelial cells (363, 365). In one study using vas deferens tissue, the neuronal accumulation of exogenous 5HT decreased by 62% and 57% after using desipramine and imipramine respectively (366); in brain slices the uptake of 5HT also declines (137, 368), the percentage inhibition after exposure to desipramine and imipramine being 40-70% depending on drug dosage (247). After exposure to these drugs, brain synaptosomes take up less exogenous 5HT in vitro (204, 369-371); in vivo, the accumulation by raphe neurones of 5HT given by intraventricular injection is also reduced (211, 370).

Accompanying this inhibition of the membrane pump for monoamines, there is a less effective recapture of 5HT released from 5HT-containing brain tissues. There is a faster efflux of unmetabolized, tritiated 5HT which had been accumulated previously by these tissues from the incubation
medium (367); similarly, imipramine inhibits the recapture and subsequent breakdown by intraneuronal MAO of the endogenous 5HT released spontaneously or when raphe neurones are stimulated electrically (340, 354).

The findings of histofluorescence microscopy provide further confirmation of these processes: with MAO inhibited, tricyclic antidepressants cause 5HT fluorescence to increase outside raphe neuronal terminals, axons and cell bodies; if reserpine is also given, a similar increase in extraneuronal fluorescence is seen, but intraneuronal fluorescence declines (200, 340, 370).

Tricyclic antidepressant drugs may also promote a decline in the raphe neuronal content of 5HT by reducing the storage function of the terminals' vesicles: these vesicles are enveloped by a membrane which contains a monoamine pump mechanism sensitive to desipramine and imipramine (372, 373).

In some studies of 5HT uptake by brain synaptosomes (369, 370), brain slices (137, 247, 368) and intact brain (177, 210, 354), the pump-blocking action of imipramine far exceeds that of desipramine; occasionally the latter drug is almost impotent (340, 374) unless given in very large doses, - for instance 50mg/kg body weight or more by intraperitoneal injection (369). In human brain imipramine is readily converted to desipramine (375); in rat brain, the rate of this conversion by N-demethylation depends on the strain of rat used (376). In the present study, in which desipramine proved the more effective inhibitor of raphe neuronal nucleolar metabolism, the degree of this conversion was not studied.
3.3.4.3 The responses of postsynaptic neurones: the reuptake of 5HT by the raphe neuronal terminals is a means of terminating the postsynaptic action of 5HT (165, 208, 377, 379) (see Introduction 1.2.7). In the snail cerebral ganglion, imipramine enhances transmission at 5HT synapses, and the responses of the postsynaptic neurones increase (378, 379). The effect of 5HT on mammalian spinal reflexes is also exaggerated by tricyclic antidepressant drugs, especially when these are used with 5HT precursors (34, 340, 380). The responses of cerebral cortical neurones to iontophoretically-applied 5HT are potentiated by imipramine (215); the effects of noradrenaline on brainstem and cortical neurones are similarly potentiated, - indeed imipramine by itself may elicit the same responses as does noradrenaline (381). Paradoxically, however, the responses of neurones to 5HT and noradrenaline decline when high extraneuronal concentrations of either monoamine are produced by large doses of imipramine-like drugs alone or by smaller doses of these drugs in conjunction with large doses of the monoamines (226, 381). It is premature to ascribe to such paradoxical electrophysiological responses of tachyphylaxis (see Introduction 1.2.7) the unexpected trend of the changes in the nucleolar dry mass of raphe neurones in the present investigation. Nevertheless it was of interest that both imipramine and desipramine decreased the nucleolar parameter of raphe neurones more effectively at their lower dosages; and that desipramine, although generally considered to inhibit 5HT uptake less than imipramine (382), still effected the more-significant nucleolar decrement.
When desipramine was given together with reserpine, there was no reduction in the nucleolar dry mass of raphe neurones. This finding may have resulted from the action of reserpine in increasing the intraneuronal breakdown of 5HT (see Discussion 3.1.4), thereby reducing the amount of 5HT released from the raphe nerve terminals; the possible importance of the synaptic or extraneuronal pool of 5HT in regulating raphe neuronal metabolism is discussed later (Concluding Discussion 4.1).

In the present study, the nucleolar dry mass of the suprachiasmatic neurones declined significantly only with the high dosage of desipramine. This change might have resulted from the accumulation of extraneuronal 5HT in the suprachiasmatic nucleus. This hypothesis is compatible with the previous finding that, when brain 5HT was depleted by the administration of reserpine, the nucleolar dry mass of suprachiasmatic neurones increased (see Results 3.1.3). The concentration of extraneuronal 5HT produced by the low doses of tricyclic antidepressants, with and without reserpine, might have been insufficient to decrease similarly the nucleolar dry mass of the suprachiasmatic neurones.

The arcuate nucleus contains few 5HT terminals, but rather more noradrenergic terminals (29). Desipramine is particularly effective in promoting the accumulation of noradrenaline outside the terminals of noradrenergic neurones (see Discussion 3.3.4.2). It was of interest, therefore, that the nucleolar dry mass of the arcuate neurones did not alter significantly during these experiments. As yet this finding cannot be explained convincingly.
The effects of lithium on raphe neurones include an altered metabolism of 5HT, an enhanced uptake of 5HT into neurones, and a reduced efflux of 5HT from neurones.

3.3.4.4 Altered 5HT metabolism: when given acutely or for up to 5 days, lithium increases the brain content of tryptophan, 5HT and 5HIAA (69, 383): lithium accelerates the rise in 5HT concentration after MAO inhibition and the rise in 5HIAA concentration after probenecid (384); by the fifth day of lithium administration, the rate of 5HT synthesis is increased by 61±82% (385). The elevation in brain 5HT and 5HIAA content after electrical stimulation of the raphe nuclei is also greater after lithium pre-treatment (270). However after 14 days on lithium the concentration and turnover of 5HT in rat brain is again normal. Thereafter there is evidence that 5HT turnover is below normal (253, 385). One report indicates that during the first 5 days of lithium administration, the rate of 5HT synthesis increases only in the raphe neuronal terminals; and that subsequently the production of 5HT is inhibited in both terminals and cell bodies (359).

3.3.4.5 Enhanced 5HT uptake: in vivo, lithium enhances the accumulation by brain tissue of isotopically-labelled 5HT given by intracisternal injection; the isotopic labelling of the deaminated metabolites of 5HT is also increased (202). Lithium affects similarly the uptake and metabolism of noradrenaline (358, 367, 388). These findings indicate that, during lithium administration, monoamines accumulating inside monoaminergic neurones are not stored there, but are broken down quickly by cytoplasmic MAO.
3.3.4.6 Decreased release of 5HT: in vitro, lithium inhibits the release of isotopically-labelled 5HT from brain slices during electrical stimulation or exposure to solutions containing high concentrations of potassium (190, 389); this inhibitory effect of lithium on monoamine release is observed also in the brain slices of lithium-pretreated animals (390). The release of noradrenaline from peripheral sympathetic neurones during electrical stimulation declines similarly after lithium (391).

The increased nucleolar dry mass of raphe neurones induced by lithium in the present study is difficult to interpret. As evidence of an increased synthesis of ribosomes, it is consistent with the many observations of an early, enhanced 5HT turnover in brain after lithium; but it is less easy to reconcile with the report that tryptophan hydroxylase activity in the cell bodies of the raphe neurones diminishes and that brain 5HT turnover decreases after 5 days of lithium. It is possible, of course, that values for 5HT turnover and tryptophan hydroxylase activity after long-term administration of lithium may conceal an interplay between the rate of synthesis of "new" enzymes, and the degree of activation of "older" enzymes concerned in forming 5HT (see Introduction 1.2.9 and Concluding Discussion 4.1). Alternatively, other facets of raphe neuronal metabolism involving an enhanced synthesis of ribosomes may increase after lithium. However the spontaneous electrical activity of raphe neurones does not alter (270), nor is there evidence that the function and synthesis of their storage vesicles are affected. An important role for MAO in regulating intraneuronal 5HT metabolism has been suggested (146) (see Introduction
1.2.4); but although an enhanced production of MAO may have been necessary during the early period of increased intraneuronal deamination of 5HT, it would not have been necessary during the subsequent decline in 5HT catabolism; moreover the inhibition of MAO by nialamide and tranylcypromine did not alter the nucleolar metabolism of raphe neurones (see Results 3.2.3). In dorsal-root ganglion neurones, a lithium-induced dilatation of Golgi vesicles and increased cytoplasmic granularity have been observed by electron microscopy; these indications of increasing neuronal metabolism have been attributed to a lithium-provoked failure of the membrane sodium pump of neurones (392-395). But so far, the only major function which is directly related to 5HT metabolism in raphe neurones and which is known to remain hyperactive during prolonged lithium administration is the facility for 5HT accumulation; it may be significant that, when by contrast this facility was reduced by tricyclic antidepressant drugs, the nucleolar metabolism of raphe neurones was inhibited (see Results 3.3.3). However no conclusions can be reached as yet about the significance of these observations.

It is also difficult to interpret the absence of nucleolar changes in the suprachiasmatic neurones after lithium. Ostensibly the diminished release and enhanced recapture of 5HT by the raphe neuronal terminals reported by other workers would tend to decrease the extraneuronal concentration of 5HT in the suprachiasmatic nucleus; the inhibition normally produced by 5HT there would be lessened thereby (see Introduction 1.2.7). Possibly the effects of lithium on cholinergic metabolism and transmission
may have resulted in the responses of suprachiasmatic neurones to 5HT being obscured.

### 3.3.5 Conclusions

The nucleolar dry mass of raphe and suprachiasmatic neurones decreased after tricyclic antidepressant drugs. This evidence is consistent with other reports that these drugs depress the metabolic activity of raphe neurones, and also promote the accumulation of 5HT outside raphe neuronal terminals.

After lithium, only the raphe neuronal nucleolar parameter changed; the increased dry mass was discussed in relation to the lithium-induced changes in raphe neuronal metabolism reported elsewhere.
3.4 The effect of various monoamines, their precursors and their analogues on the nucleolar dry mass of raphe neurones

3.4.1 Introduction

In brain tissues, the rate of 5HT synthesis may change when the concentration of 5HT rises (see Discussion 3.2.4). A decline in brain 5HT synthesis occurs in the presence of catechol amines, phenylalanine and p-chlorophenylalanine (PCPA). The compound 6-hydroxydopamine lowers the concentration of catechol amines in brain by destroying the neurones responsible for their synthesis.

Other monoamines found in brain include melatonin and histamine. Many of the behavioural and endocrinological sequelae of melatonin administration resemble those observed after giving 5HT; the effects of histamine on behaviour and 5HT metabolism are more obscure. After bilateral cervical sympathectomy, the rate at which melatonin is synthesized by the pineal gland is altered.

It was decided to investigate the dry mass response of raphe neuronal nucleoli to the administration of 5HT, melatonin, noradrenaline, histamine, 6-hydroxydopamine, tryptophan, PCPA and phenylalanine. Some of these substances were given by way of the cerebral ventricles, because the penetration of brain tissue by monoamines given by peripheral routes is uncertain. Also investigated were the effects on the raphe neuronal parameter of destroying by bilateral cervical sympathectomy the sympathetic innervation of the pineal gland and the cerebral vasculature.
FIG. 3.4.2.A. Diagram of apparatus used for infusing compounds into the lateral cerebral ventricle of rats.
3.4.2 Methods

3.4.2.1 Cerebral ventricular cannulation and continuous intraventricular infusion: the principles of this technique have been described (20, 418).

Each rat was anaesthetized with Nembutal (0.18 ml intraperitoneally); anaesthesia was maintained with ether. The vault of the skull was exposed by making a midline incision in the scalp. Using a 25g needle, the skull was penetrated at a point 3 mm posterior to the right coronal suture and 3 mm lateral to the sagittal suture. Through this aperture, the bevelled point of a stiff, narrow-bore cannula (Fortex PP10), 70-90 cm long and containing artificial cerebrospinal fluid (CSF), was introduced to a depth of 3mm, with a bias towards the midline. In this position, the opening of the cannula lay within the right lateral ventricle. The cannula was then anchored by sutures to the adjacent pericranium. A loop of the cannula was left subcutaneously, and then the cannula was sutured to the subcutaneous tissues. After its emergence through the scalp incision, the cannula was threaded through a 5cm length of wide-bore polythene tubing (Fortex PP325). The scalp incision was closed by sutures around the end of this wide tube, which in turn was held in position by further sutures. The fine cannula was supported by a counterweight slung over a pulley, and was then connected by its free end to a slow-infusion pump. The animal was placed in a case with an electrified fence (Fig. 3.4.2.A.). After the rat had recovered from the anaesthetic, it was free to move unhindered by the intraventricular cannula. At about 2 hours after the operation, the slow-infusion pump was started.
During the following 7–9 days, this pump delivered solutions of artificial CSF at a rate (1 μl/min) roughly half that of CSF formation (419).

Control rats received artificial CSF alone (for composition, see Methods 3.4.2.3). Experimental rats received solution with the substances added as described below (Methods 3.4.2.3). After 7–9 days the rats were killed, and the nucleolar dry mass of the raphe neurones was measured.

3.4.2.2 Other operative procedures: similar preparations of raphe nuclei were obtained from rats (see Footnote) which had either been subjected to bilateral cervical sympathectomy 7–9 days previously, or had been given a single injection of 6-hydroxydopamine into a lateral cerebral ventricle (250 μg in 0.05 ml artificial CSF); animals in the latter group were killed in batches up to 16 days after having received the injection; control animals received a single injection of artificial CSF intraventricularly, and were killed 10 days after.

3.4.2.3 Preparation of solutions for intraventricular infusion: a solution of artificial CSF was prepared. Its composition was: NaCl, 121.5 mM; NaHCO₃, 25 mM; KCl, 3.5 mM; CaCl₂, 1.3 mM; MgCl₂, 1.14 mM; NaH₂PO₄, 0.51 mM; urea, 3.33 mM; and glucose, 3.33 mM. Using this artificial CSF, solutions containing the following concentrations of monoamines were prepared for ventricular infusion: a) low 5HT, 50 μg/ml; b) high 5HT, 500 μg/ml; c) melatonin, 50 μg/ml; d) noradrenaline, 50 μg/ml; e) histamine, 50 μg/ml; and f) tryptophan, 1 mg/ml.

(Footnote: these tissues were made available by Professor W.E. Watson, Dept. of Physiology, Edinburgh University.)
3.4.2.4 Preparations of substances for intraperitoneal injection:

tryptophan was partly dissolved and partly suspended in Ringer lactate at a concentration of 50 mg/ml. The fluid was warmed to skin temperature and slightly acidified (2 drops of N hydrochloric acid delivered by Pasteur pipette into 5 ml tryptophan-fluid), in order to enhance the solubility of tryptophan. Control solution was prepared from which only the tryptophan was omitted. Each rat was given daily either tryptophan fluid or control solution by intraperitoneal injection. The nucleolar dry mass of the raphe neurones was measured on day 8.

Fluid containing phenylalanine was prepared similarly, at a concentration of 100 mg/ml. Each rat was given 100 mg of phenylalanine daily by the intraperitoneal route. Control animals received aliquots of control solution prepared as for the tryptophan experiment. These rats were also killed after 8 days, and the raphe neurones were studied as above.

PPLA (at a concentration of 60 mg/ml) was suspended in saline containing 1% Tween 80. In one group of rats, each animal received 1 ml of this fluid daily for 8-9 days by intraperitoneal injection. In a second group, each rat received the same injections of PPLA fluid on alternate days, but on every second day received 50 mg nialamide in 1 ml saline by intraperitoneal injection. In a third group of rats, each animal received daily injections of 1 ml control solution, which consisted of 1% Tween-saline solution. All rats were killed between days 8 and 10. The nucleolar dry mass of their raphe neurones was measured.
Fig. 3.4.3.A. Raphe neurones: columns indicate pooled mean nucleolar dry mass, with standard errors, grouped according to experimental procedure indicated. Substances were given by intraventricular infusion.

Fig. 3.4.3.B. Raphe neurones: data presented as above, but rats were given 6-hydroxydopamine by intraventricular injection.
Fig. 3.4.3.C.

Fig. 3.4.3.D.

Figs. 3.4.3.C.–E.

Raphe neurones: columns represent data as in Fig. 3.4.3.A., but rats were given intraperitoneal injections of tryptophan, phenylalanine, PCPA, or PCPA with Nialamide.
<table>
<thead>
<tr>
<th>NUCLEUS</th>
<th>PROCEDURE or DRUG (with number of animals)</th>
<th>POOLED MEAN NUCLEOLAR DRY MASS ($g \times 10^{-12}$)</th>
<th>STANDARD ERROR OF MEAN (S.E.)</th>
<th>NUMBER OF NUCLEOLI OBSERVED</th>
<th>p VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raphe</td>
<td>Control, (4)</td>
<td>8.99</td>
<td>0.32</td>
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<td>low 5HT, (6) 500ug/ml I-vent</td>
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<td>0.26</td>
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<td></td>
<td>melatonin, (5) 50ug/ml I-vent</td>
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<td>0.22</td>
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<td>high 5HT, (4) 500ug/ml I-vent</td>
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<td>sympathectomy, (3)</td>
<td>8.76</td>
<td>0.28</td>
<td>262</td>
<td>N.S.</td>
</tr>
</tbody>
</table>

| Raphe  | Control, (3)                                | 9.40                                             | 0.33                          | 198                         |         |
|        | 6-OHDA d 1-4, (4) 250ug/ml I-vent           | 9.23                                             | 0.25                          | 268                         | N.S.    |
|        | 6 d 5-7, (3)                                | 9.04                                             | 0.31                          | 187                         | N.S.    |
|        | 6 d 11-16, (7)                              | 8.95                                             | 0.17                          | 535                         | N.S.    |

| Raphe  | Control, (3)                                | 9.96                                             | 0.36                          | 155                         | 0.025-0.02 |
|        | tryptophan, (3)                             | 8.74                                             | 0.36                          | 119                         |         |

| Raphe  | Control, (2)                                | 7.68                                             | 0.46                          | 56                          | N.S. (0.3-0.2) |
|        | phenylalanine, (3)                          | 8.43                                             | 0.35                          | 152                         |         |

| Raphe  | Control, (5)                                | 7.87                                             | 0.30                          | 220                         |         |
|        | PCPA, (4) 60mg                              | 8.77                                             | 0.37                          | 205                         | N.S. (0.1-0.05) |
|        | PCPA+Nial., (3)                             | 7.50                                             | 0.39                          | 155                         | N.S.    |

(p value of PCPA+Nial. compared with PCPA alone: 0.02-0.01)
3.4.3 Results

The nucleolar dry mass of raphe neurones was reduced by the continuous intraventricular infusion of "low-5HT" and of melatonin. No changes were seen after similarly infusing "high-5HT", noradrenaline, histamine or tryptophan. The procedures involving 6-hydroxydopamine injection and cervical sympathectomy were also without effect (Figs. 3.4.3.A. & B.).

After daily intraperitoneal injections of tryptophan for 8 days, the nucleolar dry mass of the raphe neurones decreased (Fig. 3.4.3.C.). After intraperitoneal phenylalanine for 8 days, the same parameter tended to increase, but the difference was not statistically significant (Fig. 3.4.3.D.). After PCPA alone, the same parameter also tended to increase at day 8-10, although again the increase was not significant; when PCPA was given with nialamide, this tendency to increase was not observed (Fig. 3.4.3.E.).

The rats in some experimental groups displayed distinctive patterns of behaviour. Two days after the onset of intraventricular infusion with "low-5HT" or with melatonin, rats became very drowsy, and showed a reduction both in spontaneous motor activity and in reactivity to simple tactile and auditory stimuli; the animals remained thus throughout the experiment. With the infusion of "high-5HT", the rats became drowsy within minutes; but after day 1, they became hyperactive and hyperreactive; while at rest they displayed a spreadeagled stance, tail dorsiflexion, and a
coarse tremor and titubation. Rats receiving noradrenaline or tryptophan in the intraventricular infusate also were more excitable, moving around within their cages frequently and also reacting sharply to stimuli; these signs started on day 2, but were not preceded by drowsiness. For 1 hour after the single intraventricular injection of 6-hydroxydopamine, the rats were hyperactive and hyperreactive; a subsequent 12-hour phase of extreme immobility and unresponsiveness disappeared gradually, and behavioural normality returned during day 2. Those rats with cervical sympathectomy, and those receiving intraventricular histamine solution, artificial CSF alone, and the intraperitoneal injections appeared normal.

Two rats were discarded from both the noradrenaline and the tryptophan groups because of their poor condition. Apart from them, the general condition of all rats was good, and their intake of food and water was maintained.

During those experiments involving continuous ventricular infusion, the hyperactivity associated with tryptophan and noradrenaline caused coiling of the intraventricular cannulae. Despite frequent sessions devoted to reversing this process, cannulae occasionally became kinked, and the infusions therefore may have been intermittently obstructed. As a result the rate of administration of substances during these experiments may have been slightly less than the stated value.
3.4.4 Discussion

5HT given into the cerebral ventricles is taken up at low concentrations by the raphe neurones only (165, 210); at higher concentrations 5HT is also accumulated by catecholamine-containing neurones (205, 207, 208, 248, 420, 421) and other cells (209, 420, 422). The progressive rise of endogenous 5HT concentration in brain tissues after MAO inhibition is sometimes accompanied by a fall in the rate of 5HT formation from tryptophan, but not from 5HTP (237, 230–232, 336, 342); when present, this inhibition of 5HT synthesis may result from alterations in the degree of end-product inhibition of tryptophan hydroxylase by 5HT. A similar mechanism contributes to the regulation of brain noradrenaline synthesis (see Introduction 1.2.9). 5HT inhibits many aspects of brain metabolism, including glycolysis (423) and the synthesis of ribonucleic acid and protein (424–426). As yet, no 5HT-containing nerve terminals have been demonstrated in the raphe nuclei (23, 29, 427). But when 5HT is introduced by iontophoresis into the raphe nuclei, the firing of most of these neurones is inhibited (428), although some neurones are excited (213, 427); in the dorsal raphe nucleus, which is composed almost exclusively of 5HT-containing neurones (24) only the inhibitory responses to 5HT are evoked (327). In the present study, the decreased nucleolar dry mass of raphe neurones indicates that when these neurones are exposed chronically to exogenous 5HT, their production of ribosomes is inhibited; but that this inhibition ceases to operate during exposure to high concentrations of
exogenous 5HT. These results may be an indirect result of the non-specific uptake of 5HT at high concentrations (see above), or may be related to events at the postsynaptic receptors for 5HT. These results will be discussed later (see Concluding Discussion 4.1).

Melatonin may be present in the hypothalamus (55). In vitro the cerebral cortex accumulates isotopically-labelled melatonin (429). Melatonin increases the synthesis of 5HT in brain extracts; when given systemically, it raises the 5HT content of brain (430), possibly by increasing the availability of a cofactor necessary for 5HT synthesis (431). In the present study, however, melatonin infusion, like low-5HT infusion, diminished the nucleolar dry mass of raphe neurones. An interpretation of these findings will be given later (see Concluding Discussion 4.1).

Histamine is present in the central nervous system (407, 432). Its turnover increases after reserpine (433). In the present study, histamine infused into the cerebral ventricles was without effect on the nucleolar dry mass of raphe neurones.

The pineal gland is richly innervated by sympathetic nerve terminals; it also contains 5HT and melatonin, and the enzymes which synthesize and break down these compounds. After cervical sympathectomy, various aspects of the metabolism of 5HT and melatonin are altered (408). In the present study, the absence of any change in the nucleolar dry mass of raphe neurones showed that this procedure did not affect their synthesis of ribosomes.

The drug 6-hydroxydopamine causes the degeneration of noradrenergic neurones, and the brain content of noradrenaline falls rapidly in consequence (405, 434-442). However the activity of enzymes synthesizing 5HT in
brainstem tissue does not alter (406, 443), nor does the rate of conversion 
in vitro of isotopically-labelled tryptophan into 5HIAA (444). There are 
other indications that raphe neuronal function is not significantly affected 
by 6-hydroxydopamine (35, 248, 435, 436, 445-449). This view is supported 
by the unchanged nucleolar dry mass of raphe neurones observed in the 
present study.

Many changes in 5HT metabolism have been reported to follow the 
administration of catechol amines and their precursors (404, 448-451). 
Some of these metabolic interactions have been attributed to local competition 
for enzymes and cofactors (240, 279, 452-460), for transport mechanisms 
(71, 118, 177, 224, 446) and for amine storage sites (446, 454, 462-464). 
Phenylalanine can be considered as example: rats fed on a high-phenylalanine 
diet for 7-12 days show a reduced 5HT concentration and a low rate of 5HT 
synthesis in brain tissue (401, 402); in vitro, the uptake of isotopically-
labelled tryptophan by cerebral cortical slices and by whole brain synapto-
somes diminishes in the presence of phenylalanine, and the efflux of 
tryptophan from these synaptosomes accelerates (63, 65); in addition, 
phenylalanine inhibits tryptophan hydroxylase (37, 404). Noradrenergic 
terminals are present in the raphe nuclei; there are many in the dorsal 
raphe nucleus, but few in the median raphe nucleus (29). Iontophoretically-
applied noradrenaline increases or diminishes the spontaneous electrical 
activity of neurones in the raphe nuclei (427). In the present study, however, 
while the intraventricular infusion of noradrenaline produced striking
behavioural changes, there were no alterations in the nucleolar dry mass of raphe neurones. After parenteral phenylalanine, the same parameter increased, but the change was not statistically significant.

The effect of parenterally-administered tryptophan on 5HT metabolism in brain is not clear. Diets rich in tryptophan increase brain 5HT content (402), while the converse result is produced by tryptophan-poor diets (324, 465). Nevertheless the regulation of the uptake by brain tissues of plasma tryptophan is not fully understood (66, 81, 466, 467), although the degree of tryptophan-binding by plasma proteins is important (67, 468, 469).

Tryptophan given by parenteral or intracerebral injection initially increases 5HT synthesis and elevates the brain concentration of 5HT and 5HIAA (68, 81, 470-473). After an intraperitoneal injection of tryptophan (100mg/kg), 5HT fluorescence in raphe tissue of the rat increases (264). But above a certain dosage of tryptophan, which differs in various studies, progressively increasing the amount of tryptophan results in only the 5HIAA continuing to rise; very high doses of tryptophan only prolong the plateau-levels of both 5HT and 5HIAA (78, 111). Hence the evidence indicates that the increased amount of tryptophan available to brain tissues is taken up by raphe neurones and converted into 5HT (474) (see Introduction 1.2.3); and that when the vesicle-stores for 5HT are filled, excess 5HT is catabolized by MAO (176, 472, 475). It has also been suggested that more 5HT may be released from the raphe neuronal terminals on to postsynaptic receptors (201, 476-478). But there is no evidence that the administration
of tryptophan induces the production of more 5HT-synthesizing enzymes and MAO in the raphe neurones. Indeed the reduction in raphe neuronal electrical activity after tryptophan may indicate that their functional activity is inhibited (264, 478). This hypothesis is compatible with the decreased nucleolar dry mass demonstrated in the present study after intraperitoneal injections of tryptophan; this finding is discussed later (see Concluding Discussion 4.1). Unexpectedly, however, the intraventricular infusion of tryptophan produced no change in the raphe neuronal parameter. It is possible that the membrane uptake by raphe neuronal cell bodies and terminals of the tryptophan given by these two routes may have differed because of the concurrent uptake of this amino acid into dissimilar populations of other neurones and of glial, ependymal and endothelial cells (66, 99, 208, 240, 456, 461, 466, 474, 479-481). Discrepant effects on the synthesis-rate of 5HT in raphe neurones might have arisen from such different characteristics in the two routes of administration, or from the different dosages of tryptophan provided in these two experiments (see Introduction 1.2.3). However the tryptophan concentration and 5HT turnover in rat brain were not measured in this study; hence there is no basis for comparing the dissimilar values for the raphe neuronal parameter after tryptophan with those found after the intraventricular infusion of low and high concentrations of 5HT (Results 3.4.3).

After the administration of PCPA, the concentration of 5HT and 5HIAA declines slowly (482) to values dependent on the size and frequency
of the dosage (399, 483-485). A reduced turnover of 5HT is also demonstrated by the low concentration of 5HIAA in CSF (486), and by the slow rise of this concentration after probenecid (487). After PCPA, the rate of conversion of isotopically-labelled tryptophan into 5HIAA by midbrain tissue in vitro declines (444), and the accumulation of isotopically-labelled 5HT from 3H-tryptophan is retarded (488); however the retention of 3H-5HT in mouse brain is unchanged (488), or even increased (489). These reports suggest that PCPA does not increase the rate of breakdown or elimination of 5HT but rather inhibits some steps in its synthesis.

PCPA decreases the transport of many amino acids into brain (490). In synaptosomes prepared from rat brain, the uptake of isotopically-labelled tryptophan decreases while the tryptophan efflux increases (65).

In vivo the tryptophan concentration in rat brain is low after 3 days of PCPA treatment (383); however, in the 5HT-containing neurones of the snail, 14C-tryptophan uptake does not alter after PCPA, although the 5HTP synthesized from it is reduced (80). In rat brain homogenate, PCPA also inhibits the conversion of 14C-tryptophan into 5HT (404); although in vitro this PCPA-induced inhibition of tryptophan hydroxylase appears to be mainly competitive, in vivo most of the inhibition is irreversible, and appears only after a 2-day timelag (491). One deduction from this finding is that delayed inhibition is caused not by PCPA itself, but by some metabolite of PCPA (493-494). An alternative proposal is that the delayed inhibition results from the synthesis of defective tryptophan hydroxylase enzymes (493);
this may arise from PCPA being incorporated at the active sites of these enzymes as they are formed in the cell bodies of the raphe neurones (236, 495); as a result, the main inhibitory effects become apparent only as the defective enzymes reach the raphe neuronal terminals (91, 496, 497), where most 5HT is synthesized (see Introduction 1.2.3). However it is difficult to reconcile this view of PCPA-induced inhibition with other findings: for example, when the axonal transport of newly-synthesized enzymes from the cell bodies of the raphe neurones to their terminals is stopped by destroying the cell bodies themselves (498) or by transecting their axons (499), PCPA still inhibits the formation of 5HT by tryptophan hydroxylase already present in their terminals; however it may be rash to presume that 5HT-synthesizing enzymes cannot be produced in the terminals (see Introduction 1.2.3).

Whatever the mechanisms involved, PCPA-induced inhibition of 5HT synthesis is only partial (400, 482). For instance, in raphe neurones the intensity of 5HT histofluorescence produced by giving tryptophan may not alter after PCPA pretreatment (264); this finding has been challenged recently, however (500). After PCPA the amount of isotopically-labelled 5HT formed in brain from radioactive tryptophan is unaltered, both under basal conditions and when median raphe neurones are stimulated electrically (259, 400); in intact animals subjected to mild stress, 5HT synthesis may even increase despite the presence of PCPA (347). The incompleteness of the inhibition of 5HT synthesis may result from an increased activity of extant enzyme (91, 259) or it may reflect the capacity of raphe neurones
to synthesize more enzyme than can be inactivated by PCPA.

The uncertainty about how PCPA inhibits 5HT synthesis in raphe neurones makes more difficult the interpretation of the findings of the present study. These findings were that PCPA alone tended to increase the nucleolar dry mass in raphe neurones, but that when PCPA and the MAO inhibitor nialamide were given on alternate days, there was no such tendency. It is of interest, however, that the nucleolar responses of raphe neurones to the decrease in brain 5HT content caused by PCPA, phenylalanine and reserpine were qualitatively similar.

Sedation has been reported to accompany the intraperitoneal (501) or intraventricular administration of 5HT (232, 323), the intravenous or intracerebral injection of melatonin (410, 412), and the low-frequency stimulation of the raphe neurones (198). Behavioural excitation follows large doses of 5HT given intraventricularly (502), the administration of tryptophan or 5HTP with nialamide (470, 473, 503-506), nialamide with tricyclic antidepressant drugs (507), and high-frequency electrical stimulation of the raphe nuclei (198). In the present experiments the intraventricular infusion of low-dose 5HT and of melatonin induced sedation; with high-dose 5HT infusion, sedation was soon followed by agitation; with noradrenaline and tryptophan infusion, only behavioural excitation was seen. Hence only those intraventricular infusions which induced continuous sedation in the experimental animals were accompanied by decrements in the nucleolar dry mass of raphe
neurones. PCPA is reported to alter the behaviour of rats (see Discussion 3.6.4.3); in the present experiments, where only gross behavioural changes were observed and recorded, behavioural abnormalities were not seen.

3.4.5 Conclusions

Low doses of 5HT and melatonin given by intraventricular infusion for several days decreased the nucleolar dry mass of raphe neurones. No such change was seen after the intraventricular infusion of high-dose 5HT, tryptophan, noradrenaline, histamine and 6-hydroxydopamine or after cervical sympathectomy. Of the substances given intraperitoneally, only tryptophan was associated with a decreased nucleolar dry mass of the raphe neurones.
3.5 The effect of some other drugs on the nucleolar dry mass of raphe neurones

3.5.1 Introduction

The drugs which were given to rats in the experiments already described produce psychological changes in humans. MAO inhibitors, tricyclic antidepressants, oxyptetine and lithium are effective in various psychiatric syndromes, while reserpine also has psychological effects (356, 357, 508-511). The involvement in 5HT metabolism of many other psychotropic drugs is still obscure.

It was decided to investigate the effects on the nucleolar dry mass of raphe and various hypothalamic neurones of two such drugs, diamorphine and barbitone.

Changes in the turnover of 5HT have been reported during the administration of morphine (512, 513). The production of opiate analgesia, tolerance and dependence may involve brain 5HT metabolism (442, 514-520).

The phenomenon of dependence is also found after barbiturate therapy, and barbiturates are well-known for their hypnogenic properties (521); these properties are attributed also to 5HT and melatonin (482, 484, 501, 504, 522, 523). Barbiturates may influence 5HT metabolism (524).
3.5.2 Methods

In the diamorphine experiment, each rat was given intraperitoneal injections of 1mg diamorphine twice daily, at 10.00 hrs. and at 16.00 hrs. Control animals received similar injections of Ringer lactate solution. Animals from experimental and control groups were killed during the following 18 days of the experiment. Nucleolar dry mass measurements were made on raphe neurones and on neurones from the suprachiasmatic, arcuate, ventromedial and dorsomedial nuclei of the hypothalamus. These nuclei were obtained as described earlier (General Methods 2.2). The control animals were killed on days 4, 8, 10, 14 and 17; because the nucleolar values obtained from each animal did not change throughout the experiment, the results were pooled as a single control value.

Three experiments were conducted in which barbitone sodium was added to drinking water at an initial concentration of 4 gm/L. In the first experiment, rats were caged individually and were allowed free access only to their drinking solution; an activity wheel was attached to each cage. At days 10 and 17, the concentration of barbitone was raised to 8 gm/L and 10 gm/L respectively. Control animals were similarly caged, but received ordinary water to drink. The food and water intake of each rat was measured daily during the 37 days of the experiment. The reading on the counter of each activity wheel was noted daily, and each rat was weighed every second day.

In the second experiment, drinking-water containing barbitone (4gm/L) was provided for rats grouped normally, 6 to a cage. At
days 11, 14, and 23, the barbitone solution was replaced by ordinary drinking water in some cages. The other rats continued to drink the barbitone water for up to 23 days. Rats were killed at various times after barbitone-drinking had been started, and also for some days after it had been stopped. Control animals received ordinary drinking water; they were killed in batches at the beginning and end of the experiment.

In the third experiment, 4-month-old rats were housed normally and were given drinking water with the barbitone concentration maintained at 4gm/L for up to 29 days. Rats drinking ordinary water acted as controls. At days 27 and 29 of the experiment, the rats receiving the drug were killed. Control rats were killed on day 28.

While in all rats the nucleoli of raphe neurones were examined, those from the arcuate and suprachiasmatic neurones were examined in only some rats.
Fig. 3.5.A. Various neurones: columns indicate pooled mean nucleolar dry mass, with standard errors, for groups of rats; experimental animals received diamorphine for up to 18 days.

Fig. 3.5.B. Various neurones: data presented as in Fig. 3.5.A.
Fig. 3.5.C. Various neurones: data presented as in Fig. 3.5.A., but experimental animals received increasing doses of barbiturate orally for 37 days.

Fig. 3.5.D. Raphe neurones: data presented as in Fig. 3.5.A., but barbiturate was given orally, and then withdrawn at different stages during the experiment.
Fig. 3.5.E. Arcuate and suprachiasmatic neurones: data presented as in Fig. 3.5.A., but experimental animals received barbiturate orally; this barbiturate was then withdrawn at different stages during the experiment.

Fig. 3.5.F. Various neurones: data presented as in Fig. 3.5.A. but experimental animals received barbiturate orally for 29 days.
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<th>NUCLEUS</th>
<th>DRUG GROUP (with number of animals)</th>
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<th>STANDARD ERROR OF MEAN (S.E.)</th>
<th>NUMBER OF NUCLEOLI OBSERVED</th>
<th>P VALUE</th>
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</tr>
<tr>
<td></td>
<td>d 6-12, (5)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>d 15-18, (4)</td>
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<td>0.24</td>
<td>86</td>
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<tr>
<td>Ventro-</td>
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<td>0.26</td>
<td>93</td>
<td>-</td>
</tr>
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<td>medial</td>
<td>Diamorphine d 6-12, (4)</td>
<td>6.88</td>
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<td></td>
<td>1 mg b.d.</td>
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<td>0.27</td>
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<td>POOLED MEAN NUCLEOLAR DRY MASS (g x 10^{-12})</td>
<td>STANDARD MEAN ERROR (S.E.)</td>
<td>NUMBER OF NUCLEOLI OBSERVED</td>
<td>P VALUE</td>
</tr>
<tr>
<td>---------</td>
<td>-------------------------------------</td>
<td>-----------------------------------------------</td>
<td>---------------------------</td>
<td>-----------------------------</td>
<td>---------</td>
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<td>168</td>
<td>-</td>
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<tr>
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<td>0.22</td>
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</tr>
<tr>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>S.C.</td>
<td>Control, (3)</td>
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<td>0.31</td>
<td>57</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Barb. d 37, (4) 4-10g/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arcuate</td>
<td>Control, (3)</td>
<td>6.65</td>
<td>0.25</td>
<td>49</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Barb. d 37, (4) 4-10g/ml</td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Raphe</td>
<td>Control, (4)</td>
<td>8.39</td>
<td>0.29</td>
<td>191</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Barb. d 4-5, (4) 4g/ml</td>
<td>8.12</td>
<td>0.31</td>
<td>150</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>Barb. d 14, (3)</td>
<td>6.63</td>
<td>0.28</td>
<td>157</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>d 11, +6 off, (4)</td>
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<td>0.27</td>
<td>245</td>
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<tr>
<td></td>
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<tr>
<td></td>
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<td>0.30</td>
<td>180</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Barb. d 23, +7 off, (4)</td>
<td>9.11</td>
<td>0.32</td>
<td>210</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>4g/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arcuate</td>
<td>Control, (2)</td>
<td>7.29</td>
<td>0.28</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Barb. d 4-5, (4) 4g/ml</td>
<td>7.05</td>
<td>0.22</td>
<td>77</td>
<td>N.S.</td>
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<tr>
<td></td>
<td>Barb. d 14, (3)</td>
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<td>0.17</td>
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</tr>
<tr>
<td></td>
<td>d 11, +6 off, (3)</td>
<td>6.86</td>
<td>0.22</td>
<td>61</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>d 14, +7 off, (3)</td>
<td>7.10</td>
<td>0.21</td>
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<tr>
<td></td>
<td>Control, (3)</td>
<td>7.12</td>
<td>0.28</td>
<td>68</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Barb. d 23, +7 off, (3)</td>
<td>6.94</td>
<td>0.26</td>
<td>57</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>4g/ml</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NUCLEUS</td>
<td>DRUG GROUP (with number of animals)</td>
<td>POOLED MEAN NUCLEOLAR DRY MASS (g x 10^-12)</td>
<td>STANDARD ERROR OF MEAN (S.E.)</td>
<td>NUMBER OF NUCLEOLI OBSERVED</td>
<td>p VALUE</td>
</tr>
<tr>
<td>-----------</td>
<td>------------------------------------</td>
<td>-----------------------------------------------</td>
<td>-------------------------------</td>
<td>-----------------------------</td>
<td>---------</td>
</tr>
<tr>
<td>S.C.</td>
<td>Control, (3)</td>
<td>7.71</td>
<td>0.32</td>
<td>62</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Barb. d 11, +6 off, (3)</td>
<td>7.76</td>
<td>0.33</td>
<td>65</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>4gm/L d 23, +7 off, (4)</td>
<td>7.33</td>
<td>0.26</td>
<td>87</td>
<td>N.S.</td>
</tr>
<tr>
<td>Raphe</td>
<td>Control, (3)</td>
<td>10.05</td>
<td>0.41</td>
<td>165</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Barb. d 23, (4)</td>
<td>8.36</td>
<td>0.27</td>
<td>208</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>4gm/L</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Arcuate</td>
<td>Control, (3)</td>
<td>7.15</td>
<td>0.29</td>
<td>52</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Barb. d 23, (4)</td>
<td>6.53</td>
<td>0.24</td>
<td>59</td>
<td>N.S. (0.2-0.1)</td>
</tr>
<tr>
<td>S.C.</td>
<td>Control, (3)</td>
<td>7.11</td>
<td>0.29</td>
<td>40</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Barb. d 23, (4)</td>
<td>6.73</td>
<td>0.27</td>
<td>52</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>4gm/L</td>
<td></td>
<td></td>
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</tr>
</tbody>
</table>
3.5.3 Results

Between days 5 and 9 of the diamorphine experiment, the nucleolar dry mass of raphe neurones declined slightly, but not significantly. There followed a transient but significant increase in the same parameter at days 11-12 (Fig. 3.5.A.). In the suprachiasmatic neurones, the nucleolar dry mass also increased, but later than in the raphe neurones (Fig. 3.5.A.). No significant changes were seen in the nucleolar dry mass of the other neurones examined (Fig. 3.5.B., Table 3.5).

With barbitone (Figs. 3.5.C. & F., Table 3.5.), the raphe neuronal parameter was not decreased at day 5. But by day 14, a highly-significant decrease was apparent; this decrease was maintained for 30 days. The changes in dry mass values were dose-related. After withdrawing the drug at days 11, 14 and 23, the nucleolar parameter returned to normal within 7 days in each case; there was no "overshoot".

For the nucleoli of arcuate and suprachiasmatic neurones, the dry mass values decreased during barbiturate administration. The dry mass decrements of the arcuate neurones were greater than those of the suprachiasmatic neurones, in which a significant change was seen only in rats receiving barbiturate for 37 days.

In the early stages of the diamorphine experiment, there was evidence of drug toxicity: one rat was found dead on each of the first four days; and during the early experimental period, the other rats were lethargic and ate
less food. But after this initial period, the condition of all rats improved.

During the barbiturate experiments, the rats became tranquil, and responded lethargically to auditory and tactile stimuli. They appeared healthy, in that there was no soiling or eye-crusting. In the first of these experiments, rats were caged individually in order to monitor more closely their behaviour during the experimental period: the daily intake of food and water remained constant; during the first 2-3 weeks of the experiment, the weight of each animal rose by approximately 20-35 gm, but by the end of the experiment the starting values for weight were regained; lastly, although the animals were drowsy, lethargic and moderately unresponsive to stimuli when observed during daytime, the readings on the counters of the activity wheels attached to each cage provided evidence that during the nocturnal periods, normal activity was maintained throughout the experimental period.
3.5.4 Discussion

After morphine, brain 5HT concentration is usually unchanged (383, 512, 525), although it is reported to be low in one instance (526). The effect of morphine on the turnover of 5HT is more controversial (527). During the administration of morphine, the 5HT turnover in rat brain is raised at 3 days and at 3 weeks (513). During morphine dependence, the regional turnover of 5HT is elevated in brainstem and hypothalamus more than in other brain areas (518); yet conflicting results are reported (528). In other experiments, 5HT turnover rises within a few hours of the administration of morphine both in rat brain (512, 529) and in mouse brain (530, 531); but is reported to be unchanged at day 3 (532) and at day 4 (533, 534) in mouse brain, and at day 5 (535) and days 6-9 (536) in rat brain.

This conflict of evidence about 5HT metabolism may have several origins. Firstly, different dosage schedules may produce discordant results (536, 537), and the pharmacological effects of morphine given as pellet implants or by injection may also differ. Secondly, the values for 5HT turnover after morphine in mouse experiments depend on the strain of mouse used (538); the same variability may exist in rats. Thirdly, in vitro MAO is partially inhibited by morphine (539); in vivo, morphine withdrawal is followed by an increased MAO activity (540). Hence in morphine experiments, when 5HT turnover is deduced by using MAO inhibition (see Introduction 1.2.8), the results may be inaccurate.
When given intravenously, morphine does not inhibit the spontaneous firing of dorsal raphe neurones, although the firing of central noradrenergic neurones is depressed (541). Nor, when given by micro-iontophoresis, does morphine induce electrical responses in brainstem neurones which can be correlated with their responses to 5HT (542).

In the present study, the nucleolar dry mass of raphe and suprachiasmatic neurones was increased by diamorphine; these increments had a slower onset than the otherwise similar increases observed after reserpine (see Results 3.1.3). In the ventromedial neurones, too, the decline in the nucleolar dry mass values resembled the change seen after reserpine, Ventromedial neurones may be selectively involved in the development of morphine-dependence and tolerance (543, 544).

Opiates have protean effects on brain metabolism (545-553). Until further details are known about these effects, the interpretation of the nucleolar changes in the present investigation will remain difficult. Nevertheless the present results show an increased ribosomal synthesis in raphe and suprachiasmatic neurones; this finding is consistent with the reports of enhanced 5HT turnover in brain after morphine.

The concentration of brain 5HT declines within minutes of an intraventricular injection of barbiturate (554). However, between 30 minutes and 24 hours after the parenteral administration of barbiturate, brain 5HT concentration rises (333, 524, 555, 556). The turnover of 5HT in brain
is low 3 hours after the administration of barbiturate, although at this time the 5HT concentration in brain is normal (557, 558). Barbiturates given parenterally or by iontophoresis depress the electrical activity of most neurones in the central nervous system (217, 559, 560). Barbiturates also alter the transmitter actions of 5HT: the excitatory responses of various neurones to 5HT are inhibited (218), while the inhibitory effects of 5HT on other neurones may become more pronounced (218). An interaction between barbiturates and 5HT metabolism has been demonstrated during the induction and maintenance of sleep (561, 562), and in the inhibition of convulsions (563-566). Other findings indicate that barbiturates inhibit many facets of neuronal metabolism: these include noradrenaline turnover (567), acetylcholine release (568), glycolysis and oxygen utilization (569, 570).

This view of a general depression of neuronal metabolic activity is consistent with the findings in the present study, — that the nucleolar dry mass is reduced not only in raphe neurones, but also in arcuate and (less markedly) in suprachiasmatic neurones.

3.5.5 Conclusions

Two psychotropic drugs were given to rats. Diamorphine induced a delayed increase in the nucleolar dry mass of both raphe and suprachiasmatic neurones; the change in the raphe neuronal parameter preceded that in the suprachiasmatic neurones. Barbitone decreased the nucleolar dry mass of
raphe, arcuate and suprachiasmatic neurones; the change in the raphe neurones was the most pronounced, while that in the suprachiasmatic neurones was least marked. These findings were discussed in relation to the other actions of these drugs on brain 5HT metabolism.
3.6 The effect on raphe neuronal dry mass of some procedures which may alter the tissue levels of various steroid hormones

3.6.1 Introduction

Increased and decreased amounts of steroid hormones might influence the nucleolar dry mass of raphe neurones. Some of the following experiments were preliminary, and designed to observe the effect of several procedures while using relatively few animals.
3.6.2 Methods

Several experiments were performed.

Experiment I: (a) cortisone acetate (12.5mg) was given to each rat by intraperitoneal injection, daily for 14 days; (b) testosterone propionate (25mg) was given to each rat subcutaneously daily for 6 days; (c) each control animal was injected intraperitoneally with almond oil (0.5ml daily) for 8 days. When the series of injections were complete, the animals were killed and the nucleolar dry mass of raphe neurones was measured.

Experiment II: testosterone propionate (0.5mg) was injected subcutaneously into each rat on alternate days for 12-14 days. Uninjected animals were used as controls. Again the nucleolar dry mass of their raphe neurones was measured; in some animals the nucleolar dry mass of suprachiasmatic neurones was measured.

Experiment III: (a) Rats were subjected to bilateral adrenalectomy. Under ether anaesthesia, bilateral skin incisions were made in the dorso-lumbar regions of the rat, directly inferior to the rib cage and lateral to the lumbar musculature. After further deep dissection, the adrenal glands were identified in relation to the psoas muscle; the periadrenal fat was displaced, and the glands avulsed. There was little blood loss. Saline (2ml, 0.9%) was infiltrated subcutaneously before the wound was closed. Silk sutures were used for closing muscle and skin incisions.

Some of these animals subsequently received daily subcutaneous injections of cortisone acetate (12.5mg each) for up to the tenth day.
postoperatively, when they were killed; the cortisone impaired wound healing in these rats, and from day 6 onwards the wounds of some animals began to gape. The wounds of the rats not receiving cortisone healed normally, and those animals were killed between days 8 and 12 postoperatively.

Control rats were sham-operated; the only difference in procedure was that the adrenal glands were left intact; postoperatively the condition of these animals was normal. All rats were allowed free access to normal and 0.9% saline drinking water.

Experiment III: (b) At a later date, midbrain tissues containing raphe neurones were obtained from rats which had been subjected to adrenalectomy 12-15 days previously (see Footnote). Unoperated animals were used as controls. The nucleolar dry mass of the raphe neurones from both groups of animals was compared.

(Footnote: these tissues were obtained from animals provided by Professor W.E. Watson.)
Fig. 3.6.A. Raphe neurones: columns indicate pooled mean nucleolar dry mass, with standard error, of groups of rats; the rats were injected with cortisone for 14 days or with a high-dose of testosterone for 6 days, or were controls.

Fig. 3.6.B. Raphe and suprachiasmatic neurones: data represented as in Fig. 3.6.A., but experimental animals received a low-dose of testosterone by injection for 12-14 days.
Fig. 3.6.C. Various neurones: data represented as for Fig. 3.6.A., but experimental rats were subjected to adrenalectomy (d 8-12); some of these animals also received injections of cortisone.

Fig. 3.6.D. Raphe neurones: data presented as in Fig. 3.6.A., but animals were subjected to bilateral adrenalectomy (d 12-15).
<table>
<thead>
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<th>NUCLEUS</th>
<th>PROCEDURE (with number of animals)</th>
<th>POOLED MEAN NUCLEOLAR DRY MASS ($g \times 10^{-12}$)</th>
<th>STANDARD ERROR OF MEAN (S.E.)</th>
<th>NUMBER OF NUCLEOLI OBSERVED</th>
<th>P VALUE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Raphe</td>
<td>Control, (2)</td>
<td>8.67</td>
<td>0.51</td>
<td>111</td>
<td>-</td>
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<td>Cortisone d 14, (6) 12.5 mg</td>
<td>8.71</td>
<td>0.18</td>
<td>618</td>
<td>N.S.</td>
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<td>Testosterone d 6, (5) 25 mg</td>
<td>9.81</td>
<td>0.26</td>
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<td>S.C.</td>
<td>Control, (3)</td>
<td>6.27</td>
<td>0.23</td>
<td>147</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>Testosterone d 12–14, (4) 0.5 mg alt. days</td>
<td>6.91</td>
<td>0.19</td>
<td>244</td>
<td>0.05–0.025</td>
</tr>
<tr>
<td>Arcuate</td>
<td>Control, (3)</td>
<td>6.39</td>
<td>0.31</td>
<td>57</td>
<td>-</td>
</tr>
<tr>
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<td>Adrenalectomy, (4)</td>
<td>6.36</td>
<td>0.26</td>
<td>70</td>
<td>N.S.</td>
</tr>
<tr>
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<td>+ cortisone, (3) 12.5 mg</td>
<td>6.59</td>
<td>0.32</td>
<td>53</td>
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</tr>
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<td>Adrenalectomy, (4)</td>
<td>6.39</td>
<td>0.31</td>
<td>57</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>+ cortisone, (3) 12.5 mg</td>
<td>6.59</td>
<td>0.32</td>
<td>53</td>
<td>N.S.</td>
</tr>
<tr>
<td>Arcuate</td>
<td>Control, (3)</td>
<td>6.65</td>
<td>0.25</td>
<td>49</td>
<td>-</td>
</tr>
<tr>
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<td>Adrenalectomy, (4)</td>
<td>6.57</td>
<td>0.18</td>
<td>89</td>
<td>N.S.</td>
</tr>
<tr>
<td></td>
<td>+ cortisone, (3) 12.5 mg</td>
<td>6.59</td>
<td>0.23</td>
<td>66</td>
<td>N.S.</td>
</tr>
<tr>
<td>Raphe</td>
<td>Control, (4)</td>
<td>9.13</td>
<td>0.36</td>
<td>166</td>
<td>-</td>
</tr>
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<td>Adrenalectomy d 12–15, (4)</td>
<td>9.13</td>
<td>0.35</td>
<td>144</td>
<td>N.S.</td>
</tr>
</tbody>
</table>
3.6.3 Results

There were significant increases in the raphe neuronal nucleolar dry mass after repeated injections of testosterone, but not after cortisone (Figs. 3.6.A. & B., Table 3.6.). The increments after testosterone were dose-related.

After testosterone the nucleolar dry mass of suprachiasmatic neurones decreased (Fig. 3.6.B.).

After adrenalectomy there were no significant changes in the nucleolar dry mass of raphe neurones of rats killed at days 8-12 or 12-15 (Figs. 3.6.C. & D., Table 3.6.). In the former series, when adrenalectomized rats received cortisone injections daily, the nucleolar parameter of raphe neurones still did not alter. The nucleolar dry mass of arcuate and suprachiasmatic neurones was also unaffected by adrenalectomy in these experiments (Fig. 3.6.C.).
3.6.4 Discussion

3.6.4.1 Corticosteroid injections: in several studies a biphasic effect of corticosteroids on brain 5HT content has been observed. The 5HT and 5HIAA contents of rat brain decrease directly after the systemic injection of hydrocortisone (254, 571); but following the repeated injection of corticosteroids during 5 days, brain 5HT content is reported normal (572); and after very large doses of these drugs given for 20 days, brain 5HT content increases (333, 571). The capacity for 5HTP decarboxylation in brain does not change either after 5 days of corticosteroid injections or after adrenalectomy (572). Corticosteroids may reduce the synthesis of 5HT initially by an indirect mechanism: they stimulate the activity of hepatic tryptophan pyrrolase, which facilitates the conversion of plasma tryptophan to kynurenine (42) (see Introduction, Fig. 1.2.2.); kynurenine inhibits the uptake of tryptophan by brain tissue, and the synthesis of 5HT declines (40, 41, 572). However this mechanism does not account for the subsequent increase in brain 5HT content (571).

The rate of conversion of isotopically-labelled tryptophan into 5HIAA by midbrain explants from rats can be measured in vitro, thereby eliminating the effects of hepatic tryptophan pyrrolase activity: in these circumstances, the turnover of 5HT remains unchanged during 18 days exposure to corticosteroids (444).

In the present study, the nucleolar dry mass of the raphe neurones was unaltered at day 14. This observation reinforces the opinion that the changes
in brain 5HT content after the administration of corticosteroids may result, for example, from alterations in the accessibility of tryptophan to the 5HT-synthesizing enzymes in brain; changes in the production of these synthetic enzymes and in their intracellular concentration in the raphe neurones may not be involved (see Concluding Discussion 4.1).

3.6.4.2 Adrenalectomy: between 5 and 10 days after adrenalectomy, tryptophan hydroxylase activity declines in the brainstem but not in the teciencediencephalon, while in both regions the content of tryptophan and 5HT remains constant (573). This regional decline in tryptophan hydroxylase activity is accelerated by cycloheximide, but is partly counteracted by injections of corticosteroids (574). However, the precise effects of adrenalectomy on the mechanisms involved in tryptophan hydroxylase biosynthesis remain unclear. In the present studies, there were no changes in the nucleolar dry mass of raphe neurones from 8 to 15 days after adrenalectomy, or when corticosteroids were injected into adrenalectomized rats for 14 days.

Elsewhere, increases in the nucleolar dry mass of various hypothalamic neurones have been reported after adrenalectomy (21). These increases appear very early after the operation; and in some neurones they persist until day 11 post-operatively, while in other neurones they have disappeared by the fifth day. In the present study on raphe neurones, the observations were made only between days 8 and 15 postoperatively. Hence it would be necessary to investigate the response of raphe neuronal
nucleoli during the first seven days after adrenalectomy before concluding
that adrenalectomy did not affect the dry mass of raphe neuronal nucleoli.

3.6.4.3 Testosterone injections: there is some controversy about
a possible relationship between 5HT metabolism and sexual behaviour.
When MAO is inhibited, the 5HT content of rat brain rises while the sexual
activity of the rats declines; PCPA reverses the biochemical and behavioural
effects of MAO inhibition: when 5HTP is given with PCPA and MAO inhibitors,
however, the original effects are reproduced (575, 576). Again, when the
5HT content of rat brain is lowered by the administration of L-DOPA or
5-6 dihydroxytryptamine, the sexual behaviour of the rats increases; a
subsequent injection of 5HTP abolishes this activity (577, 578). The
administration of 5HTP also abolishes the decline in brain 5HT levels
and the increase in sexual activity brought about by PCPA alone (579, 580).
According to one report, however, PCPA has little effect on sexual activity
of cats although it reduces their brain 5HT content (581). Nevertheless in
most experimental animals, an inverse relationship is observed between
brain 5HT levels and the degree of sexual activity displayed by the animals.

There is some confusion as to whether this 5HT effect on sexual
behaviour is mediated in some way by endogenous testosterone. Methodo-
logical differences in the procedures of the various experiments - age of
rat, type of housing, dosage of drug and hormone, and type of sexual, social
behaviour measured - may underlie the discrepancies in the findings (575-
583).
The findings in the present study of the nucleolar dry mass of various neurones after the administration of testosterone were of interest, in that they confirmed a relationship between 5HT metabolism and testosterone: testosterone (at the high dose) increased the nucleolar dry mass of raphe neurones (see Results 3.6.3.); with PCPA a similar change was seen, although it was not statistically significant (see Results 3.4.3.). By contrast, the nucleolar dry mass of the suprachiasmatic neurones decreased; this observation was of interest because of the report that, after castration, the nucleolar dry mass of these neurones increases (21). The mechanisms which link the metabolic responses in these two groups of neurones - if indeed they are linked - remain obscure.

3.6.5 Conclusions

In these preliminary experiments, adrenalectomy and corticosteroid injections, alone or together, had no effect on the nucleolar dry mass of raphe neurones. After testosterone injections, the nucleolar parameter of raphe neurones increased while that of suprachiasmatic neurones decreased. These changes were discussed in relation to other changes in brain 5HT metabolism following these procedures.

Some of these experiments were preliminary, - designed to evaluate profitable lines of investigation into steroid-5HT interactions -; hence the findings must be interpreted with caution.
SECTION 4. CONCLUSION
4.1 Concluding Discussion

Many of the compounds used in the experiments described in this thesis have been shown by workers using other techniques to affect the metabolism of 5HT in brain; 5HT concentration, turnover or subcellular distribution may be altered. One of the aims of the present study was to identify possible relationships between changes in these indices of raphe neuronal metabolism and the alterations in nucleolar dry mass described in this thesis. In doing so, several problems had to be faced.

Firstly, the proportion of 5HT-containing neurones within the "median raphe nucleus" block of tissue excised is uncertain. At present there appears to be no technique by which this problem can be resolved with certainty. On examining preparations of raphe tissue by interference microscopy, a heterogeneity in neuronal and nuclear size was apparent; the dry mass values of the individual neuronal nucleoli were also variable. When the technique of formaldehyde histofluorescence is used (see Introduction 1.2.2), the faint yellow fluorescence, which is held to denote the presence of 5HT, is observed in most of the raphe neuronal bodies. Yet the disadvantages of this technique are, firstly, that small amounts of intracellular 5HT may not be detectable (25, 27, 584-587); secondly, that similar fluorescence may be produced by indoles other than 5HT (38, 242, 341, 588). In electrophysiological studies, when recording electrodes are placed in the median and dorsal raphe nuclei, many of these neurones - especially those in the
dorsal raphe nucleus - display slow (2 per second) firing rates: these neurones are thought to be 5HT-containing neurones, because drugs which alter 5HT metabolism also change the firing rate of these neurones, but do not affect neurones in adjacent regions of the brainstem (258, 264, 360, 377). Thus the indications are that most neurones within the median raphe nucleus contain 5HT, although the precise proportion is not known (23, 24, 589).

Secondly, many of the drugs used in these experiments have protean effects on brain metabolism, as well as effects on 5HT metabolism. Some of these effects are indicated below:

**BRAIN METABOLISM AFFECTED : References**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Catechol amines</th>
<th>Acetylcholine</th>
<th>Other</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reserpine</td>
<td>126, 255, 330</td>
<td>332-334, 599</td>
<td></td>
</tr>
<tr>
<td>MAO inhibitors</td>
<td>239, 339, 346, 590</td>
<td></td>
<td></td>
</tr>
<tr>
<td>tricyclic drugs</td>
<td>363, 370</td>
<td>334, 600</td>
<td>604, 605</td>
</tr>
<tr>
<td>lithium</td>
<td>358, 387, 390, 391</td>
<td>396-398</td>
<td>356, 395, 606-611</td>
</tr>
<tr>
<td>PCPA</td>
<td>37, 350, 399, 483, 485, 490, 491, 500, 591, 592</td>
<td></td>
<td>612, 613</td>
</tr>
<tr>
<td>morphine</td>
<td>127, 540, 541, 593-596</td>
<td>545-547, 549, 550-553, 614</td>
<td>569, 570</td>
</tr>
<tr>
<td>barbiturate</td>
<td>554, 567, 598</td>
<td>547, 568, 603</td>
<td></td>
</tr>
</tbody>
</table>

The influences of catecholamines and their precursors on 5HT metabolism have been mentioned already (see Discussion 3.4.4). It is possible, therefore, that changes in the nucleolar dry mass of raphe or suprachiasmatic neurones
which were observed in the present study were the indirect result of altered metabolism in central noradrenergic neurones, rather than the direct result of the deranged metabolism of raphe neurones. However, the intraperitoneal injections of oxypertine and phenylalanine, and the intraventricular administration of noradrenaline and 6-hydroxydopamine failed to alter the nucleolar dry mass of raphe neurones (see Results 3.3 and 3.4.3). This finding may indicate that the production of ribosomes for protein synthesis in raphe neurones is not affected significantly by drug-induced changes in the release of noradrenaline by noradrenergic neurones in brain.

Thirdly, the significance of the nucleolar dry mass changes in raphe neurones requires discussion. Changes in this parameter accompany altered metabolic demands for the ribosomal synthesis of proteins and enzymes involved in various cellular activities (see Introduction 1.1). Stimuli including drugs might be expected to alter many facets of neuronal activity. After axonal or terminal damage, for instance, cellular activities such as dendritic restructuring (615-618) and axonal sprouting take place, accompanying that accelerated synthesis of ribonucleic acid and protein which is associated with neuronal repair and regrowth (148, 179, 221, 618-622). Nevertheless, it may be justifiable to interpret more narrowly the nucleolar dry mass responses of neurones to specific stimuli which alter the functional activity of these cells (20, 21, 106); included in the functional activity of neurones are processes such as the synthesis, transport, storage, release, reuptake and catabolism of the relevant neurosecretion or neurotransmitter (109).
FIG. 4.1. SOME EFFECTS OF DRUGS ON 5HT METABOLISM (section references provided)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>5HT synthesis</th>
<th>Vesicle damage</th>
<th>MAO activity (5HT breakdown)</th>
<th>Raphe firing</th>
<th>Tryptophan Uptake</th>
<th>5HT concentration intra-, extraneuronal</th>
<th>Nucleolar Dry Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reserpine (3.1.)</td>
<td>↑</td>
<td>↑</td>
<td>↑</td>
<td>↓ or k</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
</tr>
<tr>
<td>Res./MAO inhibition (3.2.)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MAO inhibition (3.2.)</td>
<td>k or ↓</td>
<td>k</td>
<td>↓</td>
<td>↓</td>
<td>↑</td>
<td>↓</td>
<td>k</td>
</tr>
<tr>
<td>5HT (3.4.)</td>
<td>k or ↓</td>
<td>-</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↑</td>
<td>↓</td>
</tr>
<tr>
<td>Melatonin (3.4.)</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↑</td>
<td>↑ (?), -</td>
<td>↓</td>
</tr>
<tr>
<td>Tryptophan (3.4.)</td>
<td>↑</td>
<td>-</td>
<td>↑</td>
<td>↓</td>
<td>↑</td>
<td>↑ (?), -</td>
<td>↓</td>
</tr>
<tr>
<td>Phenylalanine (3.4.)</td>
<td>↓</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>-</td>
<td>k</td>
</tr>
<tr>
<td>Imipramine/</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
FIG. 4.1. (contd.)

<table>
<thead>
<tr>
<th>Drugs</th>
<th>5HT synthesis</th>
<th>Vesicle damage</th>
<th>MAO activity (5HT breakdown)</th>
<th>Raphe firing</th>
<th>Tryptophan Uptake</th>
<th>5HT concentration intra-, extraneuronal</th>
<th>Nucleolar Dry Mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipramine, and desipramine (3.3.)</td>
<td>↓</td>
<td>-</td>
<td>↓ (661)</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Res./desip. (3.3.)</td>
<td>-</td>
<td>↑</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>k</td>
</tr>
<tr>
<td>Lithium (3.3.)</td>
<td>↑ and ↓</td>
<td>-</td>
<td>↑</td>
<td>k</td>
<td>↑ (383)</td>
<td>↑ or k</td>
<td>k</td>
</tr>
<tr>
<td>PCPA (3.4.)</td>
<td>↓</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>-</td>
<td>?</td>
</tr>
<tr>
<td>PCPA/Nial. (3.4.)</td>
<td>-</td>
<td>-</td>
<td>↓</td>
<td>k</td>
<td>-</td>
<td>k</td>
<td></td>
</tr>
</tbody>
</table>

Symbols: "k" = unchanged; "?" = inferred; ( ) = not statistically significant.
In raphe neurones, the nucleolar changes may reflect drug-induced alterations in the metabolic requirements for one or more of the following enzymes: (a) those responsible for synthesizing 5HT, in particular the rate-limiting enzyme tryptophan hydroxylase (see Introduction 1.2.3), (b) those responsible for synthesizing the storage vesicles for 5HT, (c) those responsible for the catabolism of excessive amounts of intraneuronal 5HT, especially MAO, and (d) those associated with processes accompanying the electrical activity of the neurones, including the discharge and recharge of amine-storage vesicles (623) (see Fig. 4.1.).

There is no obvious relationship between the nucleolar changes observed in raphe neurones after various drug regimes and the effect of these drugs on synaptic vesicles (Fig. 4.1.). Reserpine is a drug whose actions include damage to storage vesicles (see Introduction 3.1.1 and Discussion 3.1.4); it could be argued therefore that the increase in nucleolar dry mass after reserpine alone (Results 3.1.3) reflected predominantly the increased cellular production of ribosomes necessary for the accelerated synthesis of proteins and other components of vesicles (178, 179, 317). But this argument is difficult to sustain when considering the reserpine-nialamide and reserpine-desipramine studies, in which no nucleolar changes were observed despite the action of reserpine. Moreover, nucleolar changes similar to those produced by reserpine alone were elicited by drugs such as lithium and testosterone, which do not damage vesicles, so far as is known. Hence in these experiments, additional synthesis of vesicular proteins probably could not account for the changes in nucleolar dry mass of raphe neurones.
Equally difficult to discern is a relationship between nucleolar changes in raphe neurones and putative alterations in MAO activity. It might be argued that in the lithium and reserpine experiments, the enhanced MAO activity associated with the elevated 5HIAA content of brain could be connected with the increased nucleolar dry mass of the raphe neurones. But after nialamide and tranylcypromine alone there were no nucleolar changes despite the concurrent inhibition of MAO. Moreover the administration of tryptophan and 5HT, which reportedly increases brain 5HIAA (see Introduction 1.2.3 and Discussion 3.4.4) was accompanied in the present investigation by unchanged or decreased values for nucleolar dry mass. The only detectable action of MAO inhibitors on this raphe neuronal parameter was the abolition of the increased dry mass caused by reserpine (see Results 3.2.3) - and possibly by PCPA (see Results 3.4.3); it has been shown elsewhere that in the presence of MAO inhibition, the trans-synaptic induction of enzymes in noradrenergic neurones is blocked (321).

The rate of 5HT synthesis in the raphe neurones has been related to their impulse activity (201) (see Introduction 1.2.6). In some studies, drug-induced changes in the firing-rate of raphe neurones are considered indices of short-term alterations of their metabolic activity (155). For example, it is reported that after the administration of tryptophan, reserpine, imipramine-like drugs or MAO inhibitors, the rate of firing decreases; and that after PCPA pretreatment, the inhibition of firing caused by MAO inhibitors and by imipramine-like drugs is absent (344, 360, 377). These
changes in firing rate have been considered to stem from the activation of post-synaptic 5HT receptors (264), with consequent feedback inhibition of raphe neuronal function (see below). But there are discrepancies between these electrophysiological changes, the reported changes in 5HT synthesis, and the alterations in the nucleolar dry mass of raphe neurones in the present investigations (Fig. 4.1.). After drugs such as lithium and reserpine, the rate of 5HT synthesis rises and the nucleolar dry mass increases; yet after lithium, the firing rate of raphe neurones does not increase (270), while after reserpine (given intravenously) their firing rate increases within 20 minutes, is inhibited between 18 and 24 hours after the injection, and then returns to normal values (377). Again, after MAO inhibition, the firing-rate of raphe neurones declines rapidly; yet in the present study, the nucleolar dry mass of raphe neurones was unaltered (Results 3.2.3). According to some reports, the rate of 5HT synthesis does not alter after MAO inhibition (200, 506) (Discussion 3.2.4). Moreover in those studies where a decreased tryptophan hydroxylase activity has been reported after MAO inhibition (Discussion 3.2.4), change may have resulted from altered activity of extant enzymes (43, 135) - perhaps by end-product inhibition - rather than by trans-synaptically induced changes in the rate of tryptophan hydroxylase biosynthesis (91, 259, 624). A similar dual control over the rate of noradrenaline formation by tyrosine hydroxylase has been proposed (109, 623, 625-627) (see Introduction 1.2.9). Other observations on 5HT turnover and histofluorescence in the segments
of the transected spinal cord indicate that, after MAO inhibition, 5HT synthesis is probably not controlled by mechanisms such as end-product or feed-back inhibition (200). It may be that, during MAO inhibition, the raised concentration of "free" intraneuronal 5HT inhibits raphe neuronal firing directly, through effects on the transmembrane potential of raphe neuronal terminals (86, 499), rather than through mechanisms in their cell bodies. For after MAO inhibition, there are no morphological changes in the Golgi apparatus of raphe neurones such as have been found when cellular biosynthetic activity is altered (128, 180).

Hence the discrepancies between changes in electrical activity in raphe neurones and alterations in their synthetic activity or nucleolar dry mass after drugs support the hypothesis described earlier (see Introduction 1.2.9): fluctuations of impulse activity may be linked only to short-term alterations in the rate of 5HT synthesis; these transient alterations in synthesis-rates can result from changes in the degree of enzyme activation, rather than from an altered rate of biosynthesis of new enzyme (24, 146, 155, 201, 241, 624, 627, 628). These discrepancies also support the view that changes in the nucleolar dry mass (and the accompanying alterations in ribosome synthesis) are not closely associated with those metabolic processes involved in raphe neuronal impulse activity. Other workers, too, have indicated that changes in the turnover of 5HT in raphe neurones and alterations in their electrical activity are not necessarily concomitants (201, 481, 628).
The outstanding biosynthetic relationship requiring scrutiny is that between changes in the nucleolar metabolism of raphe neurones and the altered requirements for enzymes necessary for 5HT synthesis, in particular tryptophan hydroxylase. Within monoamine neurones, the amines may be present in different subcellular compartments or pools with different rates of synthesis and destruction of the amine (259, 265, 629). Because the amines in these pools may be interchangeable over a period of time (259, 322, 475, 522, 630, 631), these intraneuronal pools have been considered as a functional entity (632, 633). In addition there exists an extraneuronal pool of 5HT (481): the 5HT molecules in this pool enter it when they are released from raphe neuronal terminals as a result of nervous activity (Introduction 1.2.6). Having entered the synaptic gap, the extraneuronal 5HT may combine with receptors on postsynaptic cell membranes; but provided the membrane pump of the raphe terminals is intact, this extraneuronal 5HT is then pumped back into the intraneuronal pool of 5HT. The size of this extraneuronal pool can vary. It can be altered directly by drugs such as the tricyclic antidepressants, or by the intraventricular infusion of 5HT and melatonin (Discussion 3.4.4); or it can be altered indirectly by substances such as reserpine or (perhaps) tryptophan which change the amount of intraneuronal 5HT available for release (478, 634) (Discussions 3.1.4 and 3.4.4). Correlations can be sought between the nucleolar responses of raphe neurones and the status of these intraneuronal and extraneuronal pools of 5HT after various procedures (Fig. 4.1.).
No relationship was discernible between the nucleolar dry mass of the raphe neurones and the probable size of the intraneuronal pool of 5HT during these investigations (Fig. 4.1.).

An inverse relationship is apparent between the amount of 5HT (or of the closely-related molecule, melatonin) probably present in the extraneuronal pool, and the nucleolar parameter of the raphe neurones. This inverse relationship is compatible with the concept of feedback control of raphe neurones, which has been proposed by many workers (222, 264, 266, 267, 327, 377, 427, 635-639). According to this concept, when receptors on neurones innervated by raphe neuronal terminals are exposed to an increased amount of 5HT released from these terminals, they activate a feed-back loop. Perhaps by means of a diminished trans-synaptic input, this loop inhibits the metabolic activity of raphe neurones, and in particular the synthesis and release of 5HT; the biosynthesis of tryptophan hydroxylase in the raphe neurones may also be inhibited, if the pattern of control of 5HT neuronal metabolism is similar to that of noradrenergic neurones (107, 623, 640) (see Introduction 1.2.9).

However, in some experiments in this study, this inverse relationship was abolished by procedures which probably grossly increased the amount of 5HT in the synaptic pool (Methods 3.3.2 and 3.4.2). Although there is no direct evidence to support the conjecture, it may be that such paradoxical findings reflect the development of receptor hyposensitivity or tachyphylaxis (see Introduction 1.2.7 and Discussion 3.3.4). For the reasons stated
earlier (see Discussions 3.1.4 and 3.4.4, Concluding Discussion 4.1) it is less likely that these findings resulted from the disordered function of nor-adrenergic neurones. However the contribution of these neurones and also of different control mechanisms (624, 641-644) to the regulation of 5HT metabolism in raphe neurones remains unclear.

The changes in nucleolar dry mass of the different hypothalamic neurones examined require cautious interpretation. These neurones have a variable quality and quantity of monoaminergic afferent input. Moreover they are not known to synthesize specific, identifiable and measurable neurotransmitters, the turnover of which would illustrate their metabolic activity. Therefore it is difficult to elucidate how the various drugs in the present study may have affected the metabolism of these neurones.

The inhibitory action of 5HT on neuronal firing has been demonstrated in the suprachiasmatic nucleus (see Introduction 1.2.7). The decreased nucleolar dry mass of the suprachiasmatic neurones after imipramine, or their increased nucleolar parameter after reserpine (see Results 3.1.3, 3.3.3) may flow indirectly from changes in the 5HT concentration in the synaptic spaces within this nucleus.

During the period when the experiments in this thesis were being performed, the baseline values for the mean nucleolar dry mass of various neurones were sometimes found to fluctuate between batches of rats, but not within batches. Similar fluctuations in this nucleolar parameter of
other groups of neurones have been reported (20, 21). In rats and mice, the metabolism of 5HT by raphe neurones may vary between different strains of these animals (124, 645, 646). In the present study, a seasonal cause for the fluctuations in nucleolar dry mass of the raphe neurones could be excluded; but it was not possible to investigate further the genesis of these fluctuations.

Some of the findings reported in the present study require to be amplified by further experimentation, designed to investigate the duration of drug-induced nucleolar changes, their dose-response relationships, and the effects of drug-withdrawal.

The effect of hallucinogenic substances on the nucleolar dry mass of raphe and suprachiasmatic neurones requires to be investigated: many of the hallucinogens, in particular mescaline and lysergic acid (264, 647-650) have been shown to alter other facets of 5HT metabolism in brain.

The suggested involvement of 5HT metabolism in thermoregulation (232, 651-653) and in the control of sex hormone activity (232, 409, 654, 655) is intriguing. In the former field, the raphe neuronal response to extremes of environmental temperature and to the manipulation of thyroid function could be observed. As for the latter field, the stages of physiological interest include pregnancy (656), lactation (657) and the neonatal period (658, 659).

It would be of interest to see whether or not the hypothesis established in this thesis, concerning the control of ribosome synthesis in raphe neurones, could be further supported by such investigations.
4.2 Summary

The nucleolar dry mass of interneurones in the brainstem and hypothalamus of the rat was measured by interference microscopy. The values for nucleolar dry mass in some neurones changed when the experimental animals were subjected to various procedures. The raphe neuronal parameter increased after the administration of reserpine, lithium, diamorphine and testosterone; it decreased after imipramine and desipramine, and after 5HT, melatonin and barbitone. The suprachiasmatic neuronal parameter increased after reserpine and diamorphine, but decreased after imipramine and desipramine, barbitone and testosterone. The arcuate neuronal parameter declined only after barbitone. The ventromedial neuronal parameter decreased after reserpine. No changes in the nucleolar dry mass of the supraoptic, dorsomedial and posterior neurones of the hypothalamus were observed.
APPENDICES

Eden Grove Bond

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4
APPENDIX I. INTERFERENCE MICROSCOPY: derivation of formulae

Full accounts of the principles of interference microscopy, and the use of this technique to measure nucleolar dry mass of neurones are available (20, 21, 662-665).

As described earlier in the text (see General Methods 2.4.2) two coherent beams of light emanate from the light source in an interference microscope. If a translucent organelle is placed in the path of one of these beams, the degree of retardation (Dw) or optical path difference (OPD) between the beams, is determined by the relationship,

\[ Dw = (N_0 - N_w)t \]  \hspace{1cm} (1)

where \( N_0 \) = refractive index of the organelle,
\( N_w \) = refractive index of water
\( t \) = thickness of organelle

The refractive index of the organelle consisting of an aqueous solution is related to the solute concentration and to the specific refractive increment of the solution:

\[ x = \frac{N_0 - N_w}{C} \]  \hspace{1cm} (2)

where \( C \) = concentration, g/ml.

\[ x = 100 \times x, \text{ and} \]

\[ X = \frac{X}{100} \]

\[ x = \text{specific refractive increment, or the increase in} \]
\[ \text{refractive index per increase in solute concentration in g/100ml.} \]

\[ x = \text{specific refractive increment, or the increase in refractive index per increase in solute concentration in g/100ml.} \]

\[ x = \text{specific refractive increment, or the increase in refractive index per increase in solute concentration in g/100ml.} \]

\[ x = \text{specific refractive increment, or the increase in refractive index per increase in solute concentration in g/100ml.} \]

\[ x = \text{specific refractive increment, or the increase in refractive index per increase in solute concentration in g/100ml.} \]
Suppose an organelle be represented as a disc of homogeneous solution, which is immersed in water,

then \( C = \frac{M}{At} \) ........ (4) when \( M \) = mass of solute in g.

\( A = \) area in cm\(^2\).

\( t = \) thickness of disc in cm.

Substituting for \( C \) in equation (3)

\[
\frac{M}{At} = \frac{N_0-N_w}{X}
\]

and \( M = \frac{(N_0-N_w)A.t}{X} \) ........ (5)

But in equation (1), \( (N_0-N_w)t = D_w \)

therefore \( M = \frac{D_w.A}{X} \) ........ (6)

that is, OPD is proportional to Mass per unit area. The value for dry mass can be calculated if the quantities \( D_w \), area, and \( X \) are measurable.

The organelle as a nucleolus

The volume of this disc-shaped organelle = \( \pi r^2 h \)

where \( r = \) disc radius

\( h = \) disc height.

A nucleolus can be considered as a sphere with a volume equal to that of the organelle,

then nucleolar volume = \( \frac{4}{3} \pi r^3 = \pi r^2 h \)

and height of disc, \( h = \frac{4}{3} r = 2/3d \), where \( d = \) diameter in cm.

The OPD imposed on a light beam perpendicular to this disc of height 2/3d will be uniform in this axis.
Hence for the nucleolar sphere, whose diameter = $d$, the mean thickness or height along which OPD will be uniform = $2/3d$.

Since $D_{w} = (N_{o}-N_{w})t$ ................. (1)

then $D_{\text{mean}} = (N_{n}-N_{w})2/3d$

and $D_{\text{peak}} = (N_{n}-N_{w})d$, or $(N_{n}-N_{w}) = \frac{D_{\text{peak}}}{d}$

By substitution, $D_{\text{mean}} = \frac{2}{3}D_{\text{peak}}$ ............. (7)

The nucleolus in media other than water

Nucleolar dry mass can be measured accurately only when its maximal imposed OPD is less than $0.2L$, where $L$ = wavelength of light used.

To ensure this situation, the nucleolus in its nucleus is placed in a medium with a refractive index greater than that of water. In this medium, the OPD, or $D_{m} = (N_{o}-N_{m})d$ ........... (8)

hence from equation (6),

$$M = \frac{D_{m},A}{X} \frac{N_{o}-N_{w}}{N_{o}-N_{m}} \text{ g.}$$ ................. (9)

The nucleolus within the nucleus

An intranuclear nucleolus is enveloped by a layer of nucleoplasm of refractive index ($nN$) which will contribute to the OPD measured over the nucleolus. The mean thickness of these layers of nucleoplasm = $(d-t)cm$,

where $d$ = mean nuclear thickness,

$\quad t$ = mean nucleolar thickness.
When \( DN = \) mean OPD for perinucleolar nucleoplasm,

\[ DN\&O = \text{mean OPD for this nucleoplasm plus nucleolus} \]

\[ Do = \text{corrected mean OPD for nucleolus alone,} \]

then \( DN\&O = Do + DN\left(\frac{d-t}{d}\right) = Do + DN(1-t/d) \]

therefore \( Do = DN\&O - DN(1-t/d) \).

From equation (1), the OPD created by this nucleoplasm can be represented as

\[ DN = (d-t)(nN-Nm) \quad \ldots \ldots \quad (10) \]

By equation (8), the OPD due to the nucleolus alone is:

\[ Dm = (No-Nm)d \]

therefore the summed OPD due to nucleoplasm and nucleolus is:

\[ Dm = (d-t)(nN-nM) + (No-Nm)d = (nN-nM)(1-t/d) + (No-Nm)d \quad \ldots \ldots \quad (11) \]

From equation (10),

the OPD due to nucleolus alone,

\[ (No-Nm)d = Dm-DN(1-t/d) \]

so from equation (9),

\[ \text{true nucleolar dry mass, } M = \frac{A}{X} \left[ Dm-Dn(1-t/d) \right] \frac{No-Nw}{No-Nm} \quad g \quad \ldots \ldots \quad (12) \]

The factors in this equation will now be considered in turn:

1) \( A \): area in \( \text{cm}^2 \) of the nucleolus normal to optical axis, can be calculated using the value for \( d \), as measured by microdensitometry.

2) \( X \): is approximately 0.18 for proteinaceous solutions, over a wide range of concentration; this value is not significantly affected by alterations of pH or temperature (662, 666). For DNA and RNA, values for \( X \) are quoted
as 0.187, and 0.168 to 0.194 respectively. For glycogen, X is around 0.15 (667). Since nucleoli are overwhelmingly proteinaceous (1, 303, 305), the value, X = 0.18 is used in calculations of nucleolar dry mass (21, 306).

3) \(DN(1-t/d)\): the contribution of the nucleus to the measured OPD is negligible, - about 1% (20); this has been ascertained by measuring the OPD of nucleoli firstly enclosed within nuclei, and then isolated, after their extrusion from the nuclear envelope.

4) \(No-Nw\): of these refractive indices, \(Nm\) was measured by refractometry and found to be 1.349; \(Nw\) is 1.333; from equation (8), \(No = \frac{Dm}{d} + Nm\), and Table 2.4.5. shows the value No calculated for 50 randomly selected neurones from each nucleus.

**Calculation of nucleolar dry mass from microdensitometric trace**

According to equation (9), \(M = \frac{DmA}{X} \cdot \frac{No-Nw}{No-Nm} \cdot \frac{1}{g} \cdot \frac{1}{g}

On a densitometer trace (Fig. 2.4.4.), it can be seen that

- \(a\) = distance in cm, from the nucleolar peak to the point of corresponding intensity on the interference band;
- \(La\) = distance in cm, between points of corresponding intensity on adjacent interference bands;
- \(a/La\) = peak nucleolar OPD as a fraction of 1 wavelength of light used, \(L = 546\text{nm} \);
- \(d\) = diameter of nucleolus;

thus nucleolar \(M = \frac{2 \cdot L \cdot a}{3 \cdot X \cdot La} \cdot \frac{No-Nw}{No-Nm} \cdot \frac{1}{(Mag)^2}

where Mag, magnification = \(4.2 \times 10^3\),

and \(\frac{2 \cdot L \cdot a}{3 \cdot X \cdot La} \cdot \frac{No-Nw}{No-Nm} \cdot (Mag)^2 = \text{constant}\)
APPENDIX II

Publications


2) 'Inhibition of Median Raphe Neurone Metabolism by Cerebrospinal Fluid (CSF) containing 5-hydroxytryptamine and Melatonin', Biochem. Pharmac. 23, 1913 (1974)
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Effects of Diamorphine on Various Neurones in the Rat Central Nervous System

Neurones of the raphe nucleus send axons to other regions of the central nervous system, especially the hypothalamus. These neurones synthesize serotonin, much of which is stored in cytoplasmic vesicles, where it is protected from the action of monoamine oxidase. Accumulations of these vesicles are found within varicosities at the raphe nerve terminals. Reserpine causes the vesicles to release their serotonin contents; once freed and thereby exposed to monoamine oxidase, serotonin is broken down. Whether or not serotonin metabolism is affected by morphine and its derivatives has remained a controversial topic.

Interference microscopy has been used to demonstrate quantitative metabolic changes in neurones with motor or neurosecretory functions; the magnitude of these changes is related to the intensity of the preceding stimulus. This technique was used to investigate three questions: first, do cells which are interneurones rather than effector neurones show similar metabolic changes; second, do interneurones specifically involved in serotonin metabolism within the central nervous system (CNS) (that is the raphe and suprachiasmatic neurones) show metabolic changes after diamorphine; third, are such changes comparable with the same neurones' responses to reserpine?

Changes in neurones from the median raphe region of the midbrain were compared with the responses of the hypothalamic suprachiasmatic neurones, which are enmeshed in large numbers of terminals containing serotonin. In addition the responses to the same stimuli were investigated in neurones from regions of the hypothalamus where serotonin terminals are scanty and where catecholamine terminals predominate, for example, ventro-medial, dorso-medial, posterior, pre-optic, anterior and posterior arcuate regions.

Fig. 1 Raphe and suprachiasmatic neurones. Columns indicate pooled mean nucleolar dry mass measurements with their standard errors, grouped into controls and according to days after starting diamorphine.

Fig. 2 Various other neurones. Columns illustrate data as in Fig. 1.
Drugs and control solutions were given by intraperitoneal injections to male albino rats (aged 3 months and weighing about 250 g). These were killed by exsanguination after light ether anaesthesia. Small blocks of tissue (0.5 mm × 1 mm) containing the relevant neurone groups were excised from slices of midbrain and hypothalamus. Specimens of isolated neuronal nuclei were prepared, and the dry mass of their nucleoli was measured by interference microscopy. Group A consisted of rats given diamorphine (1 mg twice daily) for up to 20 d (Figs 1 and 2). Group B rats received reserpine (0.5 mg daily) for up to 10 d, after which the drug was withdrawn (Figs 3 and 4). Group C rats were given reserpine (1 mg daily) for up to 9 d (Fig. 5). In all experiments control animals received appropriate volumes of Ringer lactate solution. The rats were killed in groups at various intervals during the experiments, and the dry mass response of the neuronal nucleoli from the different regions was observed.

From the figures and from the statistics shown in Table 1, the following points are evident: (1) Interneurones behaved like effector neurones in showing nucleolar metabolic changes after specific stimulation. (2) Diamorphine selectively increased the metabolism of these neurones involved in serotonin metabolism; the median raphe response preceded by some

![Fig. 3 Raphe and suprachiasmatic neurones. Columns indicate pooled mean nucleolar dry mass measurements with their standard errors, grouped into controls and according to days after starting reserpine and subsequently withdrawing the drug.](image)

![Fig. 4 Various other neurones. Columns illustrate data as in Fig. 3.](image)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Nucleus</th>
<th>Day</th>
<th>Nucleolar dry mass (g × 10⁻¹²)</th>
<th>No. of observations and animals</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diamorphine</td>
<td>Raphe</td>
<td>Control</td>
<td>9.37 ± 0.39</td>
<td>231; 5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>11-12</td>
<td>12.35 ± 0.77</td>
<td>88; 2</td>
<td>0.1-0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15-18</td>
<td>14.57 ± 0.45</td>
<td>197; 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td>S. chiasm</td>
<td>Control</td>
<td>5.04 ± 0.19</td>
<td>112; 5</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>11-12</td>
<td>4.62 ± 0.36</td>
<td>44; 2</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td></td>
<td>15-18</td>
<td>6.13 ± 0.25</td>
<td>69; 4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Reserpine (low dose)</td>
<td>Raphe</td>
<td>Control</td>
<td>7.09 ± 0.28</td>
<td>195; 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>7.84 ± 0.38</td>
<td>92; 2</td>
<td>0.2-0.1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-10</td>
<td>8.11 ± 0.34</td>
<td>184; 4</td>
<td>0.025-0.02</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+2</td>
<td>7.97 ± 0.33</td>
<td>165; 3</td>
<td>0.2-0.1</td>
</tr>
<tr>
<td></td>
<td>S. chiasm</td>
<td>Control</td>
<td>5.13 ± 0.18</td>
<td>90; 4</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-10</td>
<td>6.31 ± 0.23</td>
<td>70; 4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>+2</td>
<td>6.30 ± 0.26</td>
<td>67; 3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td>V.-medial</td>
<td>Control</td>
<td>6.06 ± 0.19</td>
<td>116; 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4</td>
<td>5.21 ± 0.23</td>
<td>47; 2</td>
<td>0.02-0.01</td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-10</td>
<td>5.28 ± 0.16</td>
<td>80; 4</td>
<td>NS</td>
</tr>
<tr>
<td>Reserpine (high dose)</td>
<td>Raphe</td>
<td>Control</td>
<td>7.55 ± 0.35</td>
<td>152; 3</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>4-5</td>
<td>8.51 ± 0.33</td>
<td>248; 5</td>
<td>0.1-0.05</td>
</tr>
<tr>
<td></td>
<td></td>
<td>6-9</td>
<td>9.34 ± 0.36</td>
<td>244; 4</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td>5-6</td>
<td>5.36 ± 0.26</td>
<td>65; 3</td>
<td>NS</td>
</tr>
<tr>
<td></td>
<td>S. chiasm</td>
<td>Control</td>
<td>5.79 ± 0.32</td>
<td>40; 2</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>8-9</td>
<td>5.72 ± 0.23</td>
<td>57; 3</td>
<td>NS</td>
</tr>
</tbody>
</table>

$$\text{Table 1}$$
Fig. 5 Raphe and suprachiasmatic neurones. Columns indicate pooled mean nucleolar dry mass measurements with their standard errors, grouped into controls and according to days after starting reserpine (high dose).

inhibited that of the suprachiasmatic neurones (Fig. 5). Those neurones not directly implicated in serotonin metabolism did not respond to diamorphine (Fig. 2); with the exception of the unexplained ventro-medial neurones' response to the low dose of reserpine, this drug also had no effect on those neurones (Fig. 4), a conclusion supported by data showing their unresponsiveness to high doses of reserpine (unpublished observations).

An increased cell nucleolar dry mass is associated with heightened nucleolar synthetic activity\textsuperscript{5,7}. Neuronal nucleolar volumes are also correlated with cell RNA levels\textsuperscript{9}; these, in turn, are linked to changes in rates of protein synthesis\textsuperscript{10}. In this study, in which nucleolar dry mass varied directly with nucleolar volume (unpublished observations), the raised raphe parameters noted during chronic diamorphine and reserpine administration may indicate accelerated synthesis of enzymes concerned in the formation of serotonin and possibly of its storage vesicles. The time lag between the raphe and suprachiasmatic responses to diamorphine and reserpine may represent the time needed for the products of the increased raphe metabolism to reach the terminals around the suprachiasmatic neurones.

I thank Professor W. E. Watson for his help. This study was supported by the Medical Research Council and by the National Fund for Research into Poliomyelitis and other Crippling Diseases.

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Inhibition of median raphe neurone metabolism by cerebrospinal fluid (CSF) containing 5-hydroxytryptamine and melatonin

(Received 25 September 1973; accepted 24 November 1973)

BRAIN 5-hydroxytryptamine is synthesized by many neurones of the raphe nucleus in the midbrain. For several hours after monoamine oxidase inhibition the rate of 5-hydroxytryptamine synthesis declines as the level of brain 5-hydroxytryptamine rises. In the present study the nuclear component of the metabolic changes in median raphe neurones was observed (a) during a prolonged (9 day) elevation of 5-hydroxytryptamine levels in cerebrospinal fluid (CSF), (b) during a similar elevation in CSF levels of other monoamines, (c) after depletion of brain catecholamines by 6-hydroxydopamine (6-OHDA), and (d) after bilateral cervical sympathectomy.

The cerebral ventricles of one group of male albino rats (aged 3 months and weighing approximately 300 g) were cannulated and continuously infused during 7-9 days, at a rate (1 μl/min) roughly half that of CSF formation. The infusate was artificial CSF alone, or with “low-dose” 5-hydroxytryptamine, melatonin, noradrenaline, histamine (all at 50 μg/ml), tryptophan (1 mg/ml), or “high-dose” 5-hydroxytryptamine (500 μg/ml). The rats were killed after 7-9 days of infusion.

In a second group of rats, 6-OHDA (250 μg in 0.05 ml artificial CSF) was given by a single injection into a lateral cerebral ventricle; controls received artificial CSF alone. The animals were killed at intervals during the following 16 days.

In a third group, bilateral cervical sympathectomy was performed and the rats killed 7-9 days thereafter.

From all rats, neuronal nuclei were isolated from the median raphe region of the brain. The dry mass of the nuclei in these nuclei was measured using the interference microscope, and the mean values are shown in Fig. 1 and Table 1.

Although the drug-induced behaviour of the rats within each group was similar, differences between the groups were seen. Two days after commencing infusion with melatonin or with “low-dose” 5-hydroxytryptamine the rats became very drowsy and showed a reduction both in spontaneous motor activity and in reactivity to simple tactile and auditory stimuli. During infusion with “high-dose” serotonin the rats were drowsy in the first day only, subsequently becoming hyperactive and hyperreactive with a coarse tremor and titubation. Rats with noradrenaline or tryptophan in the infusate showed increased spontaneous movements and hyperreactivity starting on day 2 but not preceded by drowsiness. For 1 hr after 6-OHDA injection the rats were hyperactive and hyperreactive; the subsequent 12-hr phase of extreme immobility and unresponsiveness disappeared gradually, behavioural normality returning during day 2. Those rats with cervical sympathectomy and those receiving histamine solution or artificial CSF by itself appeared normal. Assiduous attempts have been made by many workers to attribute such drug-induced changes in behaviour to concurrent changes in brain biochemistry. It is hoped that such speculations might be assisted by this study in which accompanying changes in neuronal metabolism have been noted by means of a different technique.
A cell's metabolic capacity for synthesizing protein depends largely on its complement of ribosomal RNA. The varying rate of ribosomal RNA synthesis necessary in changing circumstances can be related quantitatively to alterations in the cell's nucleolar dry mass. Thus from the measurements of the nucleolar dry mass of raphe neurones in this study (Fig. 1, Table 1) the following deductions can be drawn: (1) raphe neuron metabolism is depressed by the prolonged infusion of 5-hydroxytryptamine ("low-dose") and of melatonin, two hormones which are biochemically closely related; (2) the raphe neuronal inhibition produced during melatonin infusion is unlikely to be secondary to changes in pineal metabolism; for although the amount of melatonin synthesized by the pineal gland is altered by bilateral cervical sympathectomy, this study the same procedure does not alter the raphe parameter; (3) the inhibition induced during 5-hydroxytryptamine and melatonin infusion is not mediated by changes in the metabolism of neurones synthesizing other monoamines; this is indicated by the stability of the raphe parameter during the noradrenaline, 6-OHDA and histamine experiments; (4) the similar stability in the raphe parameter during tryptophan infusion demonstrates that an increased availability of precursor does not alter the nucleolar metabolism of raphe neurones; (5) "high-dose" 5-hydroxytryptamine infusion abolishes the inverse relationship noted here and elsewhere between the rate of raphe neurone metabolism and brain levels of 5-hydroxytryptamine.

### Table 1

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Nucleolar dry mass (g x 10^-12)</th>
<th>No. of observations and animals</th>
<th>P values</th>
</tr>
</thead>
<tbody>
<tr>
<td>Infusion control</td>
<td>8.17 ± 0.29</td>
<td>205; 4</td>
<td>—</td>
</tr>
<tr>
<td>5-Hydroxytryptamine (low dose)</td>
<td>7.12 ± 0.24</td>
<td>333; 6</td>
<td>0.01–0.005</td>
</tr>
<tr>
<td>Melatonin</td>
<td>6.98 ± 0.20</td>
<td>358; 5</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>8.23 ± 0.33</td>
<td>257; 5</td>
<td>N.S.</td>
</tr>
<tr>
<td>Noradrenaline</td>
<td>8.28 ± 0.45</td>
<td>104; 2</td>
<td>N.S.</td>
</tr>
<tr>
<td>Histamine</td>
<td>8.02 ± 0.29</td>
<td>237; 3</td>
<td>N.S.</td>
</tr>
<tr>
<td>5-Hydroxytryptamine (high dose)</td>
<td>8.24 ± 0.25</td>
<td>328; 4</td>
<td>N.S.</td>
</tr>
<tr>
<td>Sympathectomy</td>
<td>7.96 ± 0.25</td>
<td>262; 3</td>
<td>N.S.</td>
</tr>
<tr>
<td>6-OHDA Control</td>
<td>8.55 ± 0.30</td>
<td>198; 3</td>
<td>—</td>
</tr>
<tr>
<td>6-OHDA dl-4</td>
<td>8.40 ± 0.22</td>
<td>268; 4</td>
<td>N.S.</td>
</tr>
<tr>
<td>d5-7</td>
<td>8.22 ± 0.28</td>
<td>187; 3</td>
<td>N.S.</td>
</tr>
<tr>
<td>d11-16</td>
<td>7.95 ± 0.16</td>
<td>535; 7</td>
<td>(0.1–0.05) N.S.</td>
</tr>
</tbody>
</table>

N.S.—not significant.
It is not certain what physiological mechanisms decrease the tempo of 5-hydroxytryptamine production in raphe neurones when the level of brain 5-hydroxytryptamine rises. The findings in this study support the hypothesis \(^2\) \(^3\) that receptors relatively specific for 5-hydroxytryptamine are situated distal to raphe nerve endings; these receptors’ neurones control, by feedback inhibition, an excessive rate of 5-hydroxytryptamine production by the raphe neurones as evidenced by moderately raised levels of 5-hydroxytryptamine at the receptors. This study further suggests that, at very high levels of 5-hydroxytryptamine, these receptors become saturated and inactivated; in this situation the inhibitory feedback control is eliminated. Experiments now in progress are designed to test the validity of this hypothesis.

Acknowledgements—I am grateful for the help I received from Professor W. E. Watson and from Mrs. Jennifer Anderson, Miss Sheila Paton and Mr. W. Lawson. This study was supported by the Medical Research Council and by the National Fund for Research into Poliomyelitis and other Crippling Diseases.

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REFERENCES
Table 1. Species differences in lipid peroxidation, activity of ethylmorphine N-demethylase, content of cytochrome P-450 and activities of NADPH-linked cytochrome c reductase and neotetrazolium diaphorase.*

<table>
<thead>
<tr>
<th>Activity</th>
<th>Inhibitor or stimulator</th>
<th>Rat (n = 5)</th>
<th>Mouse (n = 5)</th>
<th>Guinea pig (n = 5)</th>
<th>Rabbit (n = 3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lipid peroxidation (TBA value/10 min × 10³)</td>
<td>Control</td>
<td>441 ± 14</td>
<td>206 ± 15</td>
<td>341 ± 21</td>
<td>30 ± 03</td>
</tr>
<tr>
<td></td>
<td>+ EDTA</td>
<td>16 ± 0.2†</td>
<td>0.9 ± 0.1†</td>
<td>0.6 ± 0†</td>
<td>0.8 ± 0.2†</td>
</tr>
<tr>
<td></td>
<td>+ Fe²⁺</td>
<td>694 ± 1.7†</td>
<td>51.1 ± 0.9†</td>
<td>57.0 ± 2.0†</td>
<td>20.9 ± 2.1†</td>
</tr>
<tr>
<td></td>
<td>+ Ethylmorphine</td>
<td>219 ± 0.8</td>
<td>11.8 ± 0.6</td>
<td>16.0 ± 0.9</td>
<td>2.5 ± 0.4</td>
</tr>
<tr>
<td></td>
<td>+ EDTA</td>
<td>15 ± 0.2†</td>
<td>0.6 ± 0.1†</td>
<td>0.6 ± 0.2†</td>
<td>0.7 ± 0.1†</td>
</tr>
<tr>
<td></td>
<td>+ Fe²⁺</td>
<td>63.1 ± 0.8†</td>
<td>40.5 ± 0.8†</td>
<td>32.5 ± 2.0†</td>
<td>14.2 ± 0.7†</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylation (nmoles/mg protein/10 min)</td>
<td>Control</td>
<td>484 ± 2.6</td>
<td>24.4 ± 0.5</td>
<td>37.3 ± 2.3</td>
<td>21.5 ± 2.1</td>
</tr>
<tr>
<td></td>
<td>+ EDTA</td>
<td>74.9 ± 3.4†</td>
<td>33.5 ± 1.0†</td>
<td>38.4 ± 1.8</td>
<td>22.2 ± 2.3</td>
</tr>
<tr>
<td></td>
<td>+ Fe²⁺</td>
<td>9.7 ± 0.6†</td>
<td>7.8 ± 0.1†</td>
<td>33.0 ± 1.4</td>
<td>17.9 ± 2.0</td>
</tr>
<tr>
<td>Cytochrome P-450 (nmoles/mg protein)</td>
<td></td>
<td>0.908 ± 0.016</td>
<td>0.694 ± 0.032</td>
<td>1.685 ± 0.046</td>
<td>1.272 ± 0.001</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase (nmoles/mg protein/min)</td>
<td></td>
<td>33.75 ± 2.04</td>
<td>40.50 ± 1.53</td>
<td>37.35 ± 2.61</td>
<td>31.05 ± 2.07</td>
</tr>
<tr>
<td>NADPH-neotetrazolium diaphorase (units/mg protein × 10³)</td>
<td></td>
<td>320 ± 0.5</td>
<td>22.5 ± 0.5</td>
<td>18.0 ± 1.5</td>
<td>20.5 ± 2.0</td>
</tr>
</tbody>
</table>

* Experimental details are presented in the text. Values represent mean ± S.E.
† Differs significantly (P < 0.05) from corresponding control value.