Histochemical Studies of the Nigro-striatal System of Dopamine-containing Neurones

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Summary

Title:- Histochemical studies of the nigro-striatal system of dopamine-containing neurones.

General Introduction

Reviews of previous publications.

A. The nigro-striatal system of dopamine-containing neurones.

B. The significance of the nigro-striatal system of dopamine-containing neurones in Parkinsonism.

C. The therapeutic action of L-DOPA in Parkinsonism.

D. The histochemical appearance of the nigro-striatal system of dopamine-containing neurones and of the effects of administered L-DOPA on the CNS, shown by the formaldehyde-fluorescence method.

E. The formaldehyde-fluorescence method.

Statement of the type of observations and measurements to be reported.

Section A

The formaldehyde-fluorescence histochemical method. Methodology in relation to the study of the nigro-striatal dopamine-containing neurones and of the fate of administered L-DOPA in the brain.

Introduction:- A description of methods and equipment used.

Results:-

Examination of diffuse parenchymal formaldehyde-induced fluorescence. (A-E)

A. Variations between experiments in the intensity of the formaldehyde-induced fluorescence in the neuropil of the rat nucleus caudatus-putamen, relative to autofluorescence in the neuropil of the cerebral cortex.

The intensity of diffuse fluorescence in the neuropil of the nucleus caudatus-putamen and of the cerebral cortex was measured in sections of the rat brain prepared by the formaldehyde-fluorescence method. The appearance of the fluorescence of these 2 brain regions is also described. The diffuse

fluorescence in the neuropil of the nucleus caudatus-putamen consisted of a mixture of formaldehyde-induced fluorescence, (almost entirely derived from DA in the terminal parts of nigro-striatal axons), and of tissue autofluorescence. The diffuse fluorescence in the neuropil of the cerebral cortex was almost entirely due to autofluorescence.

The ratio of fluorescence intensity in the two regions was compared for each rat in 8 different experiments and significant differences in the ratios between experiments are reported and discussed. One of the many factors which could have contributed to the differences was the period of storage of the tissue sections before they were mounted in Entellan. (See Section A; F.)

B. Diffuse autofluorescence in the neuropil of 3 regions of the rat brain prepared by the formaldehyde-fluorescence method.

The intensity of diffuse fluorescence of the neuropil of the nucleus caudatus-putamen, cerebral cortex and molecular layer of the cerebellum was measured in sections of the rat brain prepared by the formaldehyde-fluorescence method. In 2 rats the DA in the nucleus caudatus-putamen was depleted by the administration of reserpine 10 mg/kg, i.p. 4 hr. before killing.

The results indicate that the autofluorescence of the neuropil was of similar intensity in the 3 brain regions. This was confirmed in subsequent experiments. This suggests that the intensity of fluorescence in the neuropil of the cerebral cortex or molecular layer of the cerebellum can be used to infer the approximate contribution of autofluorescence to the total fluorescence in the neuropil of the nucleus caudatus-putamen, in rats not pretreated with reserpine.

C. The fluorescence intensity in the neuropil of the nucleus caudatus-putamen and cerebral cortex in tissue samples not exposed to formaldehyde.

The fluorescence intensity of the neuropil of the nucleus caudatus-putamen and cerebral cortex was measured in sections of the rat brain prepared by the formaldehyde-fluorescence method and by this method omitting the exposure to formaldehyde gas.

The results confirm that only part of the fluorescence intensity in the neuropil of the nucleus caudatus-putamen in samples prepared by the formaldehyde-fluorescence method is specific, i.e. resulting from treatment with formaldehyde.

D. The emission spectra of autofluorescence and diffuse formaldehyde-induced fluorescence derived from L-DOPA and DA.

This study reports and compares the uncorrected (i.e. distorted by the properties of the microscope and accessories) emission spectrum of autofluorescence and of the spectra of formaldehyde-induced fluorescence derived from DA and L-DOPA. It was deduced that the true (i.e. undistorted) emission maxima of these 3 spectra in the yellow/green region were not below 490 nm. Within the range 470-550 nm, which includes most of the part of an emission spectrum used for routine observation and measurement of fluorescence when the barrier filter '47' (Zeiss) is selected, there were no obvious differences between the emission spectra examined.

E. The relation between diffuse fluorescence intensity and the microtome setting for section thickness.

This study reports the relation between measured intensity of diffuse fluorescence in the neuropil of rat brain samples prepared by the formaldehyde-fluorescence method and the microtome setting for section thickness. This was investigated for each of 4 degrees of fluorophore concentration over a range of microtome settings of 6 - 16 μm.

There was an approximately linear relationship between microtome setting and fluorescence intensity for each of the 4 degrees of fluorophore concentration. This suggests that the whole of the thickness of the sections studied contributed to the measured fluorescence, i.e. that "inner-filter" quenching, (caused by insufficient excitation of the deeper parts of each section by the vertical illumination), was not significant. Thus "inner-filter" quenching is unlikely to have affected the measurement of

similar concentrations of fluorophores, in subsequent experiments, in which the sections were cut at a routine microtome setting of 8 μm. Within the range of 6 - 16 μm, the microtome setting did not appear to affect the interquartile range, (obtained by discounting the highest 25% and the lowest 25% of the range), of a series of measurements of fluorescence intensity, when the interquartile range was expressed as a percentage of the corresponding mean fluorescence intensity.

Methodological variables affecting the investigation of formaldehyde-induced fluorescence. (F-J)

F. Changes in the intensity of formaldehyde-induced fluorescence and autofluorescence during storage of tissue sections.

Changes are reported in the fluorescence intensity of formaldehyde-induced fluorescence and of autofluorescence during storage of tissue sections of the nucleus caudatus-putamen and cerebral cortex of the rat brain. Storage as 'ribbons' over 13 weeks was accompanied by a gradual increase in autofluorescence, while storage as 'ribbons' for 3 days after sectioning was associated with a marked increase in the formaldehyde-induced fluorescence of the neostriatal neuropil. The latter was prevented by mounting the sections in Entellan.

G. Variations in fluorescence intensity due to changes in the intensity of the exciting light.

Using the HBO 100 Osram mercury lamp with the Zeiss large fluorescence microscope, variations in the intensity of the light source occur within and between periods of microscopy.

A fluorescent glass standard was used in all studies reported in this thesis to detect and monitor (at 15 min intervals) variations in the intensity of the light source during each period of microscopy. This study investigated changes in the intensity of the fluorescent standard by comparing

these with corresponding changes in the fluorescence intensity of the neuropil of tissue sections, which had resulted from changes in the intensity of the exciting light produced by varying the thickness of the exciter filter.

On the basis of the results it was decided that variations in the intensity of exciting light during a continuous period of microscopy (i.e. up to 5 hrs) could be ignored when measuring areas of diffuse parenchymal fluorescence, 10 μm in diameter, in sections cut at 8 μm. However, in this and a few other subsequent experiments, unusually large variations in the intensity of the light source, during a period of microscopy, detected by use of the standard, were considered sufficient to prevent a comparison of subsequent readings with the readings taken before the change in excitation intensity.

H. The relationship between size of a dissected tissue sample and intensity of diffuse fluorescence in the neostriatal neuropil, after preparation by the formaldehyde-fluorescence method.

The relationship between size of a tissue sample and diffuse formaldehyde-induced fluorescence was examined in a region of the neuropil of the nucleus caudatus-putamen in rats pretreated with L-DOPA.

The size of a tissue sample may affect the degree of residual water in parts of a sample after freeze-drying, and the degree of exposure of the inner regions of a sample to formaldehyde gas.

The results did not demonstrate any effect of size of sample on the intensity of formaldehyde-induced fluorescence within the range of sizes used.

I. Histochemical differentiation of the formaldehyde-induced fluorophores derived from dopamine and L-DOPA.

A method of differentiating the formaldehyde-induced fluorophores derived from L-DOPA and DA is reported, based on their rates of photodecomposition during irradiation with ultraviolet light.

In a model system of 1 µl droplets containing 1% bovine serum albumin, the photodecomposition of the fluorophores derived from a range of concentrations of L-DOPA and DA in the droplet solutions was examined. After irradiation for 2 min the fluorescence derived from L-DOPA was found to have shown a greater degree of fading compared with the fluorescence derived from DA, and a similar contrast was maintained irrespective of different concentrations of L-DOPA or DA in the droplet solutions.

In addition, photodecomposition of the fluorophores derived from DA and L-DOPA was examined in tissue sections of the rat brain. The results suggest that the findings in the model system can provide a method for distinguishing the fluorophores derived from DA and L-DOPA in tissue sections.

Section B

Studies of the nigro-striatal system of dopamine-containing neurones.

Results:-

Studies of diffuse formaldehyde-induced fluorescence in the neuropil of the rat nucleus caudatus-putamen. (A,B)

A. Investigation of intersample variables affecting the intensity of fluorescence in a region of the nucleus caudatus-putamen prepared by the formaldehyde-induced fluorescence method.

This study investigated variations between tissue samples in the intensity of diffuse formaldehyde-induced fluorescence and autofluorescence in the same region of the neuropil of the nucleus caudatus-putamen on the right and left sides of 7 rats.

The mean difference in the fluorescence intensity between the 2 sides of each rat was about 4% of the mean of the values from the 14 samples. (The mean contribution of the autofluorescence to the total fluorescence of the neuropil was approximately 55% of the total fluorescence).

The differences between each pair of samples (i.e. between the right and left side of each rat) were sufficiently similar to the differences between samples from different animals so that no significant correlation between the fluorescence intensity of paired samples from right and left hemispheres could be demonstrated. (Spearman rank correlation coefficient test.)

B. Comparisons of the intensity of formaldehyde-induced fluorescence and autofluorescence between different regions of the neuropil of the nucleus caudatus-putamen.

A comparison was made between the intensity of fluorescence in several regions of the neuropil of the nucleus caudatus-putamen, in tissue sections of rat brains prepared by the formaldehyde-fluorescence method.

A significant difference was found between 2 of the regions studied. (Wilcoxon Matched-Pairs Signed-Ranks Test, $p = 0.02$, two tailed).

Functional activity of nigro-striatal dopamine-containing neurones. (C)

C. Quantitative histochemical studies of neostriatal DA depletion following DL -α-methyl-p-tyrosine administration.

This study investigated the use of a microfluorimetric histochemical method for the measurement of the depletion of DA in the rat nucleus caudatus-putamen following α-methyl-p-tyrosine (α-MT) administration. The depletion in three behavioral situations was compared with that of a control group which remained in a cage.

The results of the control group indicate that there had been a reduction of approximately 50% in the intensity of the formaldehyde-induced fluorescence derived from DA in a region of the neuropil of the nucleus caudatus-putamen during the interval (3 hr.40 min) between the administration of α-MT 300 mg/kg i.p. and killing. Disruption of conditioned avoidance response (CAR) performance after α-MT administration was confirmed, but in this study the exposure of previously trained rats to CAR performance after

∝-MT administration did not have a significant effect on the depletion of the formaldehyde-induced fluorescence of the neostriatal neuropil. The levels of -MT-induced depletion of formaldehyde-induced fluorescence in the striatal neuropil following a period of muscular co-ordination/activity and following a period of CAR training were also not significantly different from those shown by a control group.

Section C

Studies of the fate of administered L-DOPA, which is used for the treatment of Parkinson's disease, in the mammalian CNS.

Results:-

Effects of administration of L-DOPA on the rat and cat brains. (A,B)

A. A histochemical and biochemical study of the effects of L-DOPA administration.

The aim of this biochemical-histochemical study was to confirm previous evidence which had indicated that administered L-DOPA can lead to increased DA concentration in the parenchyma (i.e. excluding capillary walls) of the rat nucleus caudatus-putamen. (The therapeutic action of L-DOPA in Parkinsonism may depend on the presence of additional DA, derived from administered L-DOPA, in the neostriatal neuropil.)

The results indicate that L-DOPA, 200 mg/kg i.p. 1 hr. before killing, alone or in combination with the monoamine oxidase inhibitor nialamide or the decarboxylase inhibitor Ro4-4602, led to an increase in the concentration of DA (estimated biochemically) in the region of the nucleus caudatus-putamen studied.

In the rat, administration of L-DOPA leads to the accumulation of DA in the capillary walls of most brain regions, however the biochemical and histochemical results also suggest that the increased neostriatal DA concentration was associated with an increase in the concentration of DA in the neostriatal parenchyma.

B. Regional and species differences in the effects of L-DOPA on the CNS.

A microfluorimetric method was used for quantitative histochemical study of the diffuse formaldehyde-induced fluorescence in the neuropil of various brain regions and of the effects of L-DOPA in the rat and cat. Regional and species differences in the effects of L-DOPA on the CNS were reported and discussed.

In the rat, suitable doses of L-DOPA led to increases in the intensity of fluorescence in the neuropil of the samples of nucleus caudatus-putamen which were greater than the increases in the neuropil of the other regions studied. Marked increases in the intensity of fluorescence in the neuropil of the cat brain samples were produced by doses of L-DOPA/kg. body wt. which were too low to produce detectable increases in the fluorescence of the rat brain samples.

It is hoped that further investigation of the factors affecting the fluorescence intensity of the neostriatal neuropil following L-DOPA administration will give more information on the therapeutic activity of a dose of L-DOPA used for the treatment of Parkinsonism.

Investigation of factors which may affect regional and species differences in, and degree of, the intensity of diffuse formaldehyde-induced parenchymal fluorescence, resulting from L-DOPA administration to the rat and cat. (C,D)

C. Animal models for an enzymic blood-brain barrier mechanism for therapeutically administered L-DOPA.

Tissue slices from three brain regions of the rat and cat were incubated in a medium containing L-DOPA and nialamide and studied by the formaldehyde-fluorescence method.

Unlike the rat brain capillary walls, which showed a marked increase in fluorescence due to uptake of L-DOPA and subsequent DA formation and accumulation, the capillary walls of the cat brain samples did not show evidence of DA accumulation. This species difference may have contributed

to the species difference in the effect of administered L-DOPA which has been previously described. (Section C;B)

The nature of a blood-brain barrier to L-DOPA in man is discussed with regard to this finding. Further investigation, in animal models, of factors affecting species differences in the fluorescence intensity of the brain parenchyma after L-DOPA administration may give more information on the fate of L-DOPA used for the treatment of Parkinsonism.

D. The effects of pretreatment with the dopa-decarboxylase inhibitor Ro4-4602 on the intensity of formaldehyde-induced diffuse fluorescence, resulting from L-DOPA administration, in three regions of the rat brain.

The results indicate that pretreatment of rats with the dopa-decarboxylase inhibitor Ro4-4602 affected regional differences in the effects of L-DOPA on the intensity of formaldehyde-induced fluorescence in the parenchyma, compared with the effects of L-DOPA alone.

Inhibition of dopa-decarboxylase in the capillary walls of the rat brain may have contributed to these effects of Ro4-4602.

Further investigation, in animal models, of factors affecting formaldehyde-induced fluorescence intensity in different regions and in the same region of the brain parenchyma after L-DOPA administration, may give more information on the fate of L-DOPA used for the treatment of Parkinsonism.

The therapeutic use of L-DOPA. (E,F)

E. An investigation of the effects of therapeutically administered L-DOPA.

This study reports an investigation of the properties of a blood-brain barrier to administered L-DOPA in man. Biopsy samples, obtained during stereotaxic surgery for Parkinsonism, were incubated in a medium containing L-DOPA and a monoamine oxidase inhibitor.

Although there was insufficient data for a general conclusion to be drawn, the results suggest that the capillary walls in samples of globus

pallidus from a patient with Parkinsonism did not show evidence of accumulation of DA, (derived from L-DOPA in the incubation medium), in contrast to the majority of the capillary walls in samples of the globus pallidus from a rat brain.

The nature of a blood-brain barrier to therapeutically administered L-DOPA is discussed with regard to this finding.

F. Drugs which may potentiate the therapeutic effect of L-DOPA in Parkinsonism.

Drugs which may potentiate the therapeutic activity of L-DOPA are discussed.

Amongst these, dimethyl sulphoxide (DMSO) has been suggested as a possible potentiator of the therapeutic activity of L-DOPA, on the basis of its previously reported effects on the intensity of parenchymal formaldehyde-induced fluorescence of the rat brain following L-DOPA administration. However these effects were not confirmed in the present study.

References

General Introduction

Reviews of previous publications

A. The nigro-striatal system of dopamine-containing neurones.

(a) Monoamine-containing neurones:- The formaldehyde-fluorescence method visualizes biogenic amines and some of their precursors, by giving rise to formaldehyde-induced (i.e. specific) fluorescence in various groups of nerve cell bodies in the brain stem. (35) The cell bodies which have been demonstrated in this way can be divided into three types, containing noradrenaline (NA), dopamine (DA) and 5-hydroxytryptamine (5-HT) respectively, although recently another fluorogenic compound, which may be an indolamine (24), has been found in certain brain stem neuronal bodies. Most of the axon of a monoamine-containing neurone does not normally contain sufficient monoamine to be visualized by the formaldehyde-fluorescence method, in contrast to parts of the terminal region of each axon, which contain comparatively high concentrations of monoamine.

Several methods have been used to visualize the axons of the three main types of monoamine-containing neurone. For example, if the axons are damaged with stereotaxic lesions, the subsequent anterograde and retrograde degeneration of the neurones can be studied (54) and the accumulation of monoamine in the proximal part of the damaged axon, due to the transport of amine storage vesicles from the cell bodies, can be demonstrated by the formaldehyde-fluorescence method.

(b) Dopamine-containing neurones:- Three DA-containing groups of neurones have been described in the rat:-

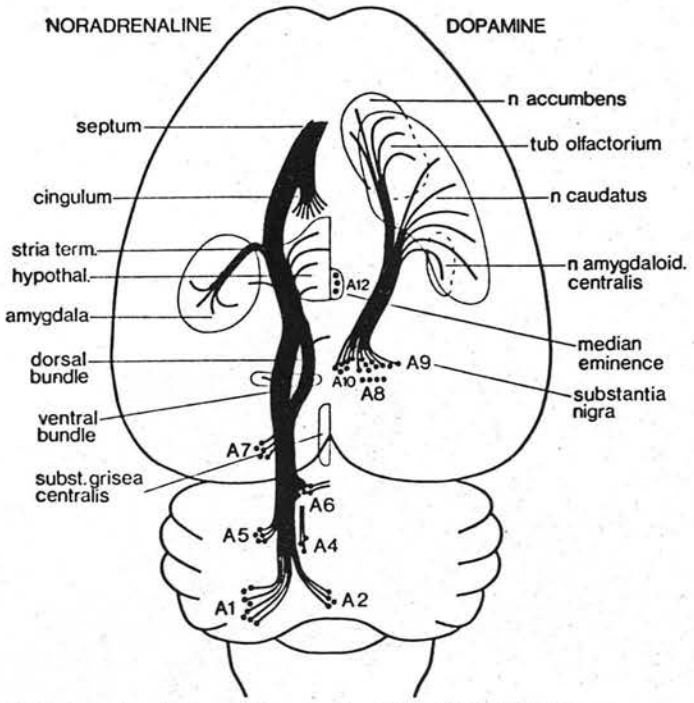


Fig 12. Horizontal projection of the ascending NA and DA pathways.

Figure 1. Acta physiol. Scand. Suppl. 367, 1971, p.28. Showing a horizontal projection of the DA-containing neurones of the rat brain.

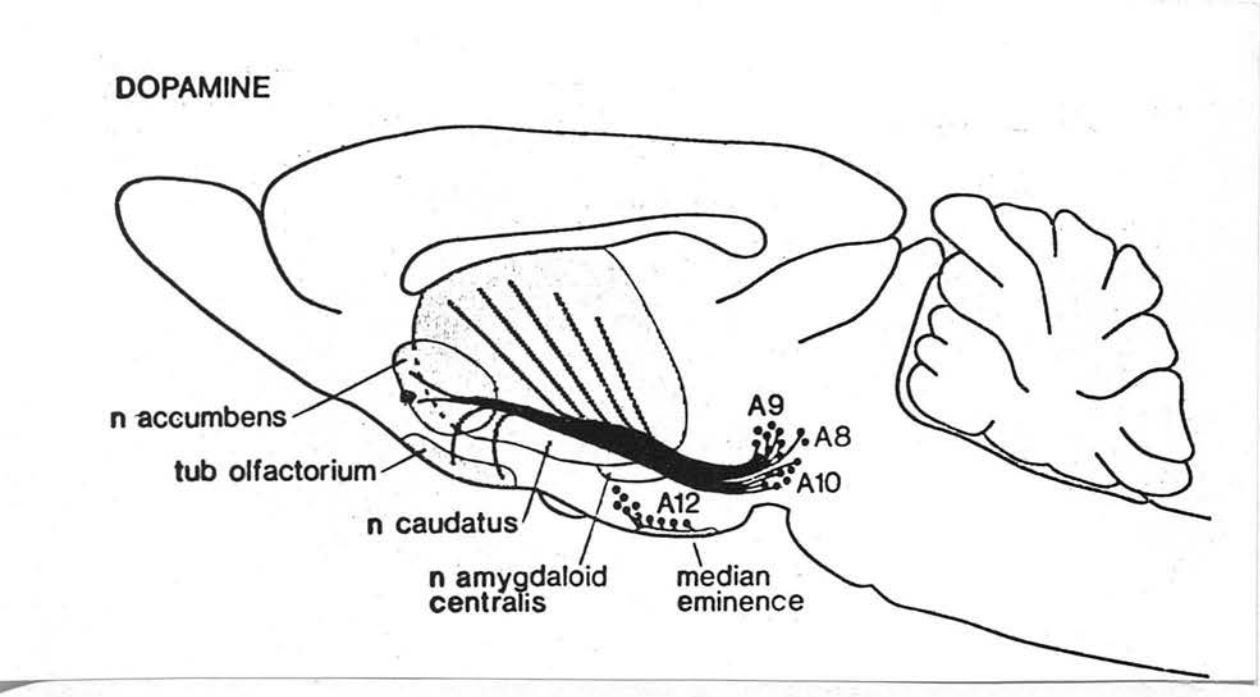


Figure 2. Acta physiol. Scand. Suppl. 367, 1971, p.27. Showing a sagittal projection of the DA-containing neurones of the rat brain.

(c) Mesolimbic DA-containing neurones. (5,37) These neurones have their cell bodies in the area surrounding the nucleus interpeduncularis. This group of cell bodies has been named group A10 by Dahlström and Fuxe (35) and is mainly in the area dorsal to the nucleus interpeduncularis (see figures 3-6). Rostrally the A10 group rapidly decreases in size after the nucleus interpeduncularis has disappeared, but some cells of group A10 are present within the nucleus interstitialis decussationis ventralis tegmenti and between the fascicula retroflexa. Both these latter structures are cranial

to the nucleus interpeduncularis (35,72). In addition, some of the cell bodies lateral to the nucleus interpeduncularis are also included in group A10. (35) Each axon of the mesolimbic system gives rise to a terminal branching network of processes in one of several regions, i.e. the tuberculum olfactorium, nucleus accumbens, the dorsolateral part of the nucleus interstitialis striae terminalis and the nucleus amygdaloideus centralis. (54) The terminal part of each neurone, which forms a branching network, has numerous varicosities distributed along the processes forming this network, and it is these varicosities which contain much higher concentrations of DA than the other parts of the neurone.

(d) Tubero-infundibular DA-containing neurones. (46,51,19) The cell bodies of this group have been named group A12 (see figures 1,2) and lie mainly in the anterior part of the nucleus arcuatus. The axons run towards the lateral border of the median eminence. The terminal parts of the axons give rise to a region containing densely packed DA-containing varicosities, in the external layers of the median eminence and of the infundibular stem. Some varicosities may also be found in the posterior lobe and in the pars intermedia of the pituitary. (54)

(e) The nigro-striatal DA-containing neurones. (2,4,92,105,63) Figures 3-6 are reproduced from Acta. physiol. Scand. 62: Suppl.232, 1964 (35) and show transverse sections of the brain stem of the rat in a caudal-rostral sequence. The plane of sectioning is approximately parallel to the frontal zero plane of König and Klippel (72). (See figure 38) The situation and classification of cells containing formaldehyde-induced fluorescence is shown in figures 3-6.

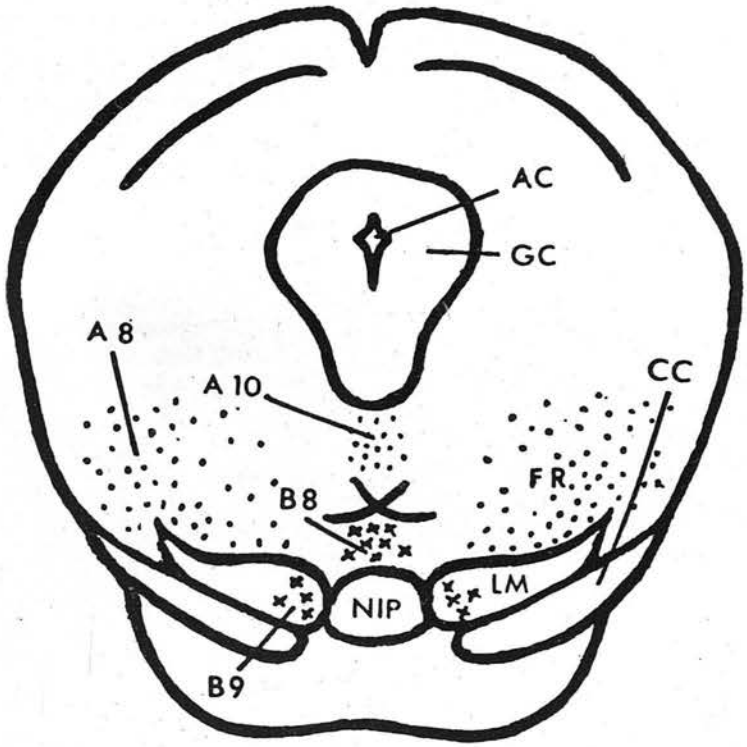


Figure 3. Transverse section through rat mesercephalon. AC = aqueductus cerebri, GC = grisea centralis, FR = formatio reticularis, LM = lemniscus medialis, CC = crus cerebri, NIP = nucleus interpeduncularis.

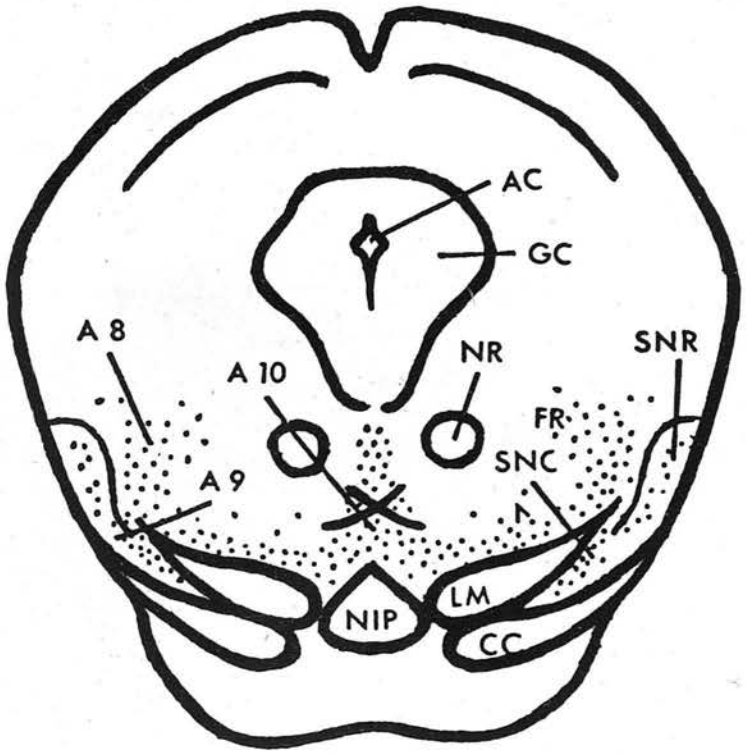


Figure 4. Transverse section through rat mesencephalon at the level of the middle third of the nucleus interpeduncularis. SNR = substantia nigra, zona reticulata, SNC = substantia nigra, zona compacta, NR = nucleus ruber. Other abbreviations see figure 3.

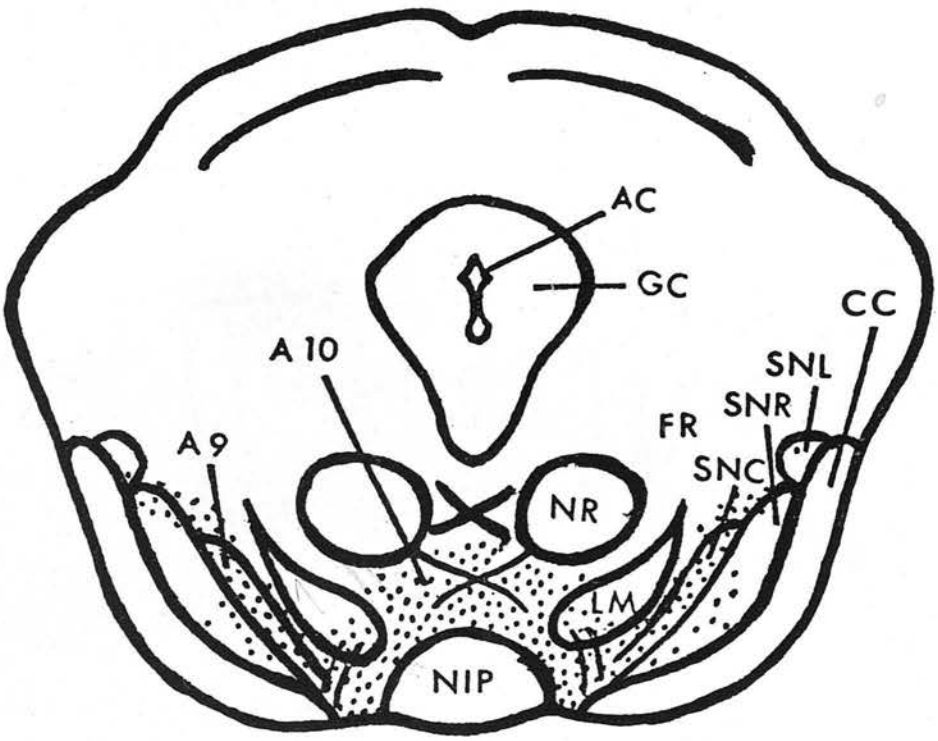


Figure 5. Transverse section through rat mesencephalon at the level of the cranial third of the nucleus interpeduncularis. SNL = substantia nigra, pars lateralis. Other abbreviations see figures 3 and 4.

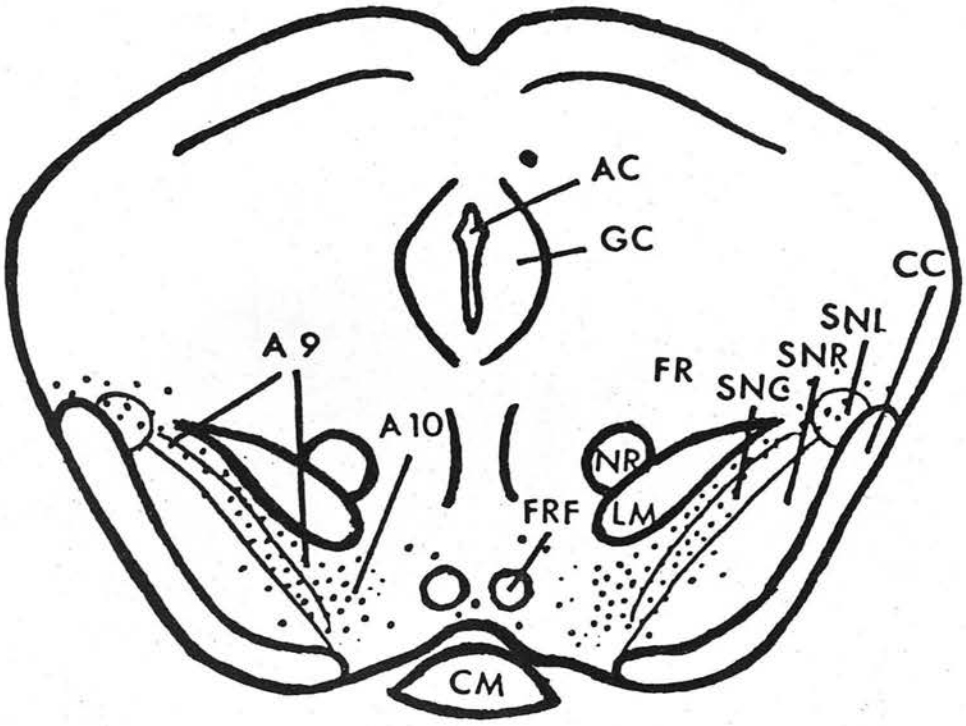


Figure 6. Transverse section through rat mesencephalon, just cranial to the nucleus interpeduncularis. CM = corpus mamillare, FRF = fasciculus retroflexus. Other abbreviations see figures 3, 4 and 5.

Cell bodies.

Most of the cell bodies of the nigro-striatal system lie in the zona compacta of the substantia nigra and are included in the cells named group A9, (figures 4-6) which also includes some cell bodies in the zona reticulata and the pars lateralis of the substantia nigra. Dahlström and Fuxe (35) noted that there was not a distinct border between the zona compacta and the area ventralis tegmenti, so that it was difficult for them to decide whether some cells of group A9 were situated in the zona compacta

or in the area ventralis tegmenti. In addition some of the cell bodies included in this group (A9) were situated within the most caudal part of the zona incerta. Group A9 was not clearly separated medially from group A10 (figures 3-6), but the borderline between these 2 groups is medial to the zona compacta and lateral to the nucleus interpeduncularis. Laterally, group A9 is not clearly separated from another group of cell bodies named as A8 (figures 3 and 4), the cells of which also contain a formaldehyde-induced fluorescence. The rostro-caudal extent of group A9, i.e. corresponding to the rostro-caudal extent of the substantia-nigra, can be seen in König and Klippel's Stereotaxic Atlas of the Rat Brain (72) which illustrates transverse sections of the midbrain, in planes parallel to their defined frontal zero plane which is shown in figure 38. König and Klippel studied rats weighing 150G, in which the substantia nigra extended approximately 1.7 mm. rostro-caudally, from their text-figure 42 to text-figure 50. Cell bodies containing formaldehyde-induced fluorescence have also been found in the substantia nigra of the cat and Cynomolgus monkey (35).

Although most of the cell bodies of the nigro-striatal system are in group A9, additional cell bodies of this system have been described. Studies after removal of the nucleus caudatus-putamen in the rat (54) reported a reduction of formaldehyde-induced fluorescence and signs of atrophy in the A8 and A9 groups of cells (figures 3 and 4). The cell bodies of group A8 lie in the ventro-lateral part of the midbrain tegmental reticular formation, mainly caudal to the red nucleus, where they lie dorsal to the medial lemniscus. At a more rostral level they lie ventro-lateral to the red nucleus. No distinct borderlines were found between groups A8, A9 and A10. At the level of a transverse section of the midbrain through the mid rostro-caudal axis of the red nucleus, (see figure 5), the cells of

group A8 had disappeared. Certain relatively large cells of group A8 have been seen to contain coarse, intensely staining Nissl granules, while the smaller cells had relatively fine, less intensely staining Nissl granules. (35) Group A8 extends from text-figure 48 to text-figure 51 in König and Klippel's Atlas (72), over a distance of approximately 740 um in a rostro-caudal direction, in rats with a weight of approximately 150 g.

It has been suggested (54) that the cells of groups A9 and A8 can be divided into medial and lateral groups which innervate the cranial medial and caudal lateral part of the neostriatum respectively.

Andén, Fuxe, Hamberger and Hökfelt (6) in a combined biochemical-histochemical study in the rat, have calculated that there are about 3,500 nigro-striatal neurones on each side, with a mean cell body diameter of 30 µm. It was calculated that each cell body contained 0.8 - 2.5 pg of DA at a concentration within the range 60-200 µg/g.

Axons.

The cell bodies of the nigro-striatal system (groups A9 and A8), on each side, give rise to an uncrossed bundle of ascending axons (figure 1), the course of which has been studied by observing their anterograde and retrograde changes after brain lesions. The non-terminal parts of the axons have amine concentrations which are normally too low to be visualized by the formaldehyde-fluorescence method, however 2 - 4 days after removal of the rat nucleus caudatus-putamen, increased formaldehyde-induced fluorescence has been visualized in the cell bodies of A8 and A9 and in the parts of the nigro-striatal axons between the cell bodies and the lesion, due to accumulation of DA (5). This method was used to trace the course of the rat nigro-striatal axons, which passed medially from groups A8 and A9 to form an ascending bundle just dorso-medial to the crus cerebri where it was situated among the outgoing fibres of the third

cranial nerve. The axons then ascended through the ventral tegmental area into the H_2 Field of Forel (54) and at the level of the posterior part of the median eminence, just anterior to the mamillary bodies, they entered the crus cerebri. The axons then passed further rostrally to join the retrolenticular part of the internal capsule, reaching the nucleus caudatus-putamen via the internal capsule and its divergent bundles (fibrae capsulae internae) which are scattered throughout the rat nucleus caudatus-putamen. The axons had diameters between 1-2 μm (61). Further information in the rat has been obtained following lesions of the substantia nigra, globus pallidus and of regions adjacent to the mamillary bodies. (5) Lesions of the substantia nigra were correlated with a reduction in neostriatal DA which was revealed histochemically and biochemically. Lesions at the level of the mamillary bodies produced similar changes in neostriatal DA on the operated side and indicated that some of the nigro-striatal axons had not entered the crus cerebri at this rostro-caudal level. The lesions at the level of the mamillary bodies also produced an increase of fluorescence in the cell bodies of groups A8 and A9 after 2-4 days, and chromatolysis, associated with reduced formaldehyde-induced fluorescence of the cell bodies, was seen after 3-4 weeks. Other lesions, in the globus pallidus and internal capsule, indicated that the nigro-striatal axons passing to the part of the neostriatum lateral to the globus pallidus run from the internal capsule through the globus pallidus to reach their destination, and that fibres to the dorsomedial part of the head of the nucleus caudatus-putamen run in the medial part of the internal capsule. The existence of the nigro-striatal pathway has been confirmed using the Nauta technique. (55)

The course of the nigro-striatal axons has been investigated in other species. The results of Poirier, Sourkes, Bouvier, Bouchet and Carabin (93)

indicate that in the monkey, a bundle of nigro-striatal fibres run in a rostral direction, dorsal to the dorso-medial lip of the substantia nigra as far as the mamillary bodies, at which level they pass laterally through the subthalamus and internal capsule towards the neostriatum. Moore, Bhatnager and Heller (82) have described a dopaminergic nigro-striatal system in the cat using the Fink-Heimer method for the demonstration of degenerating axons, resulting from electrolytic lesions of the substantia nigra and internal capsule. The effect of such lesions on the concentration of neostriatal DA was also studied biochemically. It was concluded that certain cell bodies, mainly in the pars compacta and ventral tegmental area, give rise to axons which are found in the ventro-medial part of the midbrain tegmentum and Field H of the subthalamic region, before they enter the medial part of the internal capsule and adjacent lateral hypothalamus. One part of the system was reported to pass laterally through the globus pallidus before terminating in the neostriatum, while the other part continued rostrally before terminating in the nucleus accumbens (i.e. corresponding to the mesolimbic DA-containing neurones in the rat) and the anterior part of the neostriatum. After lesions of the nigro-striatal pathway, there was loss of DA in the ipsilateral neostriatum, proportional to the extent of the lesion.

Terminal parts of the axon.

The cell bodies and axons of the three known types of monoamine-containing neurone, containing DA, NA, or 5HT, show a relatively low amine concentration, compared with the numerous varicosities which are associated with the terminal branching network of each axon. The varicosities are irregularly spaced along the terminal branches, and the number of varicosities per unit length of axon-branch varies between different systems of neurones, but has been estimated to be in the order of 15-30 per 100 μm. (48) The size and number of varicosities differ

between the different neuronal systems, e.g. NA and 5HT-containing varicosities are larger (about 1-3 μm in diameter) than those containing DA. (48)

The varicosities of the terminal parts of the axons of the nigro-striatal DA-containing neurones are distributed throughout the neuropil of the nucleus caudatus-putamen in the rat. (See figures 7-9)

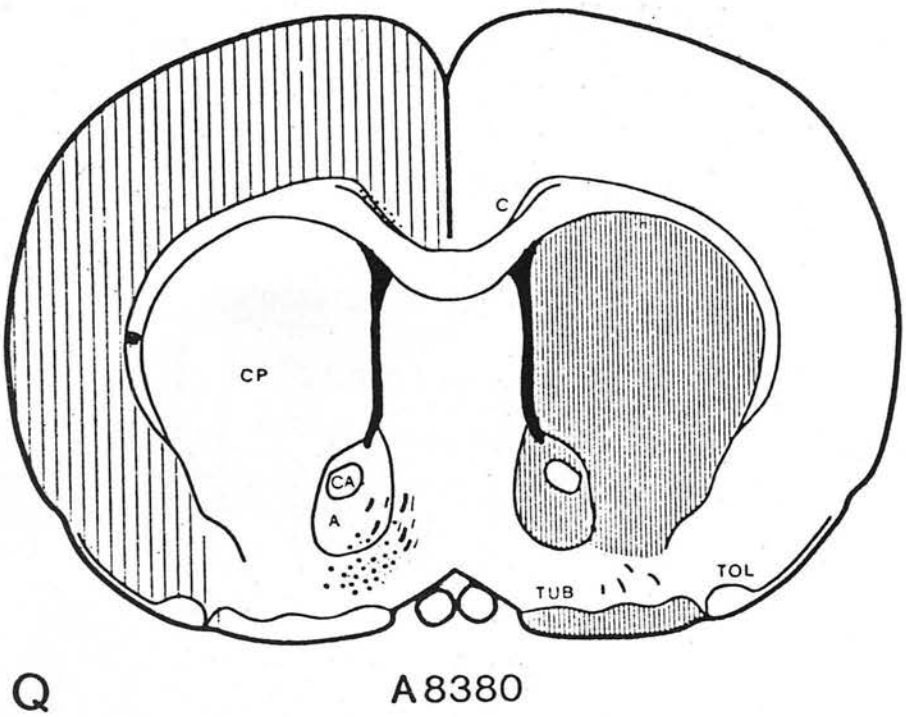


Figure 7. Acta. physiol. Scand. Suppl. 367, 1971, p.39. Frontal section of the rat brain. Drawing based on König and Klippel's stereotaxic atlas. (72) The numbers refer to the distance in µm anterior (A) to the frontal zero plane of König and Klippel (See figure 38). The more closely spaced vertical lines show the distribution of DA terminal varicosities. CP = nucleus caudatus-putamen, CA = commissura anterior. A = nucleus accumbens.

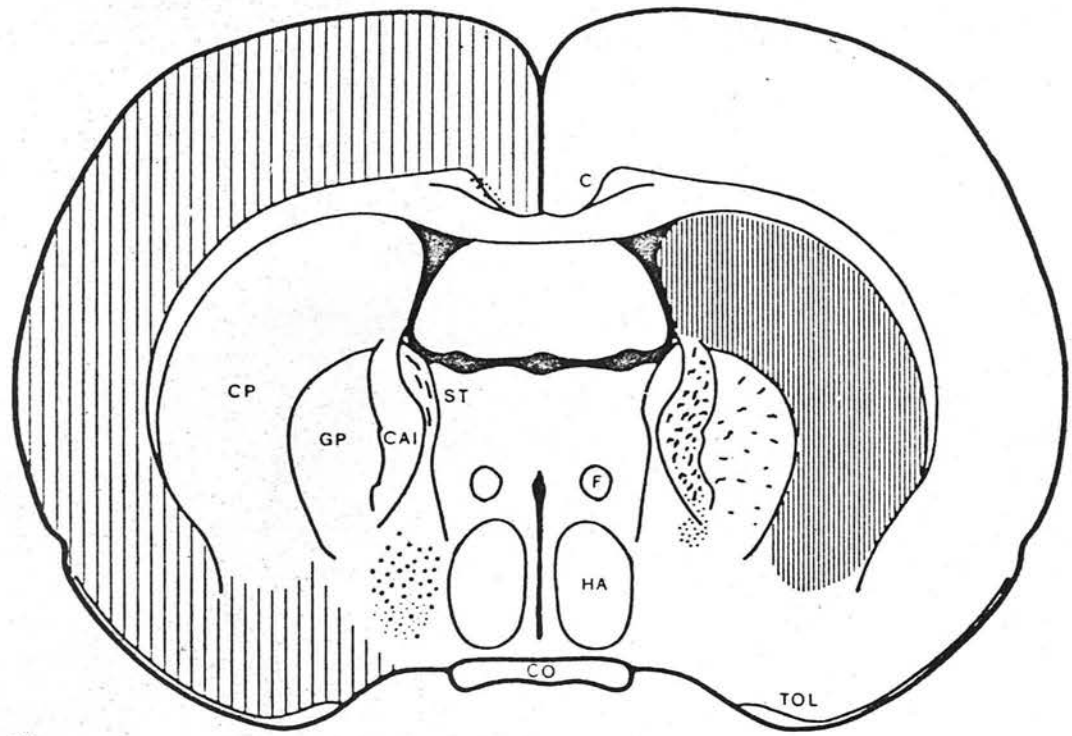


Figure 8. Acta. physiol. Scand. Suppl. 367, 1971, p.38. For explanation see figure 7. CAI = capsula interna, GP = globus pallidus.

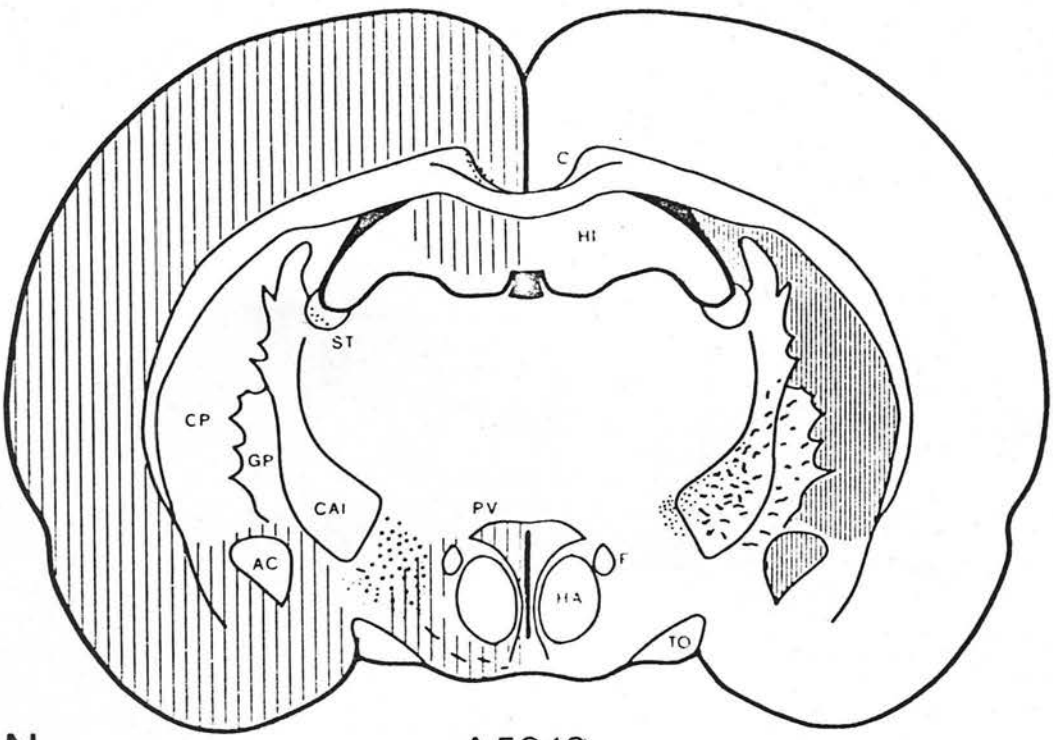


Figure 9. Acta. physiol. Scand. Suppl. 367, 1971, p.37. For explanation see figure 7. AC = nucleus amygdaloideus centralis.

Andén, Fuxe, Hamberger and Hökfelt (6) in a combined biochemical-histochemical study of the rat nigro-striatal system have calculated that the terminal, i.e. branching, part of each neurone varies between 55 and 77 cm in total length, and has about 500,000 associated varicosities. The neostriatum on one side of the brain has a volume of about 33 mm^3, and was estimated to contain approximately 1.75×10^9 DA-containing varicosities, occupying 0.3% of the neostriatal volume. The neostriatum on one side contains about 4,000,000 nerve cell bodies (not containing DA) and it was concluded that the DA varicosities of an individual neurone innervate many cells in the neostriatum, mainly by

making contact with the dendrites of the neostriatal cell bodies. The average diameter of a DA varicosity was reported to be 0.4 μm. Individual varicosities were seen in thin sections (2 μm in thickness), under "optimal" experimental conditions which prevented diffusion of DA from the varicosities. However many DA varicosities may be too fine to be seen by the "ordinary light microscope" (52) so the mean diameter of a varicosity given by Andén, Fuxe, Hamberger and Hökfelt (6) may not have taken into account many smaller terminals which were not seen. These authors calculated that an average DA varicosity contains 2.5×10^{-4} pg DA ($1pg = 10^{-12}g$), at a concentration of about 8,000 μg/g wet weight.

The varicosities of the nigro-striatal system are scattered throughout the neuropil of the neostriatum, and the electron microscope has shown (83,62) that both the smaller cell bodies (10-15 μm in diameter) and the relatively fewer larger cell bodies in the rat nucleus caudatus-putamen are associated with few axosomatic synapses, compared with numerous axodendritic synapses. The neostriatal DA-containing varicosities almost certainly are pre-synaptic structures where dopamine is stored, and released. (48) Hökfelt (62) has calculated that about 16% of the total number of varicosities in the nucleus caudatus-putamen of the rat contain DA, i.e. there is a ratio of about 400 DA varicosities to each neostriatal cell body with its dendrites.

There is uncertainty about the range of sizes of DA varicosities in the rat neostriatum. Fuxe, Hökfelt and Nilsson (52) using an electron microscope found many neostriatal varicosities with a diameter of less than 0.4 μm, i.e. down to 0.1 μm, however it was not possible to tell which of the observed varicosities contained DA. Other reports have estimated the diameters of the DA-containing neostriatal varicosities as follows:-

(i) Between 0.5 μm and 0.8 μm. (Hökfelt (62))

(ii) Between 0.5 μm and 1.0 μm. (Fuxe, Hökfelt and Ungerstedt (54))

(iii) Many less than 0.5 μm. (Fuxe (48))

(iv) Between 0.3 μm and 0.6 μm. (Hillarp, Fuxe and Dahlström (61))

(v) Many of 0.4 μm with a range of 0.3-0.7 μm. (Andén, Fuxe, Hamberger and Hökfelt (6))

The structure of the terminal parts of an axon, linking the varicosities, is also uncertain. Fuxe (48), describing catecholamine-containing neurones, stated that the segments between the varicosities have diameters down to 0.2 μm, and vary in length from 1-7 μm.

An electronmicrograph showing varicosities in the rat nucleus caudatus-putamen is shown in figure 10.

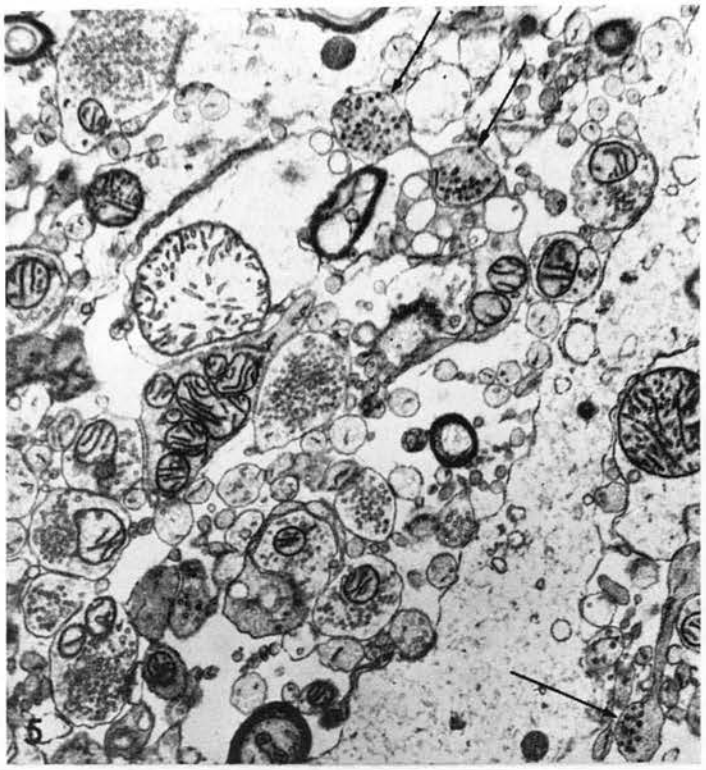


Figure 10. From Third Symposium on Parkinson's Disease, Livingstone, Edinburgh and London (1969). Electron micrograph of rat nucleus caudatus-putamen. Many varicosities are seen. One type (arrowed) contains small granular vesicles (diameter about 500 Å) and "probably represents" varicosities belonging to DA nerve terminals. (Hökfelt (62)) Two varicosities (also arrowed) containing small granular vesicles are seen connected by part of an axon. These varicosities are approximately 0.6 μm in diameter, and the thin segment is approximately 0.02 μm in width and 0.2 μm in length.

(f) DA as a neurotransmitter:- Fuxe, Dahlström and Hillarp (49) have discussed the criteria to be considered before NA, DA and 5HT can be regarded as neurotransmitters:-

(i) The amine must be located intraneuronally and concentrated in the terminal parts of the axon.

Fluorescence microscopy has shown intensely fluorescent varicosities along the length of the terminal branches of the nigro-striatal system of neurones, and the electron microscope has shown that boutons-en-passage, which are probably identical with monoamine-containing varicosities, establish synaptic junctions in the striatum (26).

(ii) The substance should be synthesized intraneuronally.

Studies in different species have measured activity of enzymes which are related to DA metabolism, and Franzen and Eysell (45) have given the following values in microgram substrate per gram tissue per hour; based on several studies of the mammalian CNS:-

	Caudate nucleus.	Midbrain.
Dopa-decarboxylase	420 *	220
Dopamine-β-oxidase	0.8	
Monoamine oxidase	900	800
Catechol-O-methyl transferase	100	110

* The value for this enzyme, needed for the decarboxylation of L-DOPA to dopamine, is higher than in any other region studied.

Anden, Magnusson and Rosengren (7) have provided evidence which suggests that dopa-decarboxylase is localized in monoamine-containing neurones. Moore, Bhatnagar and Heller (82) have measured neostriatal enzymic activity following lesions of the nigro-striatal system in the cat, and found a subsequent loss of tyrosine hydroxylase activity and of dopa-decarboxylase activity, proportional to the extent of the lesion.

(iii) There should be evidence of local inactivating enzymes for the proposed transmitter.

Monoamine oxidase (MAO) and catechol-o-methyl transferase (COMT) are present in the midbrain and neostriatum (see above), and biochemical studies have indicated that MAO is localized intraneuronally while COMT

is localized extraneuronally (49). Further histochemical evidence (49) has indicated that MAO is present in catecholamine-containing neurones. Reabsorption of released neurotransmitter by the terminal parts of the axons has been claimed to be a factor in the inactivation of released monoamine. (100)

(iv) The transmitter substance should be bound intraneuronally in an inactive form e.g. by being enclosed in, and/or bound to, protein, thus forming "granules" or "vesicles".

Several studies (49) have provided evidence that monoamines are stored in intraneuronal vesicles.

(v) The transmitter must be released from the terminal parts of the axon by nerve impulses.

Both biochemical and histochemical evidence has indicated that NA and 5HT are released from the terminal varicosities. (49) In addition, studies on peripheral neurones have shown that NA is stored in terminal varicosities and released by nerve impulses. (48,75)

(vi) The post-synaptic neurone should be sensitive to the proposed transmitter.

York (109) electrically stimulated the substantia nigra of the cat, and recorded action potentials in the putamen. He also recorded changes in neuronal discharge frequency in the putamen caused by iontophoretically applied DA, and his results suggested that DA is a neostriatal synaptic transmitter.

(vii) Drugs which interfere with the metabolism of the proposed transmitter should alter the action of the presynaptic neurone on the postsynaptic nerve cell.

Many studies which indicate that this criterion is satisfied are reviewed by Fuxe, Dahlström and Hillarp. (49) For example, there is evidence that protryptyline and cocaine give rise to an extraneuronal accumulation of monoamines which influences neuronal activity of postsynaptic neurones.

Fuxe, Dahlström and Hillarp (49) concluded that the evidence which is available "must be considered sufficiently strong to allow the statement that DA, NA and 5HT act as neuro-humoral transmitters in the CNS".

Calne (28) has summarized the experimental evidence from animal studies concerning the nigrostriatal pathway:-

(i) Experimental brain stem lesions which have resulted in degeneration of the substantia nigra have also reduced the level of striatal DA.

(ii) In addition, such lesions caused depletion of striatal enzymes concerned with the synthesis of DA, such as tyrosine hydroxylase.

(iii) After a unilateral lesion affecting the substantia nigra ipsilateral depletion of striatal DA has been reported and subsequent administration of L-DOPA led to a greater elevation of DA levels on the contralateral side compared with the ipsilateral side.

(iv) The injection of 6-hydroxydopamine into the substantia nigra causes degeneration of amine-containing neurones, and a reduction of DA in the ipsilateral striatum.

(v) Removal of the caudate nucleus and putamen led to a transient increase, followed by a subsequent decrease, in the formaldehyde-induced fluorescence of cells containing DA in the substantia nigra.

(vi) A substantial increase in output of DA, collected by irrigation of the putamen through a cannula, was produced by electrical stimulation of the substantia nigra.

(vii) It has been reported that 44% of microelectrophoretically examined neurones in the caudate nucleus were inhibited by stimulation of the substantia nigra. (A few cells (14%) displayed facilitation and the rest were unaffected.)

(viii) Recent studies with conventional histological techniques have shown degenerating nerve endings in the striatum after experimental lesions in the substantia nigra of the rat and cat.

B. The significance of the nigro-striatal system of DA-containing neurones in Parkinsonism.

Parkinsonism is a chronic neurological disorder, characterized by tremor, rigidity of the limbs and poverty of movement (hypokinesia). Initial symptoms of the idiopathic type usually develop between the ages of 50 and 70 and gradually progress, death often occurring about 10 years after onset. Known causes of Parkinsonism include viral infection (encephalitis lethargia), toxins (manganese, carbon monoxide), vascular disease (atherosclerosis) and drugs (phenothiazines, haloperidol and reserpine). In many cases however, no cause can be identified.

There have been many studies of the pathological changes in the C.N.S. associated with Parkinsonism, and there are several reports of an associated reduction in number, and depigmentation of, the pigmented cells in the pars compacta of the substantia nigra.(10) Greenfield and Bosanquet (57) examined the pigmented cells of the substantia nigra and locus coeruleus in 19 cases of idiopathic Parkinsonism (paralysis agitans), in 10 cases of Parkinsonism with a history of encephalitis lethargica and in 5 cases of Parkinsonism of uncertain aetiology. Various changes in the cells examined were reported, which varied with respect to the aetiology. Lewy's hyaline inclusions were found in all their cases of typical idiopathic paralysis agitans.

In 1960, a low DA content in the basal ganglia of Parkinsonian patients was reported by Ehringer and Hornykiewicz (39). Confirmatory studies have been reviewed by Calne (28). Barbeau (10) concluded that the symptom of akinesia in Parkinsonism is related to a low DA content in the striatum resulting from a defect in a nigro-striatal DA-containing system, and summarized the current biochemical/histological profile of Parkinsonism:-

(i) There is a marked decrease in the concentration of monoamine, particularly DA, but also NA and 5HT, in the basal ganglia.

(ii) A decrease in the concentration of DA's main metabolite, homovanillic acid (HVA), is found in the basal ganglia.

(iii) A decrease in striatal DA is found, which is related to the degree of depigmentation and loss of cells in the pars compacta of the substantia nigra. Pakkenberg and Brody (87) found a reduction of 50% in the number of neurones in the substantia nigra of 10 Parkinsonian patients, compared with 10 control brains matched for age. Cells containing melanin were more affected than pigment-free neurones, their relative depletions being 66% and 39% respectively.

(iv) There is a decreased HVA concentration in the C.S.F. which is in parallel with the decrease in striatal DA.

In addition, a reduced dopa-decarboxylase activity in the basal ganglia has been shown to be associated with Parkinsonism. (58)

This evidence, together with the knowledge of a nigro-striatal DA-containing system of neurones in several species, suggests that a lesion of a nigro-striatal dopaminergic pathway, which results in a reduction in the striatal DA concentration, is associated with Parkinsonism. However, as reductions in the concentration of 5HT and NA in the basal ganglia are also associated with Parkinsonism (10) the nigro-striatal DA-containing neurones are probably not the only neuronal system to be commonly affected in this syndrome. Denny-Brown (36) described two cases of severe Parkinsonism in whom the substantia nigra was histologically normal, and Stern (98) concluded that "the neuropathological evidence indicates that in Parkinsonism the substantia nigra is very frequently but not invariably altered and that when nigral damage is present it is usually accompanied by diffuse changes elsewhere in the neuraxis. In certain conditions other

than Parkinsonism, extensive structural changes may include the substantia nigra without Parkinsonism". Barbeau (10) raised the question whether the dysfunction of the nigro-striatal dopaminergic neurones can, in some cases of Parkinsonism, reflect a more generalized metabolic defect in DA metabolism. This idea was prompted by observation of reduced urinary DA excretion associated with Parkinsonism, however low urinary levels of DA are not specific to Parkinsonism, and Calne (28) considered that the overall metabolism of DA may be normal in Parkinsonism.

In summary, the pathology of Parkinsonism is not fully understood, however there is strong evidence that pathological changes usually affect the nigro-striatal DA-containing system of neurones.

C. The therapeutic action of L-DOPA in Parkinsonism.

There is evidence that DA mainly acts as an inhibitory neurotransmitter in the neostriatum, and that acetylcholine (Ach) is an excitatory transmitter in this region. (30) It seems that a balance may exist between the activity of Ach and DA in the neostriatum, and that in Parkinsonism there is a disequilibrium in the direction of cholinergic dominance. (9,8) Drugs which deplete the brain of DA (e.g. reserpine) or increase the concentration of Ach (e.g. physostigmine) exacerbate Parkinsonism, and drugs which reduce cholinergic function (e.g. atropine) or enhance dopaminergic activity (e.g. L-DOPA) are therapeutic. (30)

Calne (28) has reviewed the studies which have reported a reduced neostriatal DA concentration in non-iatrogenic Parkinsonism, and it seems probable that in the drug-induced Parkinsonian syndromes there is a reduction in the sensitivity of neostriatal dopaminergic receptors.

In a disease accompanied by a lack of DA in certain regions of the brain, attempts at substitution therapy were clearly indicated. The synthetic pathway of DA is as follows:- (28)

L-phenylalanine

↓ (Enzyme = phenylalanine hydroxylase)

L-tyrosine

↓ (Enzyme = L-tyrosine hydroxylase)

L-3-4-dihydroxyphenylalanine (L-DOPA)

↓ (Enzyme = aromatic L-aminoacid decarboxylase)

dopamine

Tyrosine hydroxylase is normally likely to be saturated by its substrate tyrosine (90), so it would be expected that Parkinsonism could not be effectively treated by giving an excess of L-tyrosine. L-DOPA is decarboxylated in several species by the relatively non-specific L-DOPA

decarboxylase which is now known to consist of several isoenzymes (30), however it has been difficult to demonstrate dopa-decarboxylase activity in the human brain. (30) The difficulty in demonstrating human dopa-decarboxylase may be due to technical reasons, however it is also possible that decarboxylation of L-DOPA is the rate limiting step in the formation of DA in the human nigro-striatal system. (30)

Administered DA does not cross the blood-brain barrier in most regions of the rat brain (28). A sufficient dose of L-DOPA can cross the blood-brain barrier in the rat and this precursor of DA has been used with success as a therapeutic agent in Parkinsonism. However, if degeneration affects dopaminergic axons in the neostriatum, the nerve terminals (varicosities) which are thought to contain the necessary dopadecarboxylase (107) and DA storage vesicles, would also degenerate. Nevertheless, administered L-DOPA in Parkinsonism may supply the surviving nigro-striatal axons and/or cell bodies with excess precursor and thus help to increase the rate of DA formation in the surviving varicosities.

There have been several reports that patients with mild Parkinsonism obtain greater benefit from L-DOPA than do more severe cases (28) which suggests that surviving nigro-striatal neurones are necessary for a therapeutic response. However, aromatic L-aminoacid decarboxylase is a widespread enzyme and is also concerned with 5-HT formation. Therefore even if most of the dopaminergic varicosities have degenerated, it is possible that some administered L-DOPA might be decarboxylated in the neostriatum enzyme in non-dopaminergic neurones, which could lead to an increased concentration of DA in synaptic clefts. Any activity of neostriatal DA in Parkinsonism might be enhanced at some synapses by denervation supersensitivity. (28)

Administered L-DOPA could lead to the formation of DA at extraneuronal sites such as cerebral capillaries, as has been described

in the rat and mouse. Any DA formed in this way might then diffuse to striatal neurones to exert some inhibiting effect at overactive receptor sites. However L-DOPA may exert part or all of its therapeutic effect in ways other than by compensating the reduction of neostriatal DA. (11,56) For example, part of the therapeutic effect of L-DOPA could be due to a more generalized arousal of the C.N.S. (56)

Because L-DOPA decarboxylase is so widespread outside the C.N.S. much of an administered dose of L-DOPA is metabolized at peripheral sites (i.e. outside the CNS). However, a proportion of a dose of L-DOPA is presumed to pass through the blood-brain barrier in the treatment of Parkinsonism, and a widely-held current view is that the therapeutic activity of L-DOPA derives from its conversion to neostriatal DA. (30)

Monoamine oxidase inhibitors (MOAIs) have been reported to have a beneficial effect in Parkinsonism, although other reports have denied this, and it has been reported that MOAIs also potentiate the therapeutic action of L-DOPA. (28) Calne (28) considered that these observations indicate that the therapeutic agent associated with treatment with L-DOPA is likely to be a monoamine derived from L-DOPA, e.g. DA or NA. Birkmayer and Hornykiewicz (18) have investigated the therapeutic action of dihydroxyphenylserine, which is converted to NA without the intermediate formation of DA. They gave this compound to Parkinsonian patients and failed to obtain any therapeutic response which suggests that the therapeutic action of L-DOPA is more likely to be due to increased levels of brain DA rather than NA. In addition, L-DOPA therapy leads to a marked rise in the urinary output of HVA, derived from DA, while there is only a minor increase in excretion of vanilmandelic acid, the major urinary metabolite of NA. (29)

The clinical use of L-DOPA in Parkinsonism has been reviewed by Calne and Sandler. (30) Patients with severe long standing Parkinsonism seem to derive less benefit than milder cases of recent onset, although therapeutic

successes and failures have been reported at every grade of severity. It is likely that at least 50% of Parkinsonian patients may obtain benefit from L-DOPA and in some cases the improvement will be marked. Hypokinesia is the symptom which is most responsive to L-DOPA, while tremor and rigidity are less consistently improved. At present it is not known if the course of the disease is modified by L-DOPA. If the therapeutic action of L-DOPA depends on the functioning of remaining nigro-striatal neurones then it would be expected that the drug would be less effective as the disease progressed. This trend has been described (30) but exceptions have been noted.

It is likely that the therapeutic action of L-DOPA is associated with many complex changes in the activity of more than one neurotransmitter. For example, L-DOPA probably competes with tryptophan for tissue transport mechanism (30) and with 5-HTP for decarboxylation, while brain 5-HT is depleted after L-DOPA administration. (13)

The metabolism of administered L-DOPA in Parkinsonism has been reviewed by Calne (28). It can be transaminated and 3-O-methylated as well as decarboxylated, and melanin can also be formed via quinone and indole intermediaries. Large oral doses of L-DOPA lead to large increases in urinary HVA, derived from DA, but to much less significant changes in vanilmandelic acid, formed from NA and adrenaline (A). DA formation, therefore, appears to be of major significance in the metabolism of L-DOPA.

D. The histochemical appearance of the nigro-striatal system of DA-containing neurones and of the effects of administered L-DOPA on the CNS, shown by the formaldehyde-fluorescence method.

In "normal" animals, i.e. unaffected by drugs or surgery, the terminal parts of the nigro-striatal neurones are readily demonstrated histochemically because the terminal part of each axon contains a high concentration of DA compared with the rest of the neurone. The cell bodies can also be visualized. The axons are not usually demonstrable in "normal" animals, however Andén et al (2) stated that "when the histochemical reaction was particularly successful the axons of the special cells in the substantia nigra (i.e. DA-containing cell bodies) developed a weak fluorescence and then could be seen to give rise to fibre bundles."

(a) The terminal parts of the nigro-striatal axons:- Constantinidis, Bartholini, Tissot and Pletscher (32) reported a "faint diffuse" formaldehyde-induced fluorescence in the rat striatum. Fuxe (48) stated that "in some areas a finely dotted or diffuse specific (i.e. formaldehyde-induced) fluorescence is observed", and one of these areas was the nucleus caudatus-putamen. These descriptions are in contrast with the appearance of most areas containing monoamine nerve terminals (varicosities) where discrete terminals can be visualized ranging from about 0.5 μm to 4.0 μm in diameter (48). Fuxe (48) also stated that the diffuse nature of neostriatal fluorescence is due to "the presence of very fine, partly sub-light microscopical terminals which are so closely packed that the individual terminal can only be observed under perfect conditions." He described this diffuse neostriatal formaldehyde-induced fluorescence as of "strong intensity" and noted that it was mainly due to the fluorophore derived from DA. Andén et al (2) found that the rat nucleus caudatus-putamen showed "a fairly strong green to yellow-green fluorescence due to the high DA content", and Fuxe (48) described the diffuse green fluorescence of this region as "between the nerve cell bodies". Another description (52)

noted "a fairly strong green to yellow-green fluorescence in all parts of the caudate nucleus, in which the fibrae capsulae internae stood out as dark areas."

Although the specific (i.e. formaldehyde-induced) neostriatal fluorescence usually appears diffuse, there have been several reported studies which have visualized individual DA-containing varicosities. Fuxe (48) using sections 8-10 μm in thickness stated that if the histochemical reaction was performed under "perfect conditions", discrete varicosities could be visualized. Andén et al (6) visualized individual DA-containing neostriatal varicosities in sections with a thickness of 2 μm, embedded with Araldite, and stated that in sections with a thickness of 10 μm (approximately the thickness of sections which are usually examined) "the terminals often cover each other and a diffuse green fluorescence is obtained". Fuxe et al (52) using sections with a thickness of 8-10 μm from tissue treated for one hour at 80°C with formaldehyde gas (generated from paraformaldehyde which had been stored for 2-7 days over a sulphuric acid solution giving a relative air humidity in the range 40-60%) reported that "when the histochemical reaction was perfect" individual fluorescent varicosities could be seen. These authors also stated that the neostriatal DA varicosities are "mostly too fine to be seen in the ordinary light microscope".

It seems that at least three major factors may contribute to the diffuse appearance of the formaldehyde-induced fluorescence in the neostriatal neuropil, which is the usual finding in sections with a thickness of 8-10 μm:-

(i) The high density of varicosities may make the outlines of individual varicosities difficult to observe.

(ii) The smaller varicosities may be at or beyond the resolving power of the microscope.

(iii) Diffusion of DA from some or all the varicosities may

occur during preparation of the tissue. As will be described, a critical factor affecting diffusion is the water content of the paraformaldehyde.

Fuxe (48) noted that the fluorophores of catecholamines show a greater tendency to diffuse than the fluorophore derived from serotonin.

In the above reports the factors affecting the histochemical appearance of the neostriatal neuropil are difficult to evaluate because details of the equipment used (e.g. the resolving power of the microscope), and of the methodology (e.g. details of the formaldehyde treatment, section thickness and storage of the sections) are never all available.

The nature of the neostriatal specific fluorescence:- Fuxe et al (52) reported that the diffuse formaldehyde-induced fluorescence in the rat neostriatal neuropil completely disappeared "some hours" after reserpine 5mg/kg and is due to the presence of DA. Fuxe (48) stated "the specific fluorescence in the neostriatum is due to the presence of DA-containing nerve terminals." However, Björklund, Falck and Owman (23) list 25 compounds that have given rise to a formaldehyde-induced fluorophore and note that care must be taken to identify the compound which gives rise to an observed fluorescence. If possible, histochemical analysis of the characteristics of the fluorescence, and parallel biochemical studies should be carried out.

Anden et al (6) found a mean value for the concentration of DA in the rat neostriatum of 6.7 µg/g, and Hornykiewicz (64) has reviewed the biochemical studies which have shown that DA is present in the neostriatum of all mammalian species examined in concentrations averaging 10 µg/g. Adam (1) reported a mean value of 9.9 µg/g for the DA concentration in the dog and cat neostriatum.

Fuxe (48) noted that under the usual conditions of the formaldehyde-fluorescence method only DA, NA and 5HT, of all known fluorogenic compounds,

had been found in the mammalian brain in amounts which are significant from a histochemical point of view. In Fuxe's opinion the content of adrenaline (A) in the CNS is too low to yield significant fluorescence, however varicosities containing A or unidentified monoamines may exist in the CNS. Constantinidis et al (32) gave the following values for amine concentrations in the rat nucleus caudatus-putamen:- DA 6.36 µg/g and NA 0.48 µg/g, while Hornykiewicz (64) stated that the mammalian caudate nucleus is "nearly devoid of NA (0.1 µg/g or less)". Adam (1) gave mean values for the dog and cat striatum as follows:- DA 9.9 µg/g, NA less than 0.1 µg/g and 5HT 0.7 µg/g. The relative fluorescent yield of DA, NA and 5HT in dried protein models after formaldehyde treatment for 1 hour at 80°C have been stated by Björklund et al (23) to be DA 1.0, NA 1.0 and 5HT 0.3. The distribution of the NA and 5HT in the neostriatum is not known, however from the above data it seems that, at most, about 5% of the total specific fluorescence in the striatal neuropil could be due to fluorophores derived from 5HT or NA.

In view of this data it seems a reasonable approximation to consider the neostriatal specific fluorescence as being derived from the DA in the nigro-striatal terminal varicosities. This is borne out by studies which have related lesions to the nigro-striatal pathway in the midbrain to depletion of specific neostriatal fluorescence. Andén et al (2) found "a clear correlation between the fluorescence reduction and the extent of destruction of the special catecholamine containing nerve cells of the substantia nigra." Fuxe (48) reported that unilateral lesions made in the ventral portion of the cranial part of the mesencephalon have been found practically to abolish the diffuse specific neostriatal fluorescence. However it should be remembered that such lesions may have interrupted axons ascending to the striatum from NA or 5HT containing cell bodies in the brain stem.

(b) Cell bodies of the nigro-striatal DA-containing neurones:- Dahlström and Fuxe (35) described the appearance of the cell bodies in the brain-stem which exhibited specific formaldehyde-induced fluorescence. The distribution of the cell bodies of the nigro-striatal system (groups A8 and A9) has already been reviewed. The fluorescence in these cell groups was described as green to yellow-green. It was associated with the cytoplasm of the cell bodies and often extended to the larger processes, the cell nuclei usually appearing non-fluorescent. With "perfect" freeze-drying the fluorescence was found to be diffuse and Dahlström and Fuxe suggested that it is diffusely present in the cytoplasm, or bound to structures, such as storage granules, with a widespread distribution in the cells. However it is also possible that diffusion occurred during preparation of the tissues in association with what were described as "perfect" reaction conditions, which presumably were regarded as "perfect" if diffusion had not taken place from terminal varicosities. Freeze artefacts were often seen in the cell bodies making the fluorescence uneven.

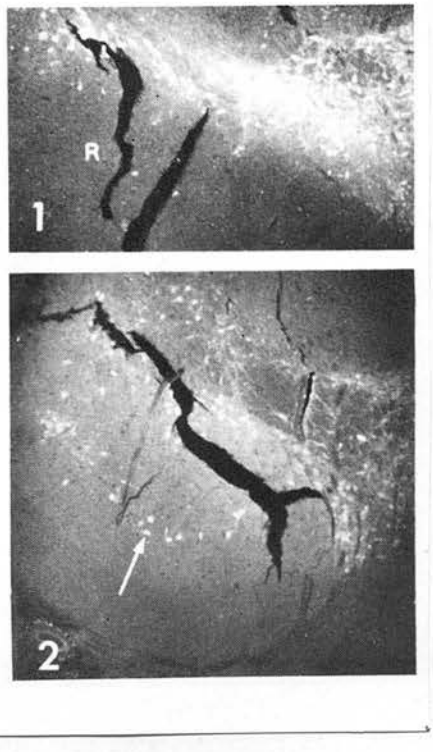


Figure 11. Third Symposium on Parkinson's Disease. 1969. Livingstone Ltd. p.41. Rat substantia nigra showing fluorescent DA-containing cell

bodies of the nigro-striatal system, as shown by the formaldehyde-fluorescence method.

1. Fluorescent cell bodies are closely packed in the zona compacta. The adjacent part of the zona reticulata (R) contains practically no fluorescent cell bodies. X 68.

2. In this section some fluorescent cell bodies are also seen in the zona reticulata (arrowed). X 68.

(c) The effects of administered L-DOPA on the formaldehyde-induced fluorescence of the C.N.S.

Bertler, Falck, Owman and Rosengren (15) gave intraperitoneal L-DOPA to mice. They found that a sufficient dose produced both a marked fluorescence in the capillary walls (due to DA accumulation, derived from the administered L-DOPA - to be discussed later) and a diffuse parenchymal fluorescence in the region studied (the cerebellum). The intensity of the diffuse parenchymal fluorescence after administration of L-DOPA was particularly marked in certain areas in which the capillary wall fluorescence was weak or absent, i.e. within and around the median eminence, the area postrema and the medial surfaces of the olfactory bulbs.

(d) Background fluorescence:- When observing diffuse formaldehyde-induced fluorescence, the background non-specific autofluorescence of the cerebral parenchyma has to be taken into account. Bertler et al (15) stated that "a greenish background fluorescence occurred throughout the parenchyma in all specimens".

E. The formaldehyde-fluorescence procedure. A review of the methodology.

(a) Introduction.

In 1962 it was reported that catecholamines could be converted to strongly fluorescent compounds when exposed to formaldehyde gas, if the reaction took place in the presence of a dried protein matrix. At first this was used to demonstrate NA in nerve terminals of air-dried preparations, and then the technique was applied to freeze-dried tissues. (67) This has led to rapid progress in research into the role of the biogenic monoamines, DA, NA, A and 5HT in the nervous system.

Pieces of tissue are dissected as soon as possible after killing, rapidly frozen in e.g. isopentane (cooled by liquid Nitrogen) before being freeze-dried, e.g. in vacuo at -35°C. The biogenic amines are not destroyed and should remain intraneuronal in the freeze-dried tissue (44). The amines can then be converted to fluorophores by exposure of the tissue samples to formaldehyde vapour (generated from paraformaldehyde). The amount of water available during this reaction is a factor which determines the yield of fluorophores and the degree of diffusion of the fluorophores. The tissue samples are then embedded in paraffin wax, sectioned and mounted in a medium such as Entellan (Merck) before their examination in a fluorescence microscope.

Fluorescence is an optical phenomenon in which radiation is absorbed by a substance (fluorophore) and is almost instantaneously re-emitted as light of a longer wavelength. The change in wavelength is known as the Stokes shift. (89) The molecules of a fluorophore become excited by absorbed light, i.e. the energy of each molecule becomes higher than its normal or ground state. When an excited molecule returns to the ground state, surplus energy can result either in heat or fluorescence, or is used in a photochemical reaction. (89) An excitation spectrum of a fluorophore is recorded by continuously changing the wavelength of the exciting light, while measuring the total intensity of the fluorescence or the intensity of a constant part of

the fluorescence. (89,97) The excitation spectrum of any fluorophore is identical with its absorption spectrum which is a plot of energy of absorbed light against wavelength. (97)

The emission spectrum is recorded by keeping the wavelength and intensity of the exciting light constant while scanning the intensity of the spectral distribution of the emitted fluorescence.

For most substances the spectral distribution of the emitted light is constant for a given molecular structure of a fluorophore, and is independent of the wavelength of the exciting light. (97) The emission can be expressed in defined units, e.g. energy or quanta per unit wavelength interval.

The Zeiss large fluorescence microscope is suitable for examination of formaldehyde-induced fluorescence. An HBO 100 Osram high-pressure mercury lamp can be used as a light source, and before the light reaches the specimen it is passed through 'exciter' filters which only transmit certain wavelengths, which are selected to correspond approximately to the excitation spectrum of the fluorophore to be examined. Systems involving transmitted light illumination or vertical illumination can be used. The emitted fluorescence passes through "barrier" filter(s) before reaching the eye. A barrier filter prevents the excitation light from reaching the eye, by only transmitting the longer wavelengths of the emitted fluorescence.

(b) Preparation of tissue for freeze-drying.

The animal is killed and the tissue samples are dissected as soon as possible. It has been reported that no differences in the fluorophores from brain amines have been observed in animals killed with a blow on the neck without previous anaesthesia, by decapitation during light ether anaesthesia, or by removing the brain from a living animal anaesthetized with Nembutal. (44) Good results have also been obtained from slaughter house preparations as much as 60 min. after death. (44) However it is clear that the time between killing and freezing of the tissue, and the conditions affecting the tissue

during this interval (e.g. temperature, or whether the brain is removed from the skull) could be important variables affecting the histochemical observations. It has been reported (69) that initial cooling of brain samples stabilizes axonal membranes, which reduces spontaneous release of monoamine transmitters.

Freeze-drying implies the removal of water from frozen tissue by sublimation (usually in a partial vacuum), at a temperature below the freezing point of the tissue.

In order to avoid the formation of ice crystal artefacts while the tissue is being frozen, the tissue is cooled rapidly to below its freezing point. This process is known as quenching. The growth of ice crystals ceases at temperatures below $-50^{\circ}C$ and if the tissue is frozen sufficiently rapidly to below this temperature, only sublightmicroscopic ice crystals are formed. (44) Quenching inhibits the postmortem chemical reactions in tissue samples known as autolysis; however histological changes such as cytoplasmic vacuolation have been described in tissue quenched within 30 secs. of killing. (88) When tissues are in a solid state, diffusion of substances is reduced. (88)

If quenching is insufficiently rapid, ice crystals of varying size, visible by a light microscope, may form from water in cell cytoplasm and in tissue spaces with subsequent precipitation of materials dissolved in such water at the boundaries of the ice crystals. However it has been shown that sufficiently small samples of quenched tissue may have no ice crystal artefacts visible by the light microscope, although the electron microscope has shown that sublightmicroscopic ice crystal formation usually occurs. (88) To obtain the minimum crystal size a large number of nuclei of crystallization are needed. The rate of formation of nuclei of crystallization in a solution varies with temperature as this is a process which increases rapidly as the temperature falls below a critical

level of -39°C. (88) The rate of cooling of a specimen depends on the thermal conductivity of the liquid in the freezing bath, into which the tissue is placed, and on the thermal conductivity of the tissue itself. This last factor depends partly on the size of the sample. It has been observed that the effects of quenching may not be uniform throughout a tissue sample, i.e. there can be an outer zone with minimal ice crystal formation which shows excellent preservation of structure, while inner zones may show relatively large ice crystals and poor preservation of tissue structure. (88) The dimensions of these zones can be altered by changing the temperature of the quenching bath. The size of the sample and the temperature and composition of the quenching bath are therefore crucial variables when considering ice crystal artefacts and preservation of tissue structure.

Another phenomenon to be noted during quenching is the development of cracks in the tissue due to a 2% contraction in volume of the sample which accompanies the cooling process. (88)

A tissue sample should not be placed directly into liquid nitrogen because the transfer of heat from the tissue is much reduced by the formation of a layer of vapourized nitrogen which surrounds the sample, i.e. the thermal conductivity of the freezing medium is low in these circumstances. Freezing is therefore carried out by the use of various liquid intermedia which are themselves cooled by liquid nitrogen and which do not boil when the tissue is introduced.

An example of such a medium is isopentane (88) which has a freezing point of -165°C. It should be noted that the temperature of liquid nitrogen is -195°C and the isopentane must be used before it solidifies. There is no need to measure the exact temperature of isopentane during the quenching process, as the point at which the isopentane becomes noticeably more viscous (at about -160°C) can be chosen for immersion of the tissue

samples. (88) An experiment with a thermocouple in the centre of tissue blocks measuring approximately 5 x 3 x 1 mm, immersed in isopentane cooled in liquid nitrogen, showed that less than 10 secs. were required to reach -150°C. (103)

Specimens as large as a mouse brain have been submitted to the process, of quenching with isopentane, however with samples as large as this severe cracks can cause problems in the future handling of the tissue. (23)

After quenching in a medium such as isopentane, the samples are rapidly transferred to storage below the surface of liquid nitrogen in a separate Dewar flask. The samples may be kept in liquid nitrogen for as long as necessary before being transferred to the freeze-drying apparatus. (44) Tissue can be stored in liquid nitrogen for "several weeks without significant loss of quality." (69)

(c) Freeze drying.

Freeze drying is the removal of water from frozen tissue at a temperature below the freezing point of the tissue. (44) Partial vacuum is usually maintained around the solid tissue samples to enhance vaporization (i.e. sublimation) of its water, and a 'trap' is provided for the continuous removal of the water molecules, e.g. a vessel containing dry phosphorus pentoxide. As has been described, rapid freezing is necessary, (e.g. to approximately -160°C), in order to minimize the formation of tissue-disrupting ice crystals. The subsequent freeze-drying of the samples takes place at higher temperatures, e.g. between -35°C and -70°C, until all except a "small, tightly-bound fraction of their water content has been removed." (88)

In the Speedivac-Pearse Model 1 Tissue Dryer (Edwards High Vacuum Ltd.) (85) the specimen carrier rests on the upper of 2 thermo-electric modules, mounted in series on a water cooled base plate. The temperature of the metal specimen carrier and metal plate on which the specimen carrier rests is measured by a thermocouple and indicated on a meter. A pyrex bell-shaped lid covers the tissues and creates a vacuum chamber.

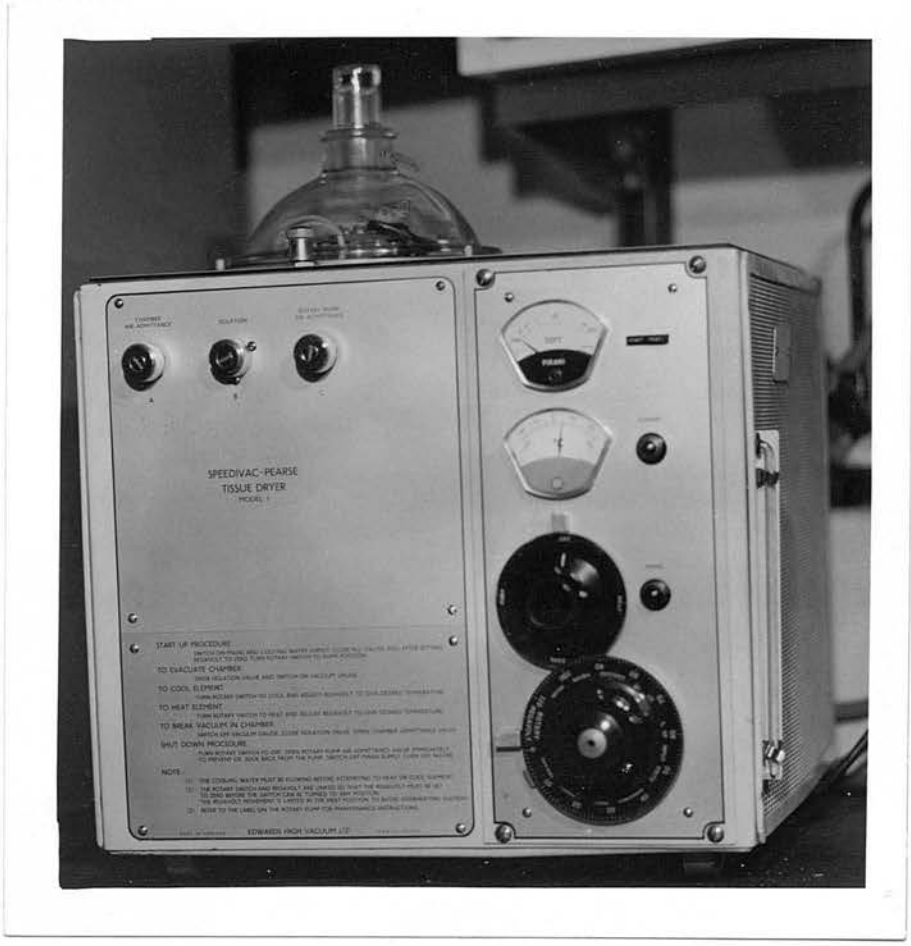


Figure 12. Edwards-Pearse Model 1 tissue dryer. The pyrex glass dome can be seen which encloses the vacuum chamber. The pressure of the vacuum chamber and the temperature of the specimen carrier are indicated on the front of the apparatus.

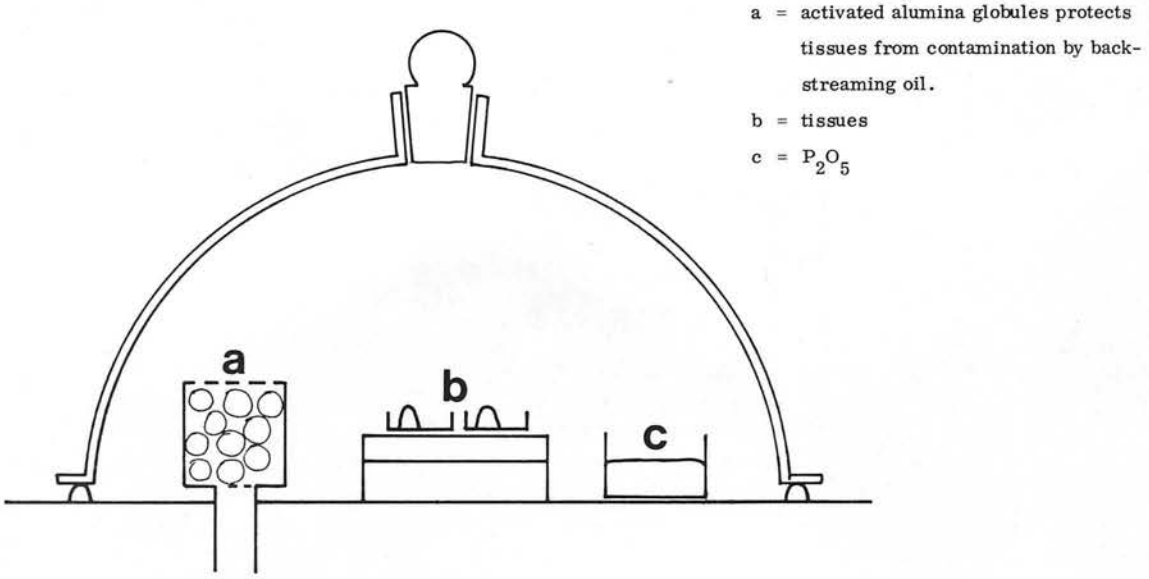


Figure 13. Diagrammatic cross section of the vacuum chamber of the Edwards-Pearse Model 1 tissue dryer. Two tissue samples, each in a specimen carrier are shown. For the sake of clarity a gap separates the specimen carriers from the underlying thermo-electric modules, but during freeze-drying the metal specimen carriers are in contact with the metal surface on which they rest. The structure containing activated alumina globules 'plugs' the opening leading to the oil-containing vacuum pump. The glass cover rests upon a rubber ring with a circular cross-section which lies in a groove (not shown) on the top of the apparatus. The rubber ring is lubricated with special grease (Edwards High Vacuum Ltd.) A vessel containing P_2O_5 which absorbs water vapour derived from the tissue samples during freeze-drying, is also shown in the vacuum chamber.

The transfer of molecules from ice to water vapour (sublimation) removes heat from the environment and unless this is replaced, the temperature of the tissue falls. (88) If it falls sufficiently far, sublimation is very much reduced. Ice sublimes more slowly at -60°C than at -40°C by a factor of X 10. (88) In the Edwards-Pearse Model 1 tissue dryer, heat is applied to the tissue samples both by conduction from the thermo-electric modules and by external radiation. A shell of dried tissue appears at the surface of the specimen and gets progressively thicker. All water vapour must diffuse through this dry barrier and the resistance to drying produced by this shell is considerable. (88) To assist drying there should be a rapid removal of water molecules from the region surrounding the tissue and this is achieved by the maintenance of a partial vacuum around the tissue during freeze-drying. In the case of the Edwards-Pearse apparatus, a vessel containing dry P_2O_5 absorbs water vapour in the vacuum chamber. The molecules of water vapour proceed by multiple collisions between roof and floor of the drying chamber until they reach the surface of the P_2O_5 trap, and the position of the P_2O_5 in relation to the tissue is not critical. (88) In addition the vacuum pump also removes water vapour from the drying chamber.

The pressure in the chamber will fall to .01 - .001 torr during the drying process (the final value will depend on the efficiency of the apparatus). A rise in pressure during the drying process may be seen if the P_2O_5 is saturated. (88) Another variable affecting the vacuum in the Edwards-Pearse apparatus is contamination of the oil in the vacuum pump which absorbs some water vapour. Air ballasting of this oil during the freeze-drying process (by passing air through the oil) improves the efficiency of the apparatus with regard to the removal of water vapour and this is reflected by a decreased vacuum pressure. (88) It has been reported (85) that there is no obvious further improvement in drying time or specimen quality if the minimum pressure falls below 0.01 torr.

A major problem is to decide when the end point of the drying process has been reached. Some of the variables affecting the period of time required are the type of tissue, the dimensions of the tissue samples, the mechanical efficiency of the apparatus, the number of tissue samples being processed, the amount and area of exposed surface of P_2O_5 in the drying chamber, any periods of air ballasting of the oil in the vacuum pump and the temperature of the tissue samples. One investigation incorporated a torsion balance in the vacuum chamber, by which the drying process could be followed until a constant weight was reached. (65) Other workers have assumed that if the vacuum remains stable after the vacuum chamber is isolated from the pump, the end-point has been reached. (88)

When the drying process is judged to be complete the temperature of the tissue samples is raised by re-setting the temperature of the thermo-electric modules of the Edwards-Pearse tissue dryer. This avoids condensation of atmospheric water upon the specimens following their removal from the vacuum chamber. Falck and Owman (44) reported that the temperature of the tissues should be raised "well above room temperature", however other workers consider that heating the samples to 20°C is sufficient. (Laszlo, personal communication.) However the importance of this and most other variables of this method is likely to vary with the nature of the problem under study. After removal of the hygroscopic tissues from the tissue dryer, they undergo exposure to formaldehyde gas. In the interval between their removal from the tissue dryer and exposure to formaldehyde they should be stored in as dry an atmosphere as possible, such as is produced by drying agents such as silica gel or P_2O_5. (35)

(d) Reaction of fluorogenic compounds with formaldehyde gas:-

Biogenic monoamines such as DA, NA, A and 5HT can be converted to fluorescent compounds by exposure of the freeze-dried samples to gaseous formaldehyde, generated from paraformaldehyde, in a closed vessel. A

suitable degree of humidity is necessary in the reaction vessel, and exposure for 1-2 hr at 80°C is a standard treatment.

Over 25 compounds are known to give formaldehyde-induced fluorescence in tissue or model systems, and it is possible that there are other uninvestigated amines which give rise to some degree of formaldehyde-induced fluorescence and which may be present in the mammalian C.N.S. (89) However only NA, A, DA and 5HT, out of the 25 compounds known to react with formaldehyde, have been found to be present in the mammalian nervous system to a sufficient extent to give rise to histochemically significant formaldehyde-induced fluorescence. The first step in the reaction between a fluorogenic amine and formaldehyde is a condensation known as the Pictet-Spengler reaction. (67) This is a condensation reaction of a β-arylethylamine with a carbonyl compound to yield tetrahydro-derivatives and is facilitated by an increased electron density at the carbon atom where the ring closure takes place. (67) This molecular requirement is fulfilled by 3-hydroxy-β-phenylethylamines and β-(3-indolyl)ethylamines.

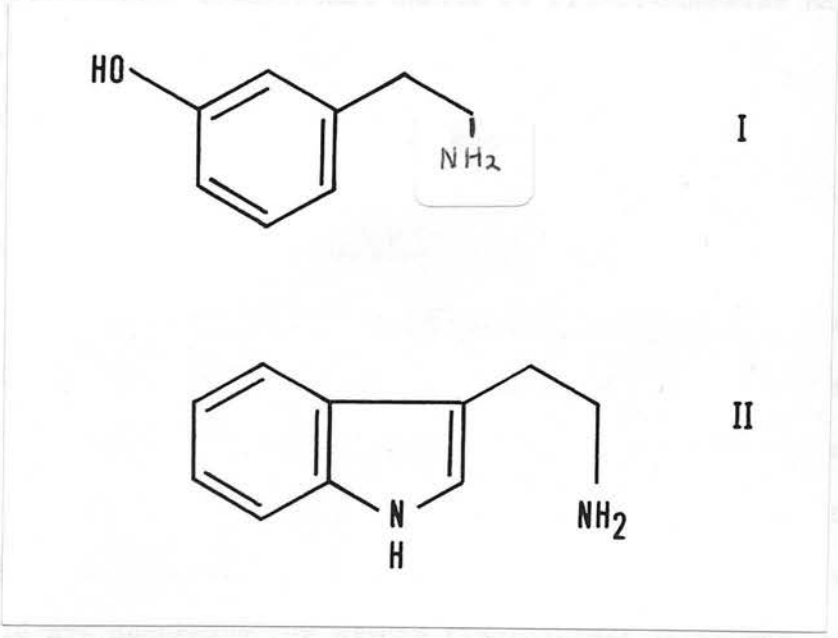


Figure 14. I 3-hydroxy-β-phenylethylamine.

II β-(3-indolyl)ethylamine.

β-phenylethylamines.

With regard to a β-phenylethylamine, an activation of the 3-hydroxy group (para to the site of ring closure) is usually required for histochemically-significant condensation with formaldehyde under the conditions (e.g. 1-2 hr. 80°C) usually employed. (67) The β-phenylethylamine must be either a primary or a secondary amine (or their corresponding amino acids) to be reactive. 3-hydroxy or 3,4 dihydroxy-phenylethylamines and their β-hydroxylated or α-methylated analogs, together with their corresponding aminoacids, react with formaldehyde to a histochemically-significant degree, while certain closely related biogenic compounds such as phenylalanine, tyrosine, tyramine and the deaminated or 3-O-methylated metabolites of catecholamines do not react with formaldehyde at all, or to an

insufficient extent to be of any known histochemical significance. (67) Phenylalanine, which lacks hydroxy groups in the benzene nucleus, and tyrosine or tyramine, which contain a 4-hydroxy group, do not undergo a histochemically-significant degree of Pictet-Spengler condensation during the reaction conditions usually used. The deaminated metabolites of catecholamines do not undergo Pictet-Spengler condensation as they are not amines, while a 3-O-methylated catecholamine gives rise to weak fluorescence compared with a 3-hydroxy β-phenylethylamine. The product of a Pictet-Spengler reaction is then dehydrogenated to its corresponding 3,4-dihydroisoquinoline during the exposure to formaldehyde. The result is a fluorophore in a pH-dependent equilbrium with its corresponding quinoidal form which is also a fluorophore. It has been shown that 6-hydroxy or 6-7 dimethoxy substitutions in the 3,4-dihydroisoquinoline molecule are necessary for strong fluorescence to appear, while the 6-methoxy-7-hydroxy substituted compound exhibits only a comparatively weak fluorescence. A 7-hydroxy group seems to quench the emission of a fluorophore in the quinoidal form. (67)

The following β-phenylethylamines (table 1) have been investigated in model systems, and gave given rise to varying yields of formaldehyde-induced fluorescence. (23,67)

DA (1.0)	N-isopropyldopamine Isoprenaline 3-methoxytyramine
NA (1.0)	Normetanephrine
A (0.4)	m-tyramine
L-DOPA (1.2)	α-me-m-tyrosine
α-me-DA (0.7)	metaraminol (1.7)
α-me-NA (1.0)	m-hydroxyamphetamine
α-me-DOPA (0.4)	4-methoxy-3hydroxyphenylethylamine
3-4,-dihydroxyphenylserine (DOPS) 0.1	3,4,5-trihydroxyphenylethylamine

Table 1. Compounds derived from β-phenylethylamine which have given rise to varying yields of formaldehyde-induced fluorescence. The figures in brackets refer to the relative fluorescent yield for some of these compounds, in dried protein-containing droplets exposed to formaldehyde for 1 hr. at 80°C (23). These values can be compared with the yields given in table 2.

β-(3-indolyl)ethylamines.

β-(3-indolyl)ethylamine reacts with formaldehyde in a Pictet-Spengler condensation to form a tetrahydro-β-carboline. This reaction is facilitated by the activation of the heterocyclic nitrogen in the indole nucleus and unsubstituted β-(3-indolyl)ethylamines are less reactive than those having an OH or CH_3O group in the 5 position of the indole nucleus. (23) The next stage in the reaction with formaldehyde is dehydrogenation of the tetrahydro-β-carboline to a fluorescent 3,4-dihydro-β-carboline. (33) The relative yield of fluorophore from a β-(3-indolyl)ethylamine is low compared with the yield from a 3-hydroxy-β-phenylethylamine, and the former requires more energetic reaction conditions (i.e. time of exposure to formaldehyde and/or temperature) than the latter to result in maximal yield of fluorophore. (67)

The following indolylethylamine derivatives have been investigated in model systems (i.e. protein-containing droplets) and have given rise to varying yields of formaldehyde-induced fluorescence. (23)

Tryptamine	(0.2)
5-hydroxytryptamine	(0.3)
5-methoxytryptamine	(0.2)
6-hydroxytryptamine	(1.3)
5-6-dihydroxytryptamine	(0,3)
α-methyl-5-hydroxytryptamine	(0.2)
N-methyl-5-hydroxytryptamine	
N-methyl-5-methoxytryptamine	
Tryptophan	(0.1)
5-hydroxytryptophan	
5-methoxytryptophan	

Table 2. Indolylethylamine derivatives which have given rise to formaldehyde-induced fluorescence. The figures in brackets refer to the relative fluorescent yield for some of these compounds, (23) and can be compared with the yields given in table 1.

In summary, the available evidence suggests that the structural requirements for a molecule to react with formaldehyde producing a histochemically significant yield of a fluorescent compound are that it must be either a 3-hydroxy-β-phenylethylamine or a β-(3-indolyl)ethylamine, or a derivative of these compounds so long as it is a primary or a secondary amine. However it should be noted that 3-methoxy derivatives of β-phenylethylamine give some yield of fluorophore, although relatively low, and that many other compounds give rise to low yields of fluorophore. Björklund and Falck (21) have stated that "a significant fluorescence that appears when tissues are treated according to the conventional histochemical procedure (reaction at 80°C for 1 hr.) will most probably be due to

substances belonging to these groups", i.e. β-(3-indolyl)ethylamines and 3-hydroxylated β-phenylethylamines. Out of the possible range of fluorogenic compounds DA, NA, 5HT, A and metaraminol (67) have been visualized in the nervous system of animals using the conventional formaldehyde-fluorescence histochemical procedure. However a fluorophore that may be derived from an unknown biogenic phenylethylamine has been observed in the pituitary gland (21), and special reaction conditions (formaldehyde and ozone) have visualized a substance thought to be tryptamine or a closely related β(3-indolyl)ethylamine, also in the pituitary. (21) It is clear, therefore, that although most of the work using the formaldehyde-fluorescence method has been concerned with the transmitter substances DA, NA and 5HT in the mammalian CNS, the formaldehyde-fluorescence method can demonstrate a wide range of aromatic amines, and if possible histochemical observations should be accompanied by chemical analysis.

(e) Further details of the chemistry of the formaldehyde-fluorescence method:-
Fluorophores of β-phenylethylamine derivatives.

If a primary CA, e.g. DA is enclosed in a dried protein layer, as in freeze-dried tissue, and treated with formaldehyde gas having a suitable degree of humidity, the Pictet-Spengler condensation results in the formation of a 6,7-dihydroxy -1,2,3,4-tetrahydroisoquinoline. (33) (This reaction can also occur in solution). (67)

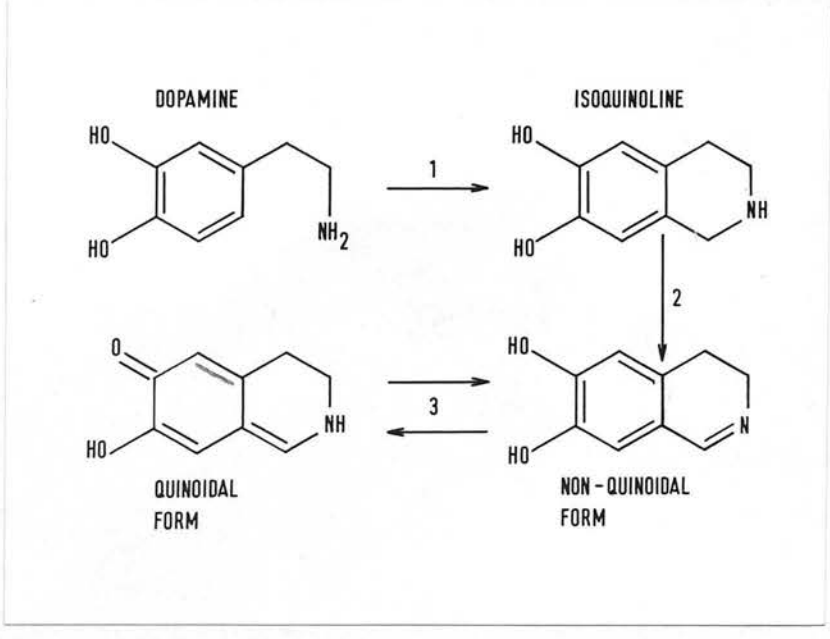


Figure 15. Chemistry of formaldehyde-induced fluorophores derived from DA. 1 = Pictet-Spengler condensation. 2 = Dehydrogenation. 3 = pH-dependent equilibrium.

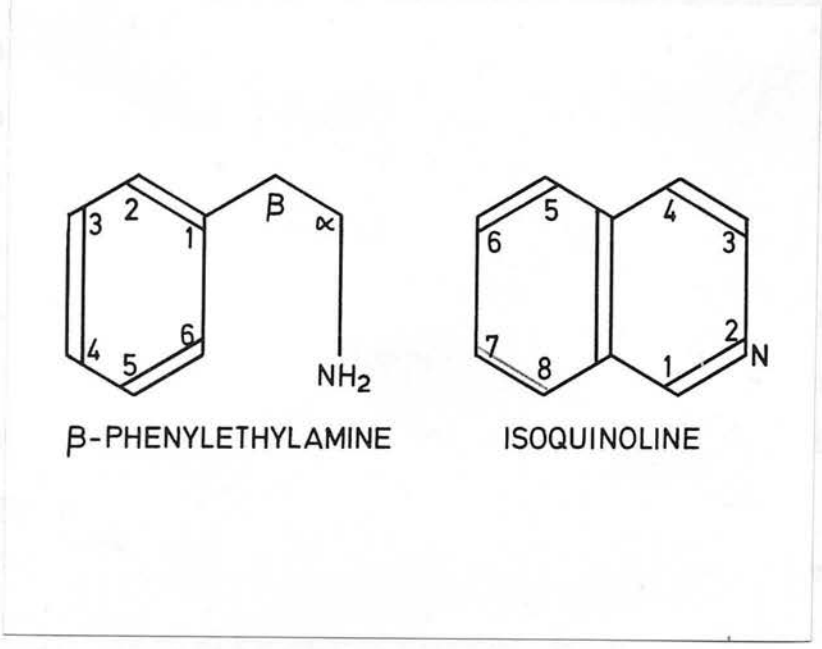


Figure 16. The numbering of chemical rings shown in Figure 15.

During continued formaldehyde treatment, the 1,2,3,4-tetrahydroisoquinoline is dehydrogenated to its corresponding 3,4-dihydroisoquinoline. This reaction is catalyzed by protein. Many proteins can act as a catalyst and this property seems to be linked to certain amino acids. Glycine and alanine have been found to be excellent catalyzers, (33) and the contact with the protein which is present in freeze dried tissue samples provides the necessary catalyzing power. (96) The dehydrogenation reaction seems to be a general reaction in that it is not linked to any specific substitution in the tetrahydroisoquinoline nucleus. (67) The reaction can occur in solution if the amino acids stated above are present.

DA gives rise to its corresponding 6,7-dihydroxy -3,4-dihydroisoquinoline, which is a fluorophore, and is in a pH-dependent equilibrium with the corresponding tautomeric quinoidal form which is also a fluorophore.

Corrodi and Jonsson (33) and Jonsson (67) reported that in the pH range 6-10, for example in freeze-dried tissue, the quinoidal form of the fluorophore derived from DA predominates, exhibiting an excitation maximum at 410 nm and an emission peak at 480 nm. When formaldehyde-treated tissue sections containing DA or NA were exposed to hydrogen chloride, the spectral properties of the fluorophores were changed (i.e. the maximal excitation and emission became 360 and 510 nm respectively), almost certainly due to transition of fluorophores to the non-quinoidal forms which were concluded to predominate in the pH range 1-4. However other reports have given different values for the spectral characteristics noted above, e.g. the quinoidal form of the fluorophore derived from DA has been reported to have emission maxima at 490 nm and 590 nm with an excitation peak at 415 nm, while excitation maxima of the non-quinoidal fluorophore derived from DA have been observed to be at 320 and 365 nm, with an emission maximum between 490-500 nm. (23)

It is interesting to note a recent report (20) that the fluorescence intensity of the fluorophore derived from DA showed a considerable increase (about X 3.5) as it was converted by HCl treatment from the quinoidal to the non-quinoidal state. This contradicts reports by Corrodi, and Jonsson and Jonsson, (33,67) that the quinoidal form of the fluorophore derived from DA, showed the maximum fluorescence intensity associated with a pH of about 8. It has also been stated (69) that an increase in the pH of a fluorophore derived from a CA above 8 results in a decrease in fluorescence intensity.

Although the spectral characteristics of the quinoidal fluorophores derived from DA and NA have been reported to be identical, (67) the non-quinoidal fluorophore derived from NA (4,6,7-trihydroxy-3,4-dihydroisoquinoline) while having identical spectral characteristics to the non-quinoidal form derived from DA, can undergo further reactions during prolonged exposure to HCl. This procedure transforms the quinoidal fluorophore derived from NA to 6,7-dihydroxy-isoquinoline with an excitation maximum at 320 nm. (20)

The 6-7-dihydroxy-3,4-dihydroisoquinoline derived from DA cannot react in this way.

Under certain conditions catecholamine fluorophores can exhibit a shift in the emission maximum from approximately 480 nm to 500-550 nm. (67) This is due to a further reaction which occurs when large amounts of catecholamine are present. It can be prevented by using milder reaction conditions (i.e. less humid formaldehyde gas, lower temperature and shorter incubation time.) Its exact nature is unknown but it may be due to a polymerization and/or oxidation. (67) The hydroxy group in position 4 of the β-phenylethylamine, which corresponds to the 7-hydroxy group in the tetrahydroisoquinoline, seems to be essential for this reaction. (69)

A secondary catecholamine such as A reacts with formaldehyde to form the N-methylated derivative, but the next step of dehydrogenation requires more severe reaction conditions (i.e. a higher reaction temperature and/or a longer incubation time of up to 3 hr. (44)) to give the maximal yield of fluorophore.

The formaldehyde-induced fluorophore derived from L-DOPA has been reported to exhibit the same spectral properties as the fluorophore derived from DA, and it was concluded that its structure is probably 3-carboxy-6,7-dihydroxy-3,4-dihydroisoquinoline and not its decarboxylated product. (67)

The m-tyramines (e.g. m-tyrosine, α-methyl-m-tyrosine, m-tyramine, α-methyl-m-tyramine and metaraminol) have a reaction with formaldehyde analogous to that between a catecholamine and formaldehyde. The fluorescent products formed from the m-tyramines can have two emission maxima, at 420 nm and 510 nm, and the acidity of the protein environment can determine which of them will predominate. Exposure to HCl vapour can change the emission maximum from 510 nm to 420 nm. (67)

Fluorophores of β-(3-indolyl)ethylamine derivatives

Tryptamine and related compounds react with formaldehyde to form tetrahydro-β-carbolines which are then dehydrogenated to a fluorescent 3,4-dihydro-β-carboline. An optimal fluorescence intensity for demonstration of e.g. 5HT is usually obtained either when the formaldehyde is more humid than that used for the demonstration of primary catecholamines or when a reaction time of 1-2 hr. at 80°C is used. (33) The fluorophore derived from 5HT is 6-hydroxy-3,4-dihydro-β-carboline, and has been reported to have maximal excitation/emission at 410/525 nm. (33) Thus 5HT-containing cells can appear yellow compared with a green appearance of the fluorescence derived from DA or NA. However this contrast also depends on several other factors which are to be discussed.

The fluorophores from 5HT or 5HTP may undergo additional reactions under more energetic reaction conditions than those stated above, which can result in changes in the emission spectrum. (67) Only 5 or 6-hydroxy or methoxy-tryptamines have been reported to give rise to strong fluorescence with the reaction conditions as outlined above. Tryptamine and tryptophan have contrasting spectral characteristics (maximal excitation/emission at 360/490 nm) compared with the fluorophore derived from 5HT. (67) Tryptophan develops a relatively very weak fluorescence compared with 5HT, but may contribute to the background fluorescence shown by tissue proteins. (67)

The fluorophores derived from catecholamines and 5HT are thought not to be bound chemically to the surrounding proteins, but enclosed in a protein "network". (67) The fluorophores are not significantly extracted by organic solvents or alcohols, and the enclosure in protein can be reinforced by prolonged exposure to formaldehyde or the use of formaldehyde with a higher humidity. (33)

The relative yields of formaldehyde-induced fluorescence intensity from several compounds in dried protein droplets exposed to formaldehyde gas for 1 hr. at 80°C were shown in tables 1 and 2, and Jonsson (66) has reported similar results.

(f) The humidity associated with the formaldehyde-fluorescence reaction:-

The reaction involving the formation of formaldehyde-induced fluorophores, by treatment of freeze dried tissue with formaldehyde gas derived from paraformaldehyde, requires the presence of water. The amount of water present during the reaction affects the intensity of the resulting fluorescence, and also the degree of diffusion of the fluorophores.

The role played by water is uncertain but it may increase the reactivity of the formaldehyde gas and may influence the pH of the surrounding proteins. (59) It is also possible that small amounts of water cause intraneuronal diffusion of biogenic amines thus reducing concentration quenching of the resulting fluorescence. (59,69)

Water for the reaction is derived from three sources:- the tissue, the air initially enclosed in the reaction vessel and the paraformaldehyde used. (60). Freeze dried tissue readily absorbs water from the air and must be stored in a dry atmosphere during the intervals between the end of freeze drying and exposure to formaldehyde, and between exposure to formaldehyde and embedding. The amount of water remaining in the tissue after freeze-drying can differ for different types of tissue. (69) Variables such as the size of the tissue sample may also affect the degree of residual water.

Water may also be derived from the air which is initially enclosed in the reaction vessel. For example the air enclosed in a vessel with a volume of 1 litre would contain about 9 mg of water at 21°C if the atmosphere had a relative humidity of 50%. (59) Effects of this variable could be minimized by controlling the relative humidity and temperature of the laboratory.

However the paraformaldehyde is the most important source of water (60,59), some of which is released by pyrolysis of the polymer. In addition, water which has been absorbed by the paraformaldehyde evaporates into the reaction vessel. During storage, paraformaldehyde takes up or loses water until an equilibrium is reached which is dependent on the temperature and relative humidity of the atmosphere of the storage vessel. The amount of absorbed water can be varied by storing paraformaldehyde, at room temperature, in closed vessels containing aqueous solutions of sulphuric acid of varying densities. The density of sulphuric acid determines the degree of relative humidity in the storage vessel. (60)

Figure 17 shows how aqueous solutions of sulphuric acid are prepared.

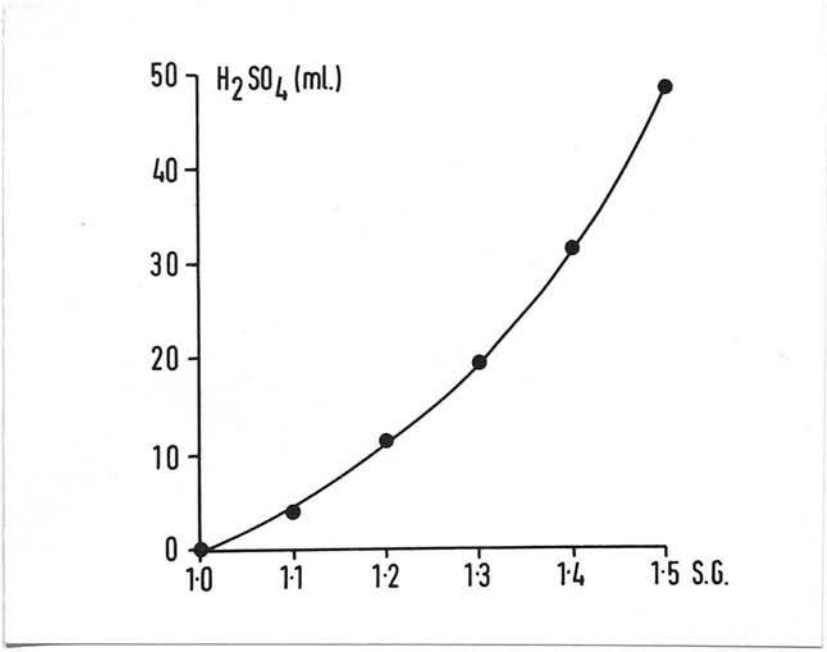


Figure 17. The volume of sulphuric acid which must be added to 50 ml of water to give solutions of specific gravity shown. (From Handbook of Chemistry and Physics. 31st Ed. Ed: Hodgman. Chemical Rubber Co.,Cleveland, Ohio, 1949).

Several reports have suggested that 5-6g of paraformaldehyde is a suitable amount for a reaction vessel of 1 litre. (59,69,89) Weighed amounts of paraformaldehyde, e.g. 5g are placed in glass containers and stored in a sealed storage vessel containing the aqueous solution of sulphuric acid needed to provide the required relative humidity. (Relative humidity is the ratio of the quantity of water vapour present in the atmosphere to the quantity which would saturate at the existing temperature.) It has been reported that when paraformaldehyde was dried for a week in a vessel containing phosphorus pentoxide, the water content was below 0.05% of the weight and after equilibration in an atmosphere of 50% relative humidity, the water content was about 0.55%. (59) However, paraformaldehyde is not usually dried before equilibration. (59) There have been various suggestions as to the length of time for the equilibration of the absorbed water content of paraformaldehyde:- i.e. at least a week (60), 10 days (59), 5-7 days (44) and 10 days or more. (89) Another study (59) showed that uptake of water by dried paraformaldehyde, over sulphuric acid at a relative humidity of 50-70% at 22°C, showed that about 90% of final water content was achieved after 5 days, but that final equilibrium had not been reached even after 25 days.

It should be noted that the temperature during equilibration is an important variable. For example an increase from 21°C to 24°C has been reported to produce an increase in the amount of absorbed water by 30% in 10 days. (59)

The amount of absorbed water in 6g of paraformaldehyde containing 0.8% water, would be about 50 mg. About the same amount can be derived from the pyrolysis of 6g paraformaldehyde at 80°C for 1 hr. (59) It is likely that paraformaldehyde is the most important source of water in the formaldehyde-fluorescence method, and since the water from the pyrolysis is probably constant, (59) the variation in absorbed water is the crucial factor. The effects ofvariation of absorbed water on the histochemical results are shown in Figure 18.

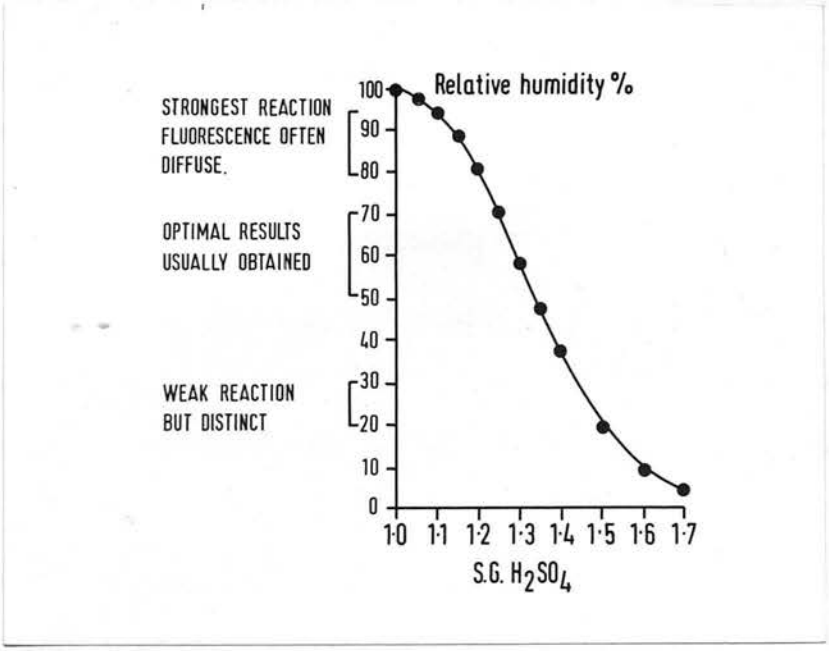


Figure 18. The relationship between specific gravity of aqueous sulphuric acid solution and the relative humidity of the atmosphere in a closed vessel containing sulphuric acid solution. (59) About 5g paraformaldehyde are equilibrated for about a week in a storage vessel containing sulphuric acid solution associated with a selected relative humidity, and used to generate formaldehyde in a reaction vessel of 1 litre. Some reported (44,60) effects of changes in the relative humidity on the intensity and diffusion of the formaldehyde-induced fluorophores derived from biogenic amines in freeze dried tissue are described.

It has also been reported (59) that a high water content of paraformaldehyde may induce additional reactions resulting in changes in the spectral characteristics of the resulting fluorophores. Another report (96) suggested that if paraformaldehyde is equilibrated with air above 80% relative humidity, the fluorescence yield may decrease.

Different types of tissues have been found to require paraformaldehyde with different water contents for optimal reaction. (60,44) Therefore the results associated with a range of absorbed water content should be examined for each region under investigation, in order to select the optimal degree of humidity in the paraformaldehyde storage vessel.

Another report has described a variant on the usual method of formaldehyde treatment. (69) An initial exposure of the tissue to formaldehyde generated from paraformaldehyde with a relatively low water content can result in the enclosure of amine and fluorophore in the protein network so that subsequent treatment with paraformaldehyde which has a higher water content will produce optimal intensity of fluorescence accompanied by less diffusion of the fluorophore than would have occurred without the pretreatment.

It has also been noted that formaldehyde-induced fluorophore in a model system of protein droplets showed the strongest intensity of fluorescence if the droplets were stored in a dry atmosphere before examination, while an increase in the humidity of the surrounding air during a period of storage decreased the intensity of fluorescence. (97,96)

(g) <u>The effects of HCl gas on formaldehyde-induced fluorophores</u>.

It has been reported (25,23) that if minute amounts of HCl gas are added to a reaction vessel containing paraformaldehyde and freeze dried tissue, the Pictet-Spengler condensation reaction is catalyzed. This

procedure was carried out by introducing air, which had been saturated with HCl, into the reaction vessel. The amount of HCl in the reaction vessel was a critical factor in determining the final fluorescence intensity, and different compounds which enter the Pictet-Spengler reaction showed different degrees of increased fluorescence following HCl catalysis. The acid-catalyzed reaction showed a good specificity for indolylethylamines (e.g. tryptamine) and 3-hydroxylated or 3-methoxylated phenylethylamines. The yield from tryptamine was increased by a factor between 20 and 200.

In addition, exposure of deparaffinized tissue sections, from formaldehyde treated tissue, to HCl gas can affect the intensity (21,20) and spectral characteristics (41,66,20,33) of formaldehyde-induced fluorescence. The intensity of fluorescence of the fluorophores derived from DA and NA has been reported to increase after acidification of the tissue sections (20), although another study using a model system of dried protein droplets, found that the fluorophores derived from a primary CA showed maximal intensity when derived from solutions at pH7-8. (66) The quinoidal form of the fluorophore from NA is initially transformed to its non-quinoidal form (4,6,7-trihydroxy-3,4-dihydroisoquinoline) during HCl treatment of deparaffinized tissue sections, but upon prolonged HCl treatment it is transformed to 6-7-dihydroxyisoquinoline (20) which has contrasting spectral characteristics and yield compared with the non-quinoidal form. This explains the differences in yield and spectral characteristics between the fluorophores derived from DA and NA after prolonged exposure of deparaffinized tissue sections to HCl gas. It has also been reported (21) that if tryptamine-containing tissue sections, which have been prepared by the formaldehyde-fluorescence method, are exposed to HCl gas, a marked increase in the fluorescence intensity of the fluorophore derived from tryptamine is produced.

The quinoidal form of the fluorophores derived from DA and NA in tissue sections have identical spectral characteristics. However prolonged exposure of the tissue sections to HCl gas can lead to the histochemical differentiation of their fluorophores which undergo different reactions as outlined above. The quinoidal form of both fluorophores have been reported to show excitation/emission maxima at 410/480 nm, while their non-quinoidal forms exhibited excitation/emission maxima at 360-70/510. (33,41) After prolonged exposure to HCl gas the spectral characteristics of the fluorophores derived from DA were unchanged, while the excitation maximum for the NA fluorophore changed to 320-30 nm. (41,20) The changes caused by exposure to HCl were reversible by exposure to NH_3 vapour. (41)

(h) The effect of ozone on formaldehyde-induced fluorophores.

It has already been noted that catalysis of the Pictet-Spengler reaction by HCl caused a marked increase in the fluorescence derived from tryptamine and several other compounds. In addition exposure of formaldehyde-treated tissue sections to HCl gas produced an increase in fluorescence derived from tryptamine.

If, during the formaldehyde treatment, the air in the reaction vessel is enriched with ozone, there is also a marked increase in the fluorescence derived from tryptamine. (21) This reaction seems to be specific for this compound (23) and in the case of a fluorophore derived from a CA or 5HT, ozone causes a reduction in the final intensity of fluorescence. (22)

(i) Photodecomposition of formaldehyde-induced fluorophores.

The formaldehyde-induced fluorophores, when excited with ultraviolet light, undergo photodecomposition with loss of fluorescence intensity. (33) The rate of fading increases with an increasing intensity of irradiation, and some recovery of the fluorescence intensity may occur if the specimens are stored in the dark, especially at low temperatures. (89) If photodecomposition leads to the production of additional fluorophores, the spectral characteristics of the fluorophore under study may change. (89)

The rates of fading for 3,4-dihydro-β-carbolines (e.g. derived from 5HT) have been reported to be approximately twice as fast as those for 3,4-dihydroisoquinolines (e.g. derived from NA or DA). (33,89) However details of the light source were not given in these reports. In another study, the fluorophore from 5HT faded faster than those from DA and NA, during illumination with a HBO 200 Osram mercury lamp. (44) Although there has been one report of a 5-10 second delay in the onset of fading of formaldehyde-induced fluorophores (96) other workers (106) have not observed any such latent period.

The rates of fading of several fluorophores in a model system of dried protein-containing droplets have been investigated following irradiation at their maximal excitation wavelength with a xenon lamp. (66) In this study, DA and NA fluorophores showed a similar rate of fading to about 90% of initial intensity after 8 min. in contrast to the 5HT fluorophore which faded to about 45% of initial intensity in this time. The fading of the fluorophores of several synthetic 3,4-dihydroisoquinolines and 3,4-dihydro-β-carbolines was also investigated. 6,7-dihydroxy-3,4-dihydroisoquinoline (derived from DA), 6-hydroxy-7-methoxy-3,4-dihydroisoquinoline and 6-methoxy-7-hydroxy-3,4-dihydroisoquinoline, showed slower fading rates than that from 6,7-dimethoxy-3,4-dihydroisoquinoline. All these fluorophores faded more slowly than those from 6-hydroxy-3,4-dihydroisoquinoline, 3-methyl-6-hydroxy-3,4-dihydroisoquinoline, 6-methoxy-3,4-dihydro-β-carboline and 1-methyl-6-methoxy-3,4-dihydro-β-carboline.

Using a model system of dried protein-containing droplets, the fading rates of other fluorophores have also been investigated, (20) during irradiation with a XBO 75 Xenon lamp at the maximal excitation wavelength of each fluorophore. The quinoidal and non-quinoidal forms of the fluorophore derived from DA showed a similar degree of fading, while the 6,7-dihydroxyisoquinoline formed from NA (by prolonged exposure of the formaldehyde-treated specimen to HCl gas) showed a faster fading rate than

the quinoidal fluorophore derived from NA.

It should be noted that the fading rates of 2 fluorophores with different excitation spectra, following irradiation at each maximal excitation wavelength using a xenon lamp, will differ from those obtained using a mercury arc lamp, (which has an emission spectrum different from a xenon lamp) with a constant exciter filter.

(j) The effect of sodium borohydride on formaldehyde-induced fluorescence.

The specificity of formaldehyde-induced fluorescence derived from β-phenylethylamines and β-(3-indolyl)ethylamines can be tested by treating tissue sections with an alcoholic solution of sodium borohydride. (77) Fluorophores derived from the above compounds are reduced to nonfluorescent derivatives, which can be reconverted to fluorophores by a second exposure to formaldehyde gas. Autofluorescence in tissue sections does not show any marked changes associated with sodium borohydride treatment. (67) The proof of the specificity is the regeneration of fluorescence after reduction, i.e. extraction of the fluorophores by the solvent must be excluded as the cause of the loss of fluorescence.

(k) Autofluorescence.

Most tissues contain structures which exhibit autofluorescence which has varying spectral characteristics. (44) Connective tissue (collagen, elastin) and lipofuscin show strong autofluorescence and proteins containing tryptophan, tyrosine and phenylalamine, if excited in the range 250-280 nm also show autofluorescence. (89) The autofluorescence of collagen and elastic fibres is reported to have emission peaks between 460 and 470 nm and between 470 and 480 nm respectively before and after formaldehyde treatment. (97) Differentiation of (non-specific) autofluorescence from formaldehyde-induced (specific) fluorescence may be attempted in several ways:- (44)

(i) It has been said that autofluorescence tends to be more stable when exposed to ultraviolet light compared with specific fluorescence, although there are exceptions. (77,44)

(ii) Autofluorescence is shown in specimens which have not been treated with formaldehyde, and may be diminished by treatment with formaldehyde gas. (44)

(iii) Autofluorescence remains unaffected by drugs which affect the concentrations of biogenic amines.

(iv) Sodium borohydride treatment does not have any significant effect on autofluorescence.

(1) Embedding.

The tissue samples are impregnated with de-gassed paraffin as soon as possible after exposure to formaldehyde. This is carried out in a partial vacuum which results in a more complete and rapid penetration of the tissue. (23) Paraffin wax of various melting points have been recommended, e.g. 50°C (89) 52-54°C (23) and 56°C (77). The wax should not be heated to above 60°C (77). The samples are still hygroscopic after formaldehyde treatment, and should be kept in a dry atmosphere in the interval prior to embedding. (44) It has been reported that hot paraffin wax can extract some of the formaldehyde-induced fluorophores in certain regions. (23) The time needed for evacuation and infiltration of the tissue samples is likely to be affected by the apparatus used, and the nature and size of each sample. One report (77) suggests an evacuation period of 30 min and an infiltration time of 10 min. Until an equilibrium between tissue and the partial vacuum is obtained, the longer a tissue sample is evacuated, the shorter is the infiltration time. It would seem that the embedding time should be constant if comparisons are to be made between tissue samples, due to the possibility of some extraction of fluorophores.

(m) Sectioning and mounting.

After each sample is contained in a 'block' of paraffin wax, it is serially sectioned into 'ribbons', usually at a microtome setting for section thickness in the range 7-15 μm. (77) However "a microtome setting cannot be relied upon to give more than a very approximate indication of section thickness". (89) If holes occur in the sections during cutting, it may be due to poor impregnation with wax. (77)

Representative sections are placed onto clean glass slides. Water cannot be used to flatten the sections as it has an immediate quenching effect on formaldehyde-induced fluorescence (77) and several alternative methods for flattening the sections have been suggested. One method is to partially flatten the sections using a camel hair brush before placing the slides on a hot plate at a temperature "very near the melting point of the wax" (77) e.g. 50°C. (23) The paraffin wax is allowed to soften thus further flattening the sections. However it has been stated that the sections should not be heated too strongly as this appears to increase the intensity of the autofluorescence. (77) The paraffin wax is allowed to reharden before mounting. Other workers have floated the sections in several liquids, e.g. acetonitrile or liquid paraffin (77,31) to produce the necessary flattening. When it is planned to use the sections for further histochemical processing, requiring firm adhesion of the section to the slide, the slides must be smeared with albumin solution and allowed to dry before receiving the sections. (77)

The flattened sections can be studied under certain low power objectives without mounting but sections are usually mounted under coverslips. (77) Various mounting media have been suggested, e.g. Entellan (Merck) and De Pex (Gurr). Liquid paraffin and Fluormount (Gurr) have also been used. (77) If the fluorophore derived from A is to be studied, the sections must not be mounted in a medium containing xylene which extracts the fluorophore. Liquid paraffin can be used in this situation. (44)

It has been suggested that the newly-mounted slides should be returned to the hot plate for 20 min if the sections have been mounted in Entellan or for 30 min if liquid paraffin has been used as a mounting medium. (44,23,35) This is designed to dissolve the paraffin wax in the mounting medium, (Xylene can be added to Entellan and has been reported to assist this process).

(n) Storage of formaldehyde-treated tissue, following embedding.

The effects of storage of the embedded tissue before examination by the fluorescence microscope are not well understood and may vary depending on the nature and distribution of the fluorophore under study.

It has been stated (35) that paraffin wax blocks can be stored in the dark and in a "dry place" for at least 2-3 months without significant effect on the histochemical observations, and that sections which have not been de-paraffinized may be stored in a "dry place", in darkness, for a few weeks. (44,35) There are conflicting reports on the effect of storage after the sections have been mounted. A considerable decrease in fluorescence intensity after 24 hrs. has been reported (44) but it has also been stated that mounted sections can be stored in darkness for 5-7 days without an obvious decrease in fluorescence intensity. (35) It seems that whatever the state of the tissue, any storage should be in darkness, as exposure to light causes a decrease in fluorescence intensity. (45)

Another report (23) states that paraffin wax blocks of CNS tissue can be stored for a few weeks before examination and that formaledhyde-induced fluorescence in mounted sections shows fading over a period of a few days, associated with an increase in non-specific background fluorescence. These changes were reported to be diminished by storage of blocks or sections in darkness and at a reduced temperature.

(o) Fluorescence microscopy

The source of exciting light

A Xenon lamp (e.g. Osram XBO 1600W) and high pressure mercury lamps (e.g. Osram HBO 100 or HBO 200) have been used as light sources. (66,44)

Mercury lamps have strong emission at certain specific wavelengths and have sufficient arc stability for use in experiments involving photometric measurements of the fluorescence. (80) Zeiss (Oberkochen/ West Germany), recommend the HBO 100 super-pressure mercury lamp as being particularly well-suited as a light source for photometric measurements of fluorescence. (80) Mercury-pressure lamps require a warming up period of about 15 min before full emission intensity is reached. (110) A Xenon lamp together with a prism or grating monochromator is suitable for studies of excitation spectra. (89)

Other light sources such as a quartz-iodine lamp, a cadmium lamp and a carbon arc have also been used. (110)

Filters

Excitation filters are placed between the light source and the specimen, while barrier filters are placed in the light path between the specimen and the eye.

Glass filters are of two types, one type usually having a bell shaped transmission curve, which is suitable for exciter filters. (89) The second type, which contains crystals within the filter, has a longwave region of high transmission and a shortwave region of opacity separated by a cut-off region. A filter of this type is suitable for use as a barrier filter and is subject to some inconsistency in manufacture. (89)

In addition, interference filters may form part of the optical arrangement in the fluorescence microscope, acting as exciter and/or barrier filters. This type of filter has an interference coating which transmits mainly in a narrow band and which usually has small holes in the coating which transmit some unwanted light. An interference filter can be made for all wavelengths, whereas glass filters have a limited range of transmission characteristics. (89)

Method of illumination

Both transmitted illumination and epi-illumination may be employed in the fluorescence microscope. Epi-illumination causes the exciting light to reach the specimen through the objective, and has advantages compared with transmitted illumination for quantitative studies of the intensity of fluorescence:- (89)

(i) Correct focusing of the objective guarantees correct focusing of the condenser. Using transmitted light, variations in focusing the condenser may cause marked differences between sections in the intensity of the exciting light and therefore in the intensity of fluorescence.

(ii) Only the areas being examined need be irradiated. Unnecessary irradiation increases the possible error in the recorded intensity of fluorescence due to photodecomposition.

An epi-illumination system is illustrated in Figure 32. A beam-splitter is placed above the objective and reflects part of the exciting light to the specimen as well as transmitting part of the emitted fluorescence to the observer. If the beam-splitter has 50% transmission and 50% reflection at all wavelengths, only 50% of the exciting light is transmitted. For greater optical efficiency the beam-splitter should be a dichroic mirror which has variable transmission depending on the wavelength of the incident light. It has been calculated that if a dichroic mirror has 100% reflectivity with regard to the exciting light and 100% transmission of the fluorescence, it would increase the intensity of the observed fluorescence by a factor of 4 compared with a system using a conventional beam-splitter. In practice a factor of about 3 can be obtained using a dichroic mirror. (89)

Pseudofluorescence

Attention has been drawn (108,97) to the fact that, under the fluorescence microscope, apparent fluorescence (pseudofluorescence) often

occurs associated with surfaces or edges of crystals and along the edges of air bubbles. It was found that this was not always fluorescence, but could be due to reflection or diffraction of visible light which had been transmitted by the exciter filters. Although exciter filters are designed to absorb visible light and transmit a band in the ultraviolet range, they are not as efficient as claimed, and it has been reported that "all filters available let pass some visible light". (108) Although the UV light transmitted is much more intense than the visible light transmitted, fluorescence is thermodynamically an inefficient process, and it was suggested that fluorescence and any reflected or diffracted visible light could show a similar degree of intensity.

These observations imply that interfaces (e.g. membranes) separating phases of different refractive indices may show pseudofluorescence and it has been suggested that if fluorescent material is distributed along a membrane (e.g. in a synaptic vessicle) the intensity of adjacent pseudofluorescence may be affected. "It is possible that different apparent intensities of fluorescence might reflect different spatial arrangements rather than a difference in concentrations." (108)

Colour of emitted fluorescence

Although several reports distinguish the colours of the fluorophores derived from a CA and 5HT, the apparent colour of a fluorophore can depend on several factors:-

(i) The eye may not appreciate the true colour if the intensity of the fluorescence is too low. (89)

(ii) If the intensity of fluorescence is altered, while its emission spectrum remains unchanged, the eye can interpret this as a change in colour. This is known as the Bezold-Brücke effect. (97) It has been shown that with the exception of light at three wavelengths (476, 508 and 570 nm), the eye interprets an increased intensity as a positive or negative change in the

wavelength of the light. At high intensities yellow-green or green fluorescence seems to be yellower or yellow. (44,97) This phenomenon is probably responsible for the fluorescence of certain strongly fluorescent CA-containing cells in the superior cervical ganglion of the rat having been misinterpreted as fluorescence derived from 5HT. (97)

(iii) Adaptation of the eye to a certain colour gives an after-image of the complementary colour, which is known as the "sucessive contrast effect." Thus a glance at a blue light may result in the distortion of a colour seen soon afterwards by a sensation of yellow. (97)

(iv) If a colour is seen against a background of the complementary colour, the contrast between the two colours will be exaggerated. This is known as the "simultaneous contrast effect." (97)

(v) The colour will depend on the properties of the barrier filter(s) employed.

Thus if possible, the emission spectrum of fluorescence should be measured to confirm an observed colour of fluorescence. However, the concentration of fluorophore may affect emission spectra, as low and high concentrations of fluorophores derived from CA have been reported to differ in their emission spectra by a few nanometers. (97)

The recording of excitation and emission spectra of fluorophores

An emission spectrum is recorded by keeping the wavelength and intensity of the exciting light constant while scanning the intensity of the spectral distribution of the emitted fluorescence. The 'true' (i.e. undistorted during recording) emission spectra is a defined physical entity characteristic for a fluorophore, and can be expressed in energy or quanta per unit wavelength interval. However the 'true' emission spectrum may be deformed by several factors:- (97)

(i) The transmission of the barrier filter(s) may vary with the wavelength.

(ii) The transmission of other parts of the optical pathway between the specimen and photomultiplier may vary with the wavelength.

(iii) The response of the photomultiplier may vary with the wavelength.

(iv) If photodecomposition of the fluorophore occurs during registration of the spectrum, the emission spectrum may be affected.

(v) Reabsorption of fluorescence will occur in the specimen when absorption and emission spectra overlap, or if a non-fluorescent substance with absorption in the emission range is present in the specimen.

Any distortion due to the factors i-iii can be corrected if an instrument calibration curve is prepared. This can be done by measuring the emission spectrum of a reference fluorophore and comparing it with its known 'true' emission spectrum. (97)

An excitation spectrum would be found by continuously changing the wavelength (but not the energy per unit frequency interval) of the exciting light, while measuring the total intensity of fluorescence or the intensity of emission at a constant wavelength. However, the 'true' excitation spectrum may be affected by variations in the intensity of the light source at different wavelengths. In addition, any variation in the transmission of the optics between the light source and the specimen, at different wavelengths, or any variation in the band width of the exciting light produced by a monochromator, would cause a distortion of the excitation spectrum. (97)

(p) Quantitative aspects of fluorescence microscopy.

Quantitation of fluorogenic compounds by measuring the emitted fluorescence requires approximately linear relationships between fluorogenic compound and yield of fluorophore and between fluorophore concentration and fluorescence intensity. However there are many factors affecting the yield and spectral characteristics of a fluorophore and the relationship between measured fluorescence intensity and fluorophore concentration:-

Reabsorption of emitted fluorescence

Quantitation of fluorogenic compounds is impaired if the emitted fluorescence does not reach the photomultiplier but is reabsorbed, e.g. by the fluorophore, if there is overlap between the excitation and emission spectra. This error can be reduced by measuring the fluorescence at a wavelength where the fluorophore and the rest of the specimen have minimal absorption. (96)

Excessive absorption of exciting light

Quantitation is also impaired if the concentration of fluorophore is so high that the exciting light is completely absorbed before it has reached every fluorophore molecule. This is known as the "inner-filter effect". (95) This error can be minimized by reducing the thickness of the specimen, by reducing the concentration of the fluorophore, (96) or by increasing the intensity of the exciting light. (104)

Concentration-dependent quenching of fluorescence

Concentration-dependent quenching causes a reduction in the intensity of fluorescence as a result of an increase in fluorophore concentration. (96)

The energy of exciting light can be lost during collisions between excited and non-excited fluorophore molecules in solution, while in the solid state, energy may migrate from an excited fluorophore molecule to

adjacent, non-excited, molecules of fluorophore or non-fluorescent material. In addition, energy may migrate from an excited fluorophore molecule before being absorbed by non-fluorescent (or less efficiently fluorescent) dimers or polymers of the fluorophore. This type of absorption is usually accompanied by a change in the excitation spectrum.

Model experiments, using a system of dried protein-containing or sucrose-containing droplets (96,97) have illustrated marked concentration-dependent quenching of formaldehyde-induced fluorophores in the solid state. It was concluded that this phenomenon was caused by energy migration between excited and non-excited fluorophore molecules at a critical concentration. The threshold concentration, above which concentration-dependent quenching occurred, of a fluorophore derived from a CA, was lower than that of the fluorophore derived from 5-HT. When the concentration of NA, DA or L-DOPA in dried protein microdroplets was gradually increased, the resulting formaldehyde-induced fluorescence intensity per unit droplet volume increased proportionally up to concentrations of CA of about 4.5×10^{-2}M (moles per litre of dried droplet volume). Above this level the fluorescence yield decreased, and the linear relationship between amine concentration and fluorescence intensity was destroyed. As the concentration continued to increase, a level was reached above which the fluorescence per unit volume of dried droplet showed a decreasing intensity associated with further increase in fluorophore concentration. The concentration of 5HT at which quenching appeared was about 9×10^{-2}M. The threshold concentrations in the droplets were calculated to be equivalent to 8×10^{3} µg/g wet weight of tissue for CA, and 16×10^{3} µg/g wet weight of tissue for 5HT.

These threshold concentrations of fluorogenic substances in the droplet solutions or in tissue are associated with a nearly maximal yield of fluorescence. It should be noted that the same concentration of amine

treated with a milder set of reaction conditions would give rise to a lower yield of fluorophore and a different value for the threshold concentration. Thus reducing the yield of fluorophore could in certain circumstances abolish quenching. (69)

Effects of changes in concentration of fluorogenic compounds on the yield and spectral characteristics of emitted fluorescence.

Any change in an emission spectrum would be likely to affect the measured fluorescence intensity. As previously described, if large amounts of CA are present during the formaldehyde-fluorescence reaction, additional reactions can occur leading to a shift in the emission peak from about 480 nm to 500-550 nm. (67) In addition, although no evidence of such additional reactions was found in the droplet experiments reviewed in the section on concentration-dependent quenching (96), there was a displacement of emission maxima of spectra derived from catecholamines by about 5 nm towards longer wavelengths, associated with increasing CA concentration in the droplet solutions. (96)

Photodecomposition of fluorophores

Measurements of fluorescence intensity should be made as quickly as possible after illumination to minimize error due to photodecomposition of the fluorophore. Measurement after a fixed period of irradiation has also been suggested as a method of reducing this error. (89)

Quantitative histochemical studies of the monoamine-containing neurones

Several studies of sympathetic NA-containing neurones have been reported, and many of the findings are valid for other monoamine-containing neurones. (69) Electron-microscopical studies have shown that a sympathetic NA-containing neurone contains granules or vesicles which are called "dense core" granules. The largest concentration of these granules is found in "boutons", which are the varicosities associated with the

terminal parts of the axons. There are two types of granule, a smaller predominant type with a diameter of about 500Å and a larger type with a diameter of about 1000Å. (69) An important factor in deterimining the degree of fluorescence quenching is to what extent the fluorophore is concentrated in the granules when finally examined. The fluorescence due to fluorophore which is concentrated in granules could be quenched to a much greater extent compared with a specimen where the fluorophore was dispersed in the cytoplasm.

Changes in the intracellular amine distribution may contribute to the increase in the intensity of intraneuronal fluorescence which is associated with an increase in the water content of the paraformaldehyde. An increase in the humidity of the formaldehyde vapour can cause a diffusion of amine from the granules. (97) (It is possible that the distribution of amine within the neurone may affect the yield of fluorophore, although concentration of amine did not effect the yield of each fluorophore in experiments with protein-containing droplets. (96))

Thus, two important variables may affect quenching of intraneuronal fluorescence:-

(i) The distribution of fluorogenic compound within the neurone before formaldehyde treatment.

(ii) Any subsequent changes in the distribution within the neurone (e.g. from storage granules to intracellular space) or from within the neurone to outside the neurone, as a result of diffusion during the formaldehyde-fluorescence procedure.

A study of NA in the terminal varicosities of sympathetic nerves in the rat iris (68) involved depletion of the endogenous amine stores by reserpine or by α-methyl-p-tyrosine before incubation in H^3-NA. Two types of varicosity were produced experimentally, i.e. where the replenished amine (H^3-NA) was mainly stored in the granules and also where the replenished amine was mainly situated extragranularly in the axoplasm.

In both types of H^3-NA distribution, the fluorescence was found to be proportional to the NA concentration up to a value between 30-40% of the endogenous level, above which concentration quenching of the fluorescence occurred. When the H^3-NA was situated mainly in the intraneuronal storage granules the quenching was associated with a lower concentration when compared with the mainly extragranular distribution. However it was pointed out that it was not known to what extent the distribution of the fluorophores changed during the histochemical procedure.

If concentration quenching is associated with a given set of reaction conditions, it might be reduced in subsequent experiments by using a more humid formaldehyde gas which could cause increased fluorophore diffusion. Alternatively, a drier formaldehyde gas might give a lower yield of fluorophore which could result in an increased distance among the fluorophore molecules, thus reducing the quenching threshold.

However the above study of NA-containing varicosities in the rat iris could not be used to predict the degree of quenching, following similar reaction conditions, in other systems of CA-containing neurones. A linear relationship between fluorescence intensity and NA content up to their normal concentration has been reported for NA-containing varicosities in the cerebral cortex, in contrast to NA varicosities in the hypothalamus which were observed to be associated with a degree of quenching. (69) It has also been reported that the quenching threshold in nerve terminals of the rat nucleus caudatus-putamen and median eminence was found to be higher than in the varicosities of the rat iris. (69) It is possible that amines in different neuronal systems have different degrees of affinity to their storage granules. (69)

Quantitative histochemical studies of formaldehyde-induced fluorescence have also been reported on 5HT in isolated peritoneal mast cells, on NA in cell bodies of sympathetic neurones and on DA in nerve cell bodies. In these studies, concentration quenching did not seem to occur. (23)

The significance of a measured change in fluorescence intensity

It has been shown (67) that within a certain concentration range, certain reaction conditions for fluorophore production can produce an approximately linear relationship between concentration of fluorogenic compound and intensity of emitted fluorescence. At concentrations above this range concentration quenching accounts for the following possibilities:

(i) An increase in fluorescence intensity could represent a decrease, as well as an increase, in fluorophore concentration. (See above, "Concentration-dependent quenching of fluorescence.")

(ii) A decrease in fluorescence intensity could represent an increase, as well as a decrease, in fluorophore concentration. (Above the concentration-quenching threshold an increase in fluorophore concentration can cause a decrease in fluorescence intensity).

(iii) Concentration quenching may mask a change in concentration, i.e. no change in fluorescence may be detected despite a change in fluorophore concentration.

In summary, if a change in fluorescence intensity is observed, it probably reflects a change in fluorophore concentration (assuming the distribution is not changed) although the direction of the change does not necessarily reflect the direction of the change in concentration. However, a linear relationship between fluorophore concentration and fluorescence intensity is possible within a concentration range of fluorophore molecules, for a given set of reaction conditions. This concentration range may

differ between different fluorophores. (69)

Sensitivity of the formaldehyde-fluorescence method

The sensitivity of the method will depend on such factors as the reaction conditions, the optical arrangements, the distribution of the fluorophore and the degree of background autofluorescence. (69)

In protein-containing droplets, primary CAs and 5HT have been demonstrated in concentrations of 0.005-0.001% (w/v), and it has been shown that less than 1 pg NA, evenly distributed in 10,000-20,000 μm^3, of perinuclear cytoplasm, could be readily detected. (33) The amount of NA in the body of a nerve cell in the superior cervical ganglion of the cat, which exhibited strong fluorescence, was calculated to be about 0.4×10^{-6} µg, corresponding to a concentration of 0.1×10^{3} µg/g. wet weight. (33)

Fluorophore "Standards" for microfluorimetry

As the yield of fluorophore depends on many variables, a constant yield is difficult to reproduce in different experiments. In order to determine the intensity of fluorescence which corresponds to a particular amine concentration (within the concentration range giving a linear relationship between fluorophore concentration and fluorescence intensity) reference "standards" of fluorophore have been used. (23) Freeze-dried albumin-containing droplets or freeze dried gelatin cylinders, containing a known amine concentration, have been used as reference standards which undergo processing with tissue samples in each experiment. However both these types of standard may be associated with a different yield of fluorophore, compared with tissue samples.

Fluorescence of uranium glass is subject to fading, but has been found adequate for short term standardization (i.e. hours to days). A fluorescence standard of uranyl glass (Schott GG17) has been used to detect variations in the intensity of the exciting light during microscopy. (95)

(q) Differentiation of formaldehyde-induced fluorophores.

Many differences between the various fluorophores have already been described. Several criteria exist for histochemical differentiation between

the fluorophores derived from CAs and that derived from 5HT. For example:-

(i) There is a difference between their emission maxima. However, a yellowish fluorescence does not always indicate the presence of 5HT, since CAs under certain conditions can be involved in additional reactions with formaldehyde, resulting in maximal emission between 500-550 nm. In addition, although an emission maximum may be at 480 nm., the fluorescence may appear yellow, because of differences in the observed colour due to changes in the intensity of fluorescence. (67)

(ii) There is a difference between their rates of photodecomposition.

(iii) Exposure of tissue sections to hydrogen chloride produces spectral changes typical of the fluorophores derived from CAs.

Recent work has succeeded in differentiating NA and DA in tissue sections on the basis of spectral changes due to exposure of tissue sections to HCl. (20) A primary CA (DA, NA) can be differentiated from secondary CA (A) by several methods (67) e.g. on the basis of their different reaction kinetics with formaldehyde. (67)

In addition, pharmacological analyses can be used for differentiation, e.g. by the administration of drugs which selectively interfere with monoamine metabolism. (59,35)

Statement of the type of observations and measurements to be reported

Several studies in this thesis investigated the nigro-striatal system of dopamine-containing neurones in the mammalian CNS, using the formaldehyde-fluorescence method. In addition, the effects of administered L-DOPA on the CNS and the functional activity of the nigro-striatal system were also examined. The main type of result which is reported consists of measurements of diffuse formaldehyde-induced fluorescence in the neuropil of several brain regions. Tissue samples of the nucleus caudatus-putamen, cerebral cortex and cerebellum from the rat and cat were examined.

In addition the "blood-brain barrier" to administered L-DOPA was investigated in the rat, cat and man. In some experiments, biochemical estimations of DA in the CNS, or of blood levels of administered L-DOPA, were carried out in addition to histochemical observations. Several methodological problems were also examined.

Section A

The formaldehyde-fluorescence histochemical method. Methodology in relation to the study of the nigro-striatal dopamine-containing neurones and of the fate of administered L-DOPA in the brain.

Examination of diffuse parenchymal formaldehyde-induced fluorescence. (A-E)

Methodological variables affecting the investigation of formaldehyde-induced fluorescence. (F-J)

Introduction

A description of the methods and equipment used.

Killing of animals and dissecting of tissue

Rats and cats were killed by decapitation under chloroform or pentobarbitone sodium anaesthesia. The vault of the skull was then removed with bone forceps thus exposing the brain which was extracted. Dissection was carried out without prior cooling of the tissue samples. A varying delay of up to 2 hr. occurred between killing of the animals and freezing of the tissue samples, depending on the number of animals which had to be dissected. However, all the animals in each experiment were killed over a short period (less than 5 min.) and all the dissected samples were frozen simultaneously. Thus the interval between killing and freezing of the samples was almost identical for all the samples in each experiment, although the interval between removal of the brain from the skull and freezing of the samples was not constant.

The dissected samples were placed in stainless steel trays and stored in a closed vessel, containing moist filter paper, so that the surface of the tissue would remain moist until quenching. The thickness of the coronal slices of the rat cerebral hemisphere was approximately 3 mm.

Quenching

The tissue samples were arranged in two stainless steel trays, specially made to fit onto the platform in the vacuum chamber of the Edwards-Pearse Model 1 tissue dryer. The two trays were subdivided by metal strips so that each tray had four compartments. Two samples were placed in each compartment so that a total of sixteen tissue samples could be processed in one experiment.

The trays were then placed on a specially-made tissue holder, prior to immersion in isopentane, cooled in liquid nitrogen.

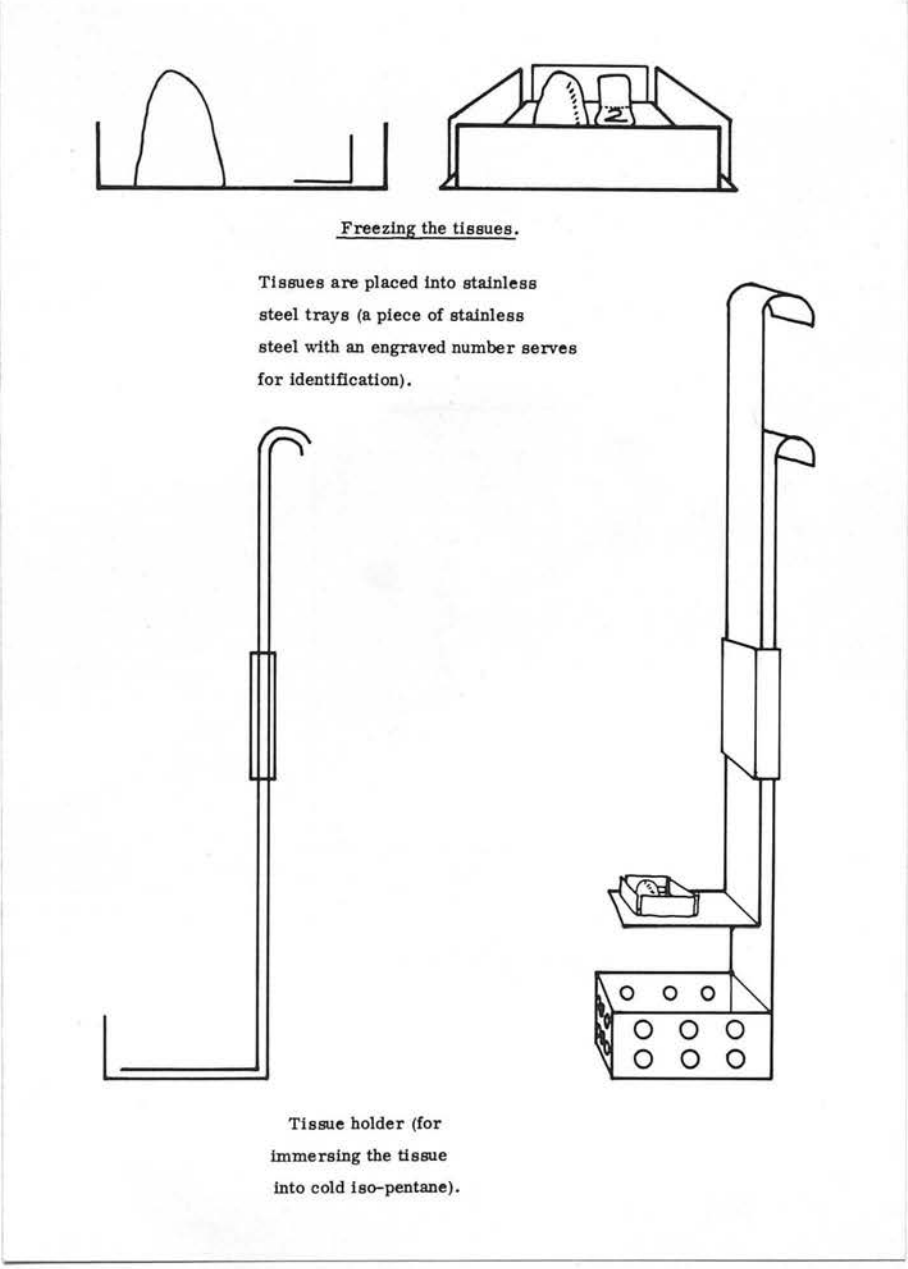
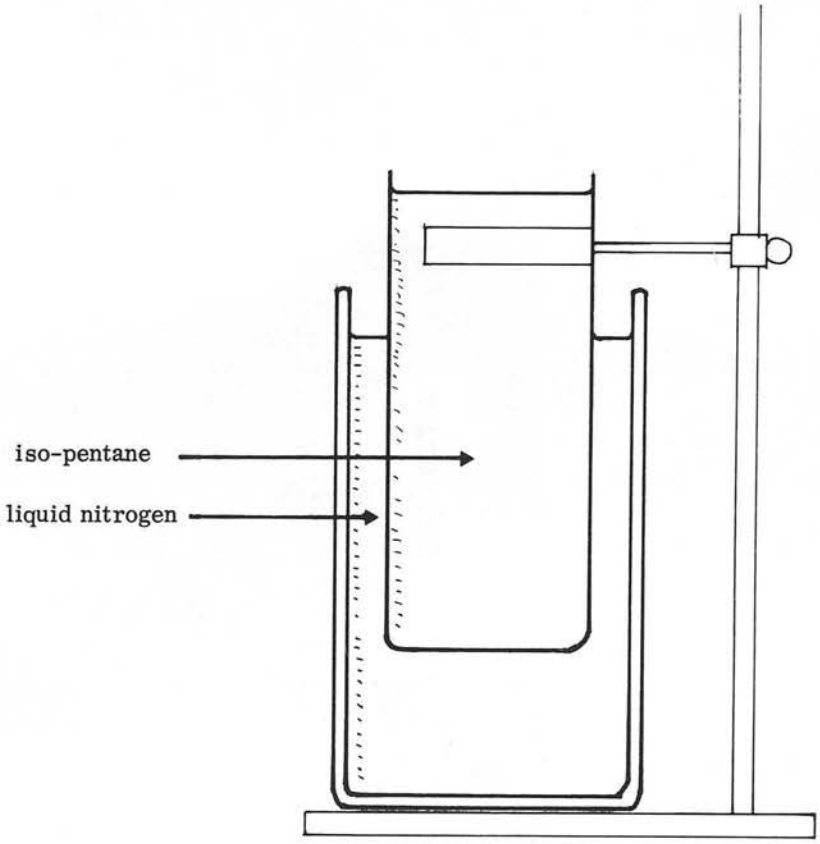


Figure 19. Diagram of a tray containing a tissue sample and of a tissue holder for immersing the tissue in isopentane. (A tray for a single sample is illustrated.)

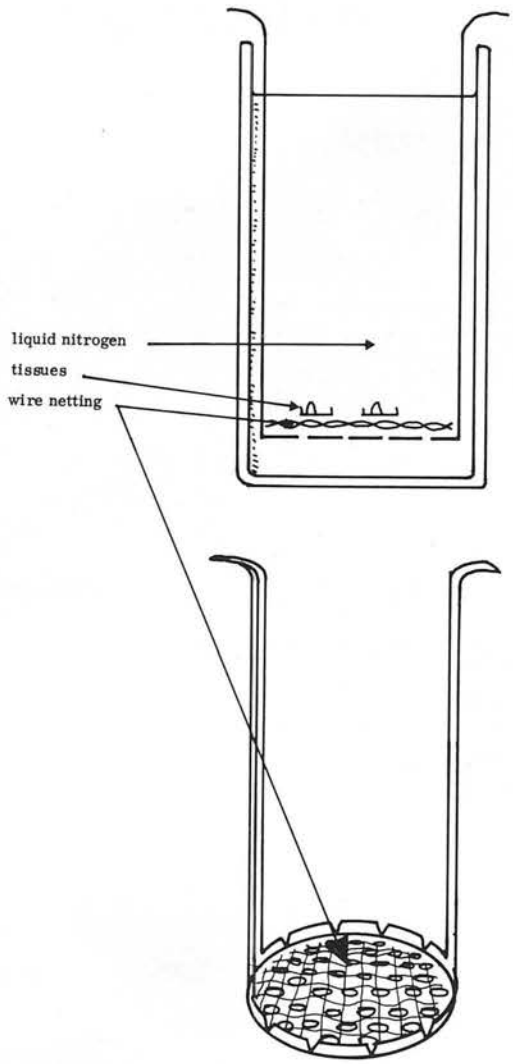


iso-pentane cooled
with liquid nitrogen.

Figure 20. Method of cooling isopentane in liquid nitrogen.

About 50 ml of isopentane were placed in a pyrex glass container and lowered into liquid nitrogen contained in a Dewar flask. This produced boiling of the surrounding liquid nitrogen, and when this had subsided the trays containing the tissue samples were lowered, by means of the tissue holder, into the isopentane. After immersion for approximately 15 seconds, the trays were removed and placed on another specially-made tissue holder

(figure 21), prior to immersion in liquid nitrogen contained in another Dewar flask.



tissues in liquid nitrogen in a Dewar flask (Wire netting diminishes the adhesive effect of frozen iso-pentane between the metal surfaces, which may cause delay during the removal of trays from the Dewar flask.

Figure 21. Tissue holder for storage of trays, containing tissue samples, in liquid nitrogen.

The construction of the trays containing the tissue samples is shown in figure 19. Spaces were provided at the corners where the isopentane in the tray could drain out as soon as the tray was removed from the quenching medium. The time between removal of the tray from isopentane and immersion in liquid nitrogen was about 5 seconds. The trays were stored in liquid nitrogen for varying periods, up to 2 hrs., before transfer to the Edwards-Pearse Tissue Dryer.

Freeze-drying

The thermo-electric modules of the Edwards-Pearse Model 1 Tissue Dryer (figures 12,13) were cooled to -35°C before the trays were transferred from storage in liquid nitrogen onto the platform provided in the vacuum chamber. Evacuation of the chamber was immediately started and was continued for varying periods, between 4 and 5 days, depending on the number of tissue samples in the experiment. At the end-point of the freeze-drying process a vacuum of 0.001 torr had usually been achieved. The end-point was taken to be after 4 days if 0.001 torr had been reached after 3 days, otherwise freeze-drying took place for 5 days.

At the end of the freeze-drying process the thermo-electric modules were gradually brought to a temperature of 20°C over a period of 1½ hrs. After a further period of 30 mins., the trays were removed from the Tissue Dryer and rapidly transferred to a glove box containing air at a relative humidity of about 10% (figure 22).

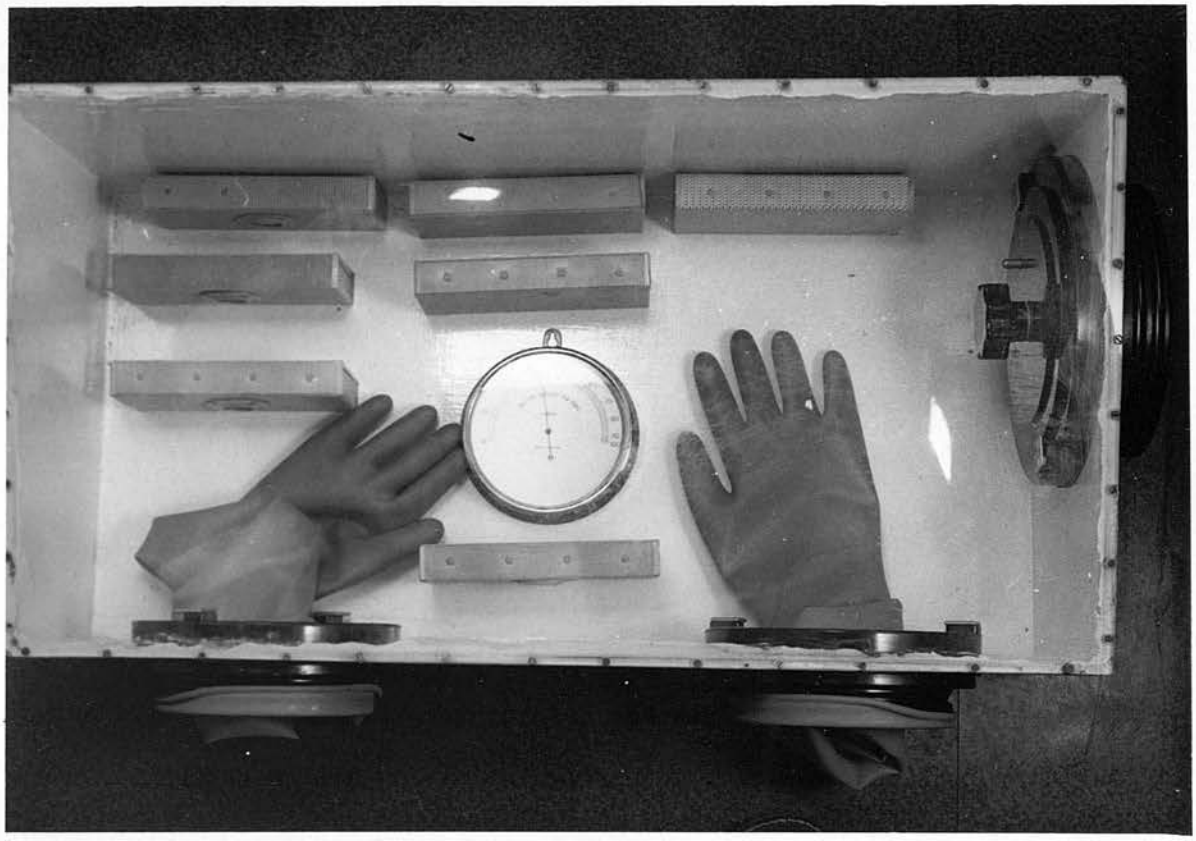


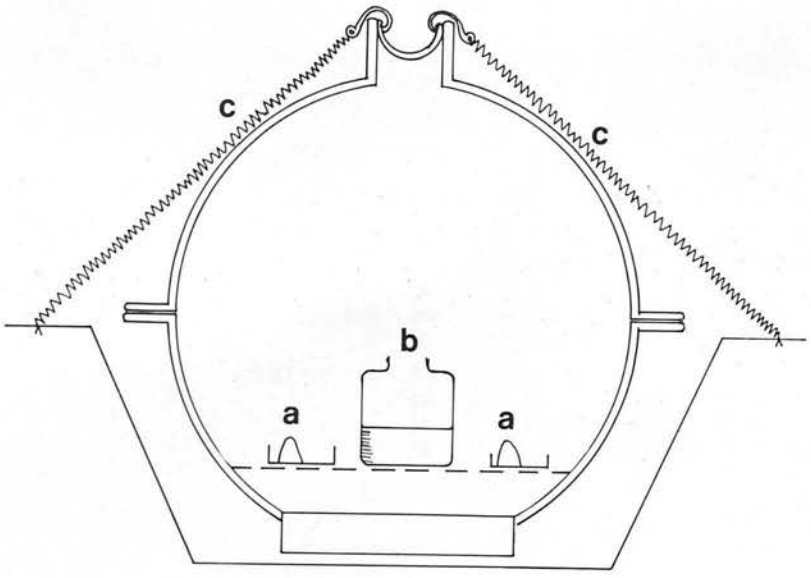
Figure 22. Glove box with hygrometer and containers filled with previously dried silica gel which produced a relatively dry atmosphere for storage of the hygroscopic tissue samples.

As soon as possible, i.e. usually within 5 minutes, the samples were exposed to formaldehyde gas.

Exposure of tissue samples to formaldehyde gas

The tissue samples were placed in compartments created by plastic cylinders sewn onto a nylon mesh (which was attached to a wire frame) in a reaction vessel which contained a measured amount of paraformaldehyde. The reaction vessel was not introduced into the glove box, therefore the air enclosed in the reaction vessel had the relative humidity of the atmosphere in the laboratory at the time.

Figure 23. Desiccator containing compartments for tissue samples, together with a measured amount of paraformaldehyde.



Formaldehyde treatment.

a = tissues
b = paraformaldehyde sample
c = top of the desiccator in which the formaldehyde treatment is performed, secured with springs during heat treatment.

Figure 24. Diagram of reaction vessel for the exposure of freeze-dried tissue to formaldehyde gas.

Several reports have recommended the use of 5g of paraformaldehyde for each litre of the reaction vessel. The above reaction vessel had a volume of 215 cc. and each measured amount of paraformaldehyde was 1.075g. The paraformaldehyde had been routinely stored (at room temperature, approximately 20°C) for a minimum of 10 days, in a dessicator containing sulphuric acid diluted to give rise to a relative humidity in the contained air of 58%. (See General Introduction) The effect of this level of relative humidity had been compared with those of relative humidities of 88% and 26%. The relative humidity of 58% was selected for routine use because it gave a greater yield of fluorescence in the striatal neuropil, compared with the other levels.

In each experiment all the tissue samples were contained in the same reaction vessel while being exposed to formaldehyde gas generated from the paraformaldehyde during heating in an oven at 80°C for 1 hr. The top of the reaction vessel was secured by springs (figure 24) during heat treatment, to minimize sudden movements of the reaction vessel due to escape of enclosed gas.

The tissue samples were placed on nylon mesh so that each specimen would have the maximal surface area exposed to formaldehyde gas.

After formaldehyde treatment the samples were replaced in the glove box, where they were stored for periods up to 1 hr. before embedding in paraffin wax.

Embedding

The next stage involved infiltration of each tissue sample, in turn, by paraffin wax (Gurr) with a melting point of 54°C.

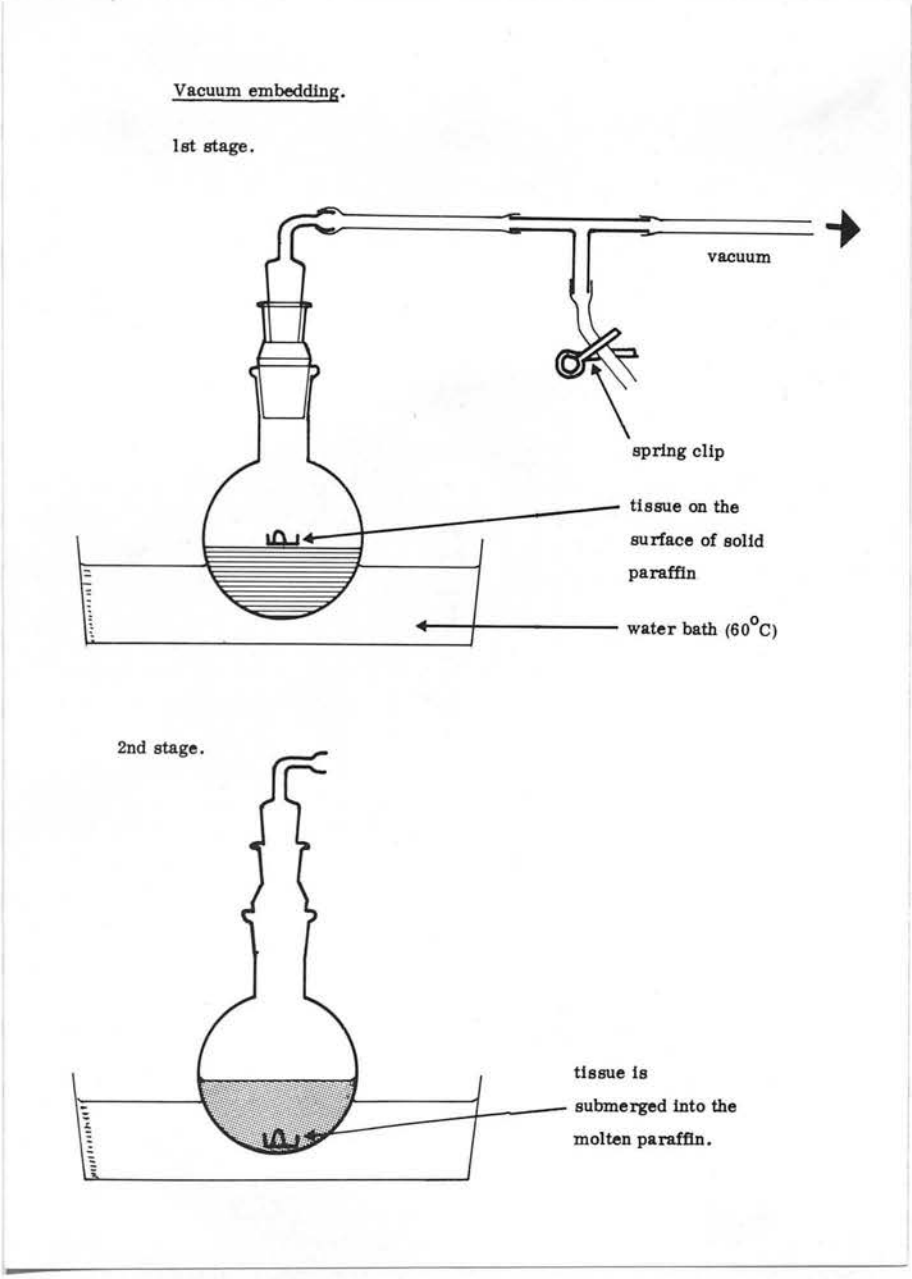


Figure 25. Vacuum embedding.

Each tissue sample was placed on the surface of solid paraffin wax contained in a flask which was then partially evacuated for 2 minutes by an oil-less vacuum pump. This initial evacuation decreased the time needed to infiltrate the tissue with paraffin wax.

The paraffin wax was then melted as shown in figure 25, causing the tissue to be submerged. Approximately 5 min. were needed for infiltration of each tissue sample which was then incorporated into a paraffin wax block. The blocks were stored in darkness before sectioning.

Sectioning, storage of sections and mounting.

Each block was sectioned on the day after embedding. A Leitz microtome was used, usually having a setting of 8 um for the section thickness. The ribbons of adjacent sections were stored between two adjacent cardboard slide trays (i.e. in darkness) which were stacked inside non-airtight plastic boxes. The time between sectioning and mounting the sections varied but the sections were mounted within 5 days of sectioning unless otherwise stated.

As the sections could not be flattened using a water bath, 2 or 3 adjacent sections were placed on a glass slide and trimmed as shown in figure 26. The slide was then put onto a hot plate for a few seconds. The length of time on the hot plate had to be judged so that the paraffin wax became moderately soft but did not melt. The next step involved flattening the softened sections by rolling them with a stainless steel rod.

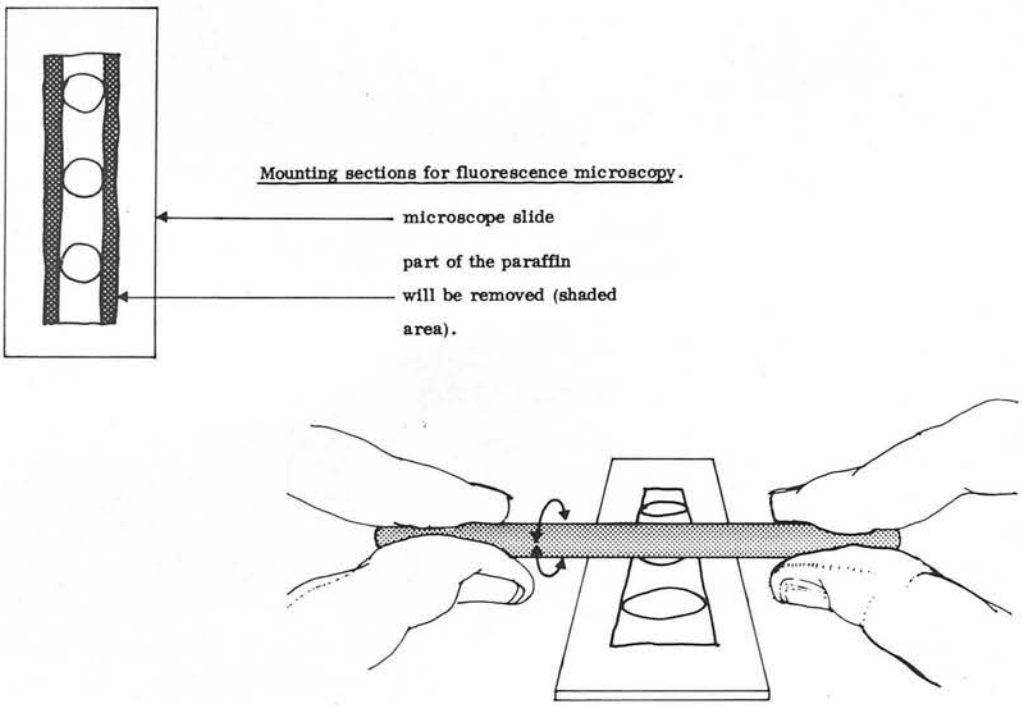


Figure 26. Flattening sections during mounting.

The slides were then returned to the hot plate until the paraffin wax was seen to melt, thus increasing the flattening of the sections.

The sections were then mounted in Entellan (Merck) as shown in figure 27.

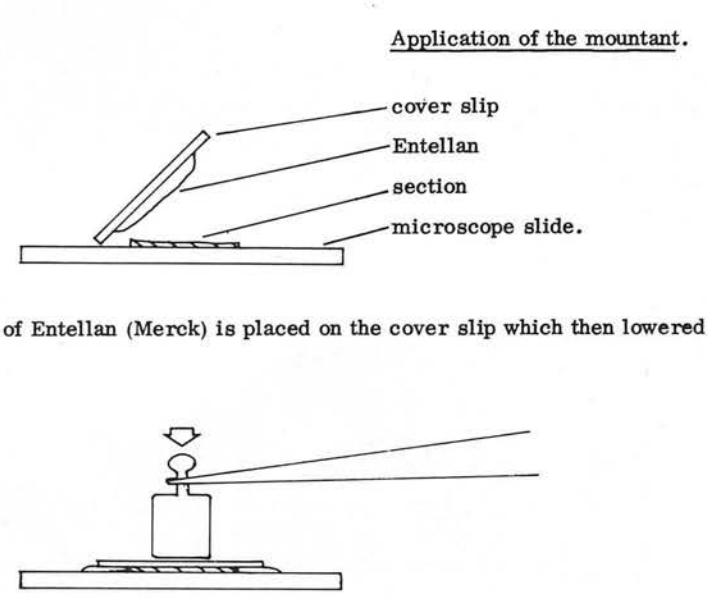


Figure 27. Mounting sections.

Entellan contains about 75% by weight of toluene and some workers have added xylene before using Entellan as a mounting medium. However satisfactory results can be obtained by using Entellan without added xylene (Eränkö, reported personal communication) and xylene was not added to Entellan in the studies to be reported.

Although it has been suggested that mounted sections should be returned to a hot plate for 15-30 minutes, I found that this procedure did not produce any detectable change in the appearance or measured intensity of formaldehyde-induced fluorescence or autofluorescence. This step was therefore omitted. (It should be noted that this procedure has been reported to affect the intensity of non-specific 'background' autofluorescence.)

The sections, mounted in Entellan, were stored for 1 day (unless otherwise stated), in darkness, before examination with the fluorescence microscope.

Staining selected sections with Luxol Fast Blue and Cresyl Violet

Figure 28 is an illustration, from König and Klippel's Stereotaxic Atlas of the rat brain, of a coronal section of the hemisphere about 0.5 mm. behind the mid-sagittal part of the anterior commissure. The myelin sheaths are reproduced in white and can be contrasted with the darker grey matter.

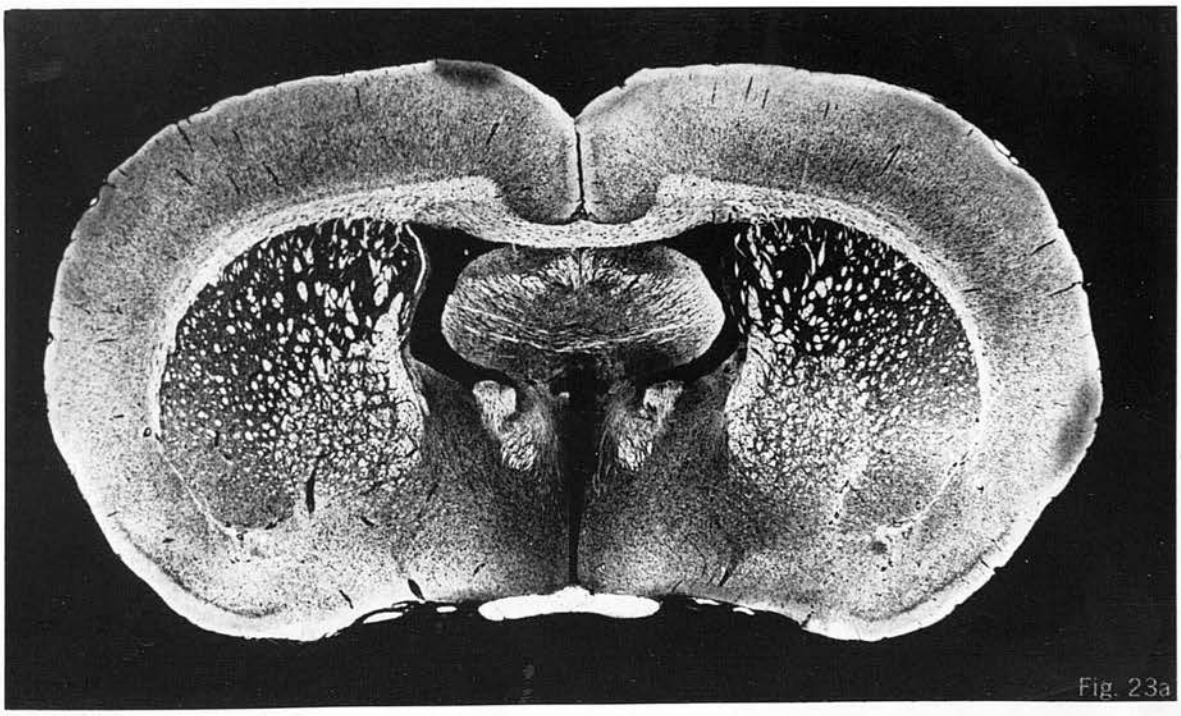


Figure 28. Coronal section of rat brain. Illustration from König and Klippel's Stereotaxic Atlas of the rat brain. The sections had been stained with Sudan Black B and the photographic negatives were reproduced so that the myelin sheaths are reproduced in white.

In the studies to be reported, the myelin sheaths were distinguished from the grey matter by staining with Luxol Fast Blue and Cresyl Fast Violet. After a tissue sample had been serially sectioned, several sections representing different parts of the sample were stained so that identification of the different parts of the sample was possible, using König and Klippel's Stereotaxic Atlas.

Glass slides were covered with a thin layer of adhesive solution prepared from equal parts of egg albumin solution and glycerine. (The albumin solution was prepared from 0.5g dried albumin, 0.5g NaCl and 100 ml. distilled water. (76)) The tissue sections were placed in contact with the slides using a water bath at 40°C for flo_tation, and the slides were then tilted from the horizontal which promoted the drainage of excess water from beneath the sections. The excess water was absorbed using filter paper. The slides were then placed in a vertical position in a staining rack and air dried for a further 1 hr. before staining with Luxol Fast Blue and Cresyl Fast Violet. (79) The myelinated fibres appeared greenish-blue and darker than the less heavily stained reddish coloured grey matter. (figures 29,30)

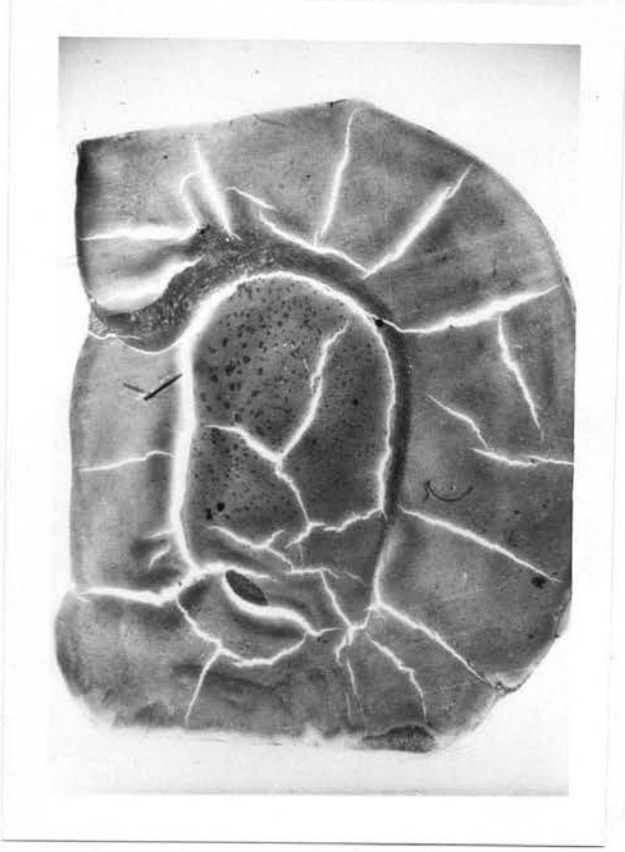


Figure 29. Coronal section of rat cerebral hemisphere. The tissue section, 8 μm in thickness, was prepared by the formaldehyde-fluorescence method, and stained by Luxol Fast Blue and Cresyl Violet. The section corresponds to figure 14 in König and Klippel's Stereotaxic Atlas. The corpus callosum and anterior commissure are clearly shown as heavily stained areas. The corpus callosum surrounds the nucleus caudatus-putamen in which the intensely stained bundles of fibres of the internal capsule are clearly seen. X 18.

Figure 30. Coronal section of rat cerebral hemisphere. Prepared as in figure 29. The section corresponds to figure 33 of König and Klippel's Stereotaxic Atlas. The corpus callosum, internal capsule, fimbria and mamillo-thalamic tract can be seen as more heavily stained areas. The part of the nucleus caudatus-putamen in this section can be seen lateral to the internal capsule in the lower left region of the figure. X 18.

Thus, different parts of each serially-sectioned tissue sample were identified.

The fluorescence microscope

The Zeiss Large Fluorescence Microscope was used, with microphotometric accessories.

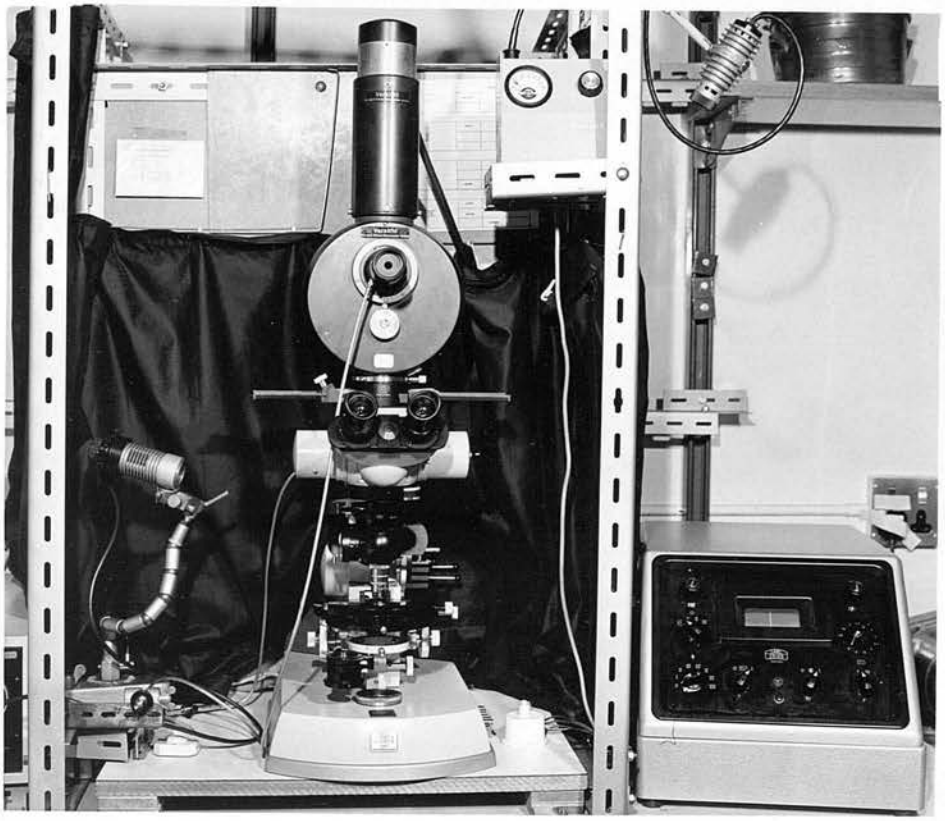


Figure 31. Zeiss Large Fluorescence Microscope with microphotometric accessories. The display unit which registered the intensity of emitted fluorescence is on the right of the photograph.

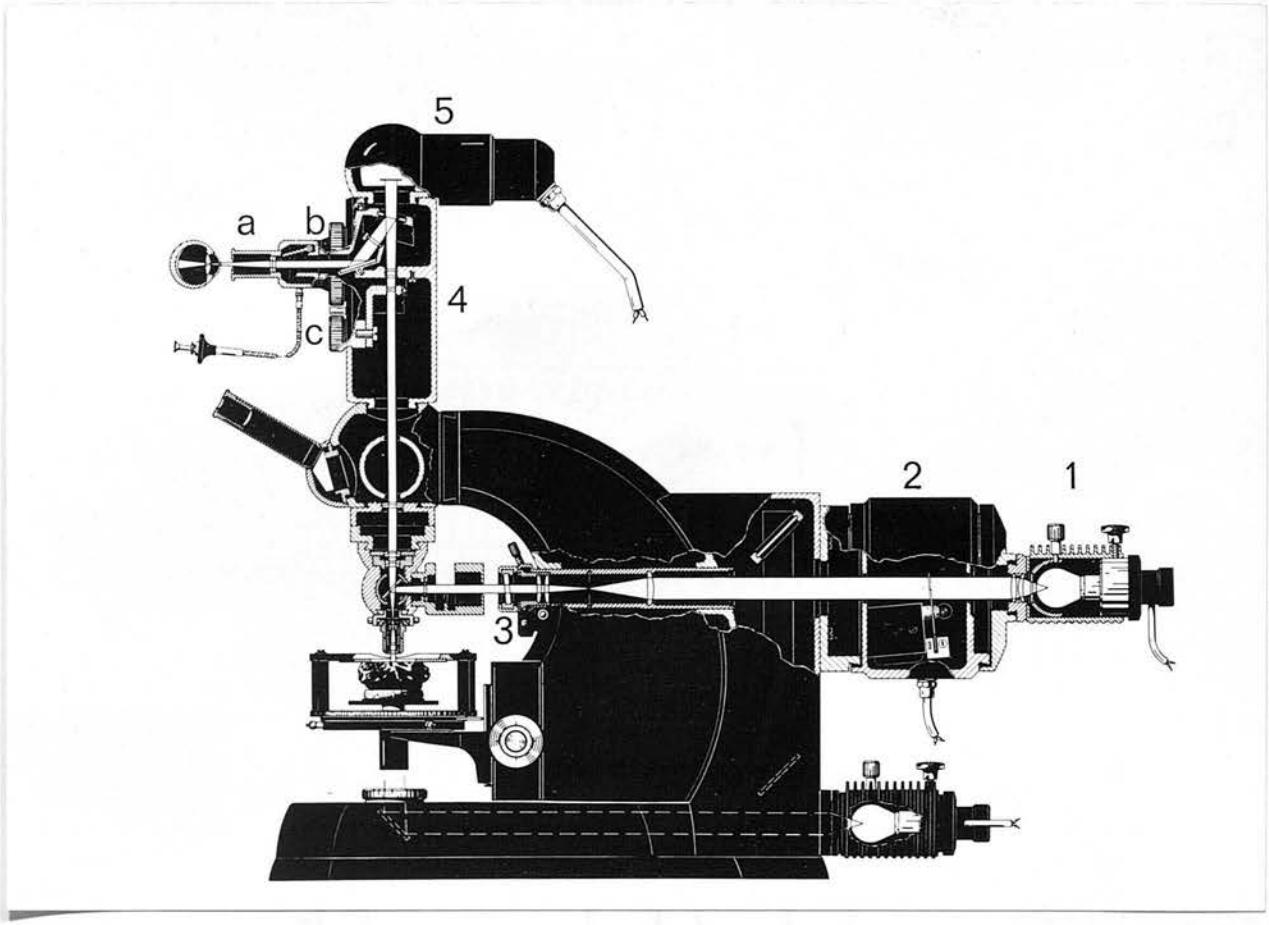


Figure 32. Zeiss Large Fluorescence Microscope with microphotometric accessories. Path of rays with vertical illumination.

The instrument shown in figure 31 had the following characteristics which differ from those illustrated in figure 32:-

(i) An HBO 100 Osram mercury lamp was the light source.

(ii) Before reaching a point equivalent to "3" (figure 32) the course of the light beam had been modified because of the presence of an automatic camera in the body of the microscope.

(iii) The photomultiplier was not that which is shown in figure 32.

However, figure 32 illustrates the main features of the optical system which was used. The exciter filters were situated between "3" (figure 32) and the beam splitter above the objective, while the barrier filters were situated above the beam splitter.

The light source was a stabilized HBO 100 W/2 Osram mercury arc lamp with a mean life of 200 hrs. This has been described (80) as having an "extremely good arc stability, being particularly well suited as a light source for photometric measurements of fluorescence." (The high tension unit had a magnetic voltage stabilizer.) After switching on the lamp there was a delay of about 15 min. before it became fully "warmed up".

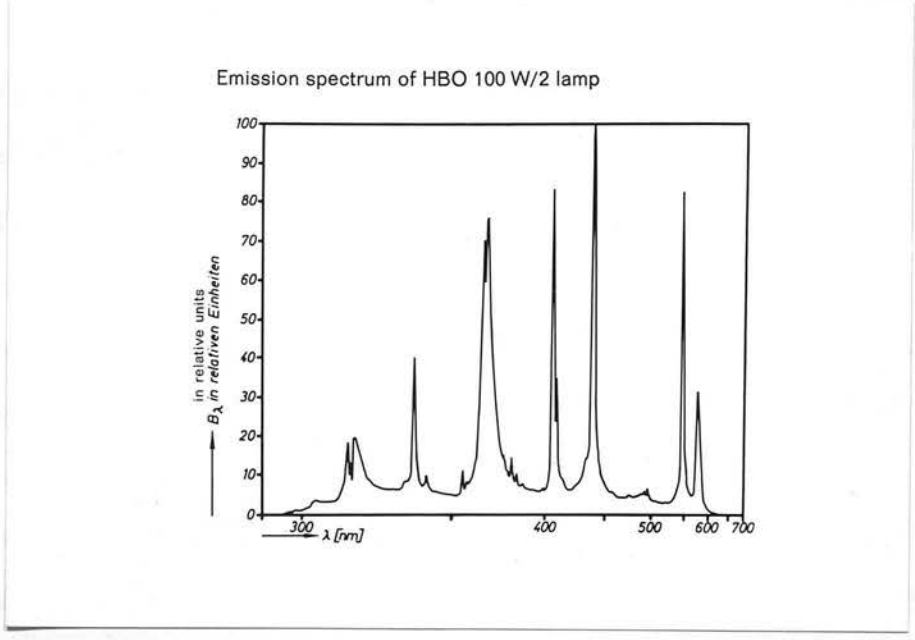


Figure 33. Emission spectrum of HBO 100 W/2 mercury lamp.

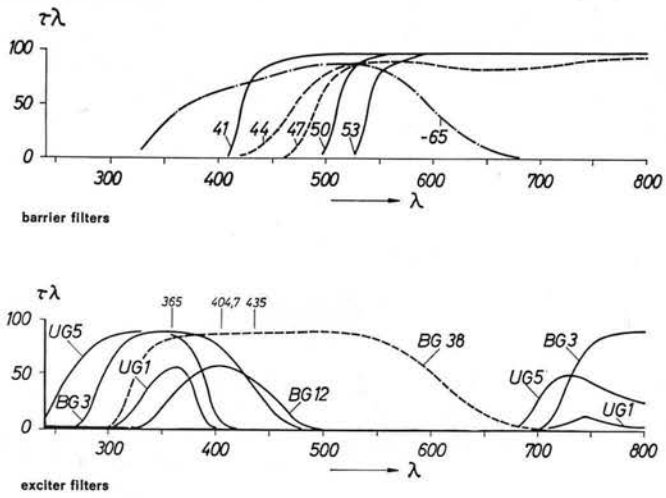


Figure 34. The relation between transmission and wavelength for barrier and exciter filters.

The filter BG 38 2.5 mm (Schott) (figure 34) was in the light path between the exciter filters and the light source, and acted as a heat-absorbing filter. The exciter filter UG5 was selected for routine use and BG 38 also had the effect of practically eliminating the transmission of that part of the HBO 100 emission above 680 nm which would otherwise have been reflected by the specimen thus contaminating the emitted fluorescence. (Although the emission of the HBO 100 lamp is of relatively low intensity above 600 nm, it is possible that such contamination could have had a significant effect on the observed colour of the fluorescence although it would have had no effect on measurements of fluorescence, because of the sensitivity of the photomultiplier which was used. (See below))

The fluorophores of DA and L-DOPA exhibit maximal excitation at about 410 nm (67). Clearly, if the maximal degree of absorption of exciting light by these fluorophores is to be obtained, the emission peak of the

HBO 100 lamp at 404.7 should be transmitted by the exciter filters. Of all the filters shown in figure 34, BG3 is the obvious choice for this purpose, however the selection of BG3 would have led to the selection of the barrier filter 50, as the barrier filter 47 would have also transmitted some of the exciting light (reflected) as well as the emitted fluorescence. Thus the fluorescence would have been contaminated if the combination of BG3 and 47 had been selected. However barrier filter 50 would not have transmitted an important part of the fluorescence derived from DA and L-DOPA, which has an emission maximum in tissue sections at about 480 nm. (67) In view of this, I selected UG5 2 mm (Schott) as the exciter filter, in combination with the barrier filter 47. (Zeiss) This combination provided a satisfactory intensity of fluorescence in the experiments to be described and, in theory, eliminated transmission of any reflected exciting light by the barrier filter. However this phenomenon cannot be completely avoided with available filters. (See General Introduction) If the selected combination had not given satisfactory results another possibility would have been to use an interference exciter filter with a narrow band of transmission including the 404.7 emission peak. Such a filter e.g. AL406 (Schott) was not readily available, so that the results of its use were not investigated.

The filter UG5 transmits in the range 220-420 nm, with a flat peak from about 300-365 nm. In addition it transmits above 680 nm. The barrier filter 47 is a sharp cutting long-wave pass filter which transmits wavelengths above 470 nm. It has a transmittance of about 10% at 470 nm and below this wavelength very little light is transmitted. Above 470 nm the degree of transmission rises steeply.

Immediately before passing through the exciter filters, the exciting light passed through an iris diaphragm. This enabled the intensity of the exciting light to be reduced while areas for fluorimetry were selected, thus minimizing photodecomposition before the measurements of fluorescence intensity could be taken.

The beam splitter above the objective is shown in figure 32. A proportion of the exciting light was reflected to the specimen via the objective which acted as a condenser. The emitted fluorescence which entered the objective was partly reflected by the beam splitter, and thus lost for the purposes of observation or measurement while the rest was transmitted to the barrier filter.

The Zeiss oil-immersion objective Apochromat X40.NA1.0 was used.

The Veril S 200 graduated interference filter (Schott), 200 mm in length, was placed in the light path between the objective and the photomultiplier in order to measure emission spectra. During such measurements, the barrier filter 47 was removed from the light path. The transmittance of the Veril S 200 is "nearly constant" over the whole spectral range of 400-600 nm and a change of wavelength by 1 nm is achieved by displacing the filter 0.42 mm. (111) The situation of the filter is shown in figure 31.

The EMI 6256B photomultiplier was used.

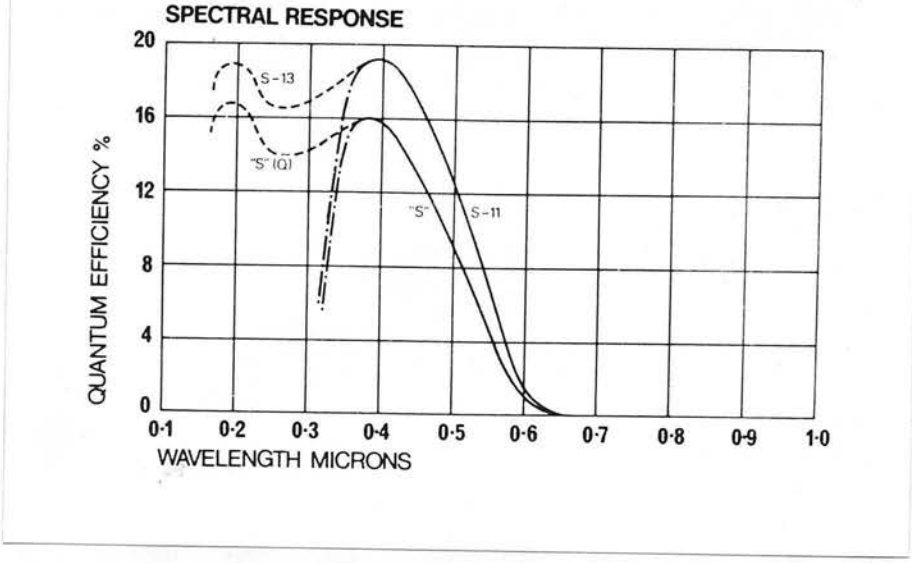


Figure 35. The spectral response of the EMI 6256B photomultiplier is shown by the curves labelled S-13 and S-11. (Information provided by EMI as a booklet "Photomultiplier Tubes.") The quantum efficiency of the photocathode to light of a specified wavelength is the number of photoelectrons emitted from the photocathode per incident photon. The ratio is expressed as a percentage. The varying sensitivity to incident light at different wavelengths is shown.

In the General Introduction the differences between a true (undistorted) emission spectrum and a measured (uncorrected) emission spectrum have been discussed. Using the above apparatus (figure 31), a comparison was made between the emission spectrum of the HBO 100 mercury lamp (see figure 33), which reached the objective using a transmitted illumination system, and the measured (uncorrected) emission spectrum of the HBO 100 lamp using the Veril S 200 mm. graduated interference filter. The filters 47 and UG5 were removed from the light path, however BG38 remained, as it was built-in to the microscope.

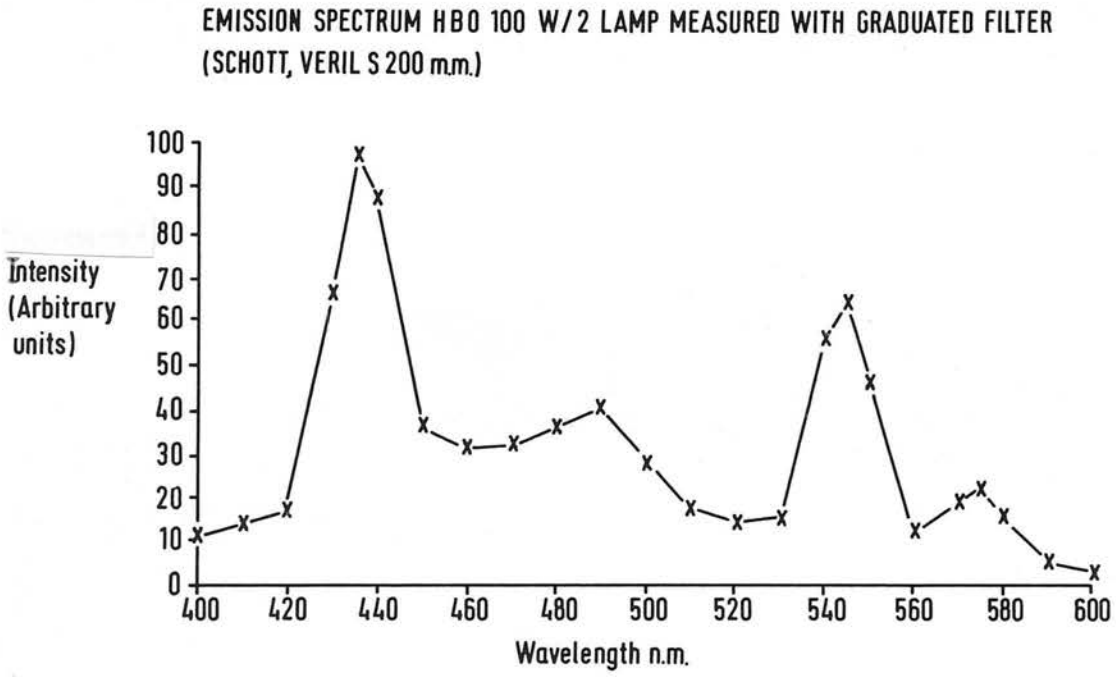


Figure 36. The measured (uncorrected) emission spectrum of an HBO 100 mercury lamp, using a graduated interference filter. The exciter filter BG38 remained in the light path. The emission peaks at about 435 nm and 545 nm are approximately equivalent to corresponding maxima in figure 33.

The photometer head was attached to the tube head of the microscope. In the photometer head, i.e. immediately below the photomultiplier, there were three mirrors, containing central holes, on a revolving disc by means of which they could be introduced into the light path. These circular openings varied in size so that the amount of light reaching the photomultiplier could be varied. In the studies to be reported the largest opening (5mm in diameter) was selected. Several projection lenses were available in the photometer head, and the X10 magnification was

chosen. The display unit (figure 31) contained the high-voltage power supply unit for the photomultiplier, the amplifier for the photoelectric current and a mirror galvanometer for indicating the measured value for the fluorescence intensity. If the primary voltage changed by 10% of its nominal value, the deflection on the display unit would have changed by less than 0.2% of the indicated value. (111)

A delay occurred between irradiation of the specimen and the registration of the intensity of the resulting fluorescence on the display unit. The degree of damping of the mirror galvanometer could be varied, and the minimum damping which was needed to stabilize over-swing of the recorded intensity was associated with a delay of about 2 seconds between irradiation and registration.

Using the objective and photometer head settings as described, the intensity of fluorescence was measured from circular areas each with a diameter of 10 μm.

Unless otherwise stated, a series of measurements which were compared were obtained during a continuous period of microscopy. As will be described elsewhere, although there were variations in the intensity of the light source during a period of microscopy, these were usually found to be sufficiently small to be ignored. Thus, the fluorophore concentration corresponding to an arbitrary unit of fluorescence intensity was usually considered to be constant during each period of microscopy. (Section A; G) However, there were often marked variations in the intensity of the exciting light between different periods of microscopy.

In addition, it should be noted that the selected amplification of the current from the photomultiplier often differed between different periods of microscopy so that this factor often contributed to differences in the fluorophore concentration corresponding to an arbitrary unit of fluorescence intensity, between different experiments.

Unless otherwise stated the microscopy for each experiment was carried out on the same day, during a continuous period of microscopy.

An automatic camera was situated in the body of the microscope. The exposure could be automatically determined by taking into account either the emitted fluorescence from the whole field, or from a selected part. Ilford FP4 film, DIN 22 was used, and it was found that the best results could be obtained by setting the automatic exposure meter to a DIN value of 26.

Orientation of tissue sections

The first step in the selection of a region of a tissue section for microscopy was to view the section through a low power objective. The section was illuminated by an oblique light, from above, derived from the filament lamp shown on the left of figure 31.

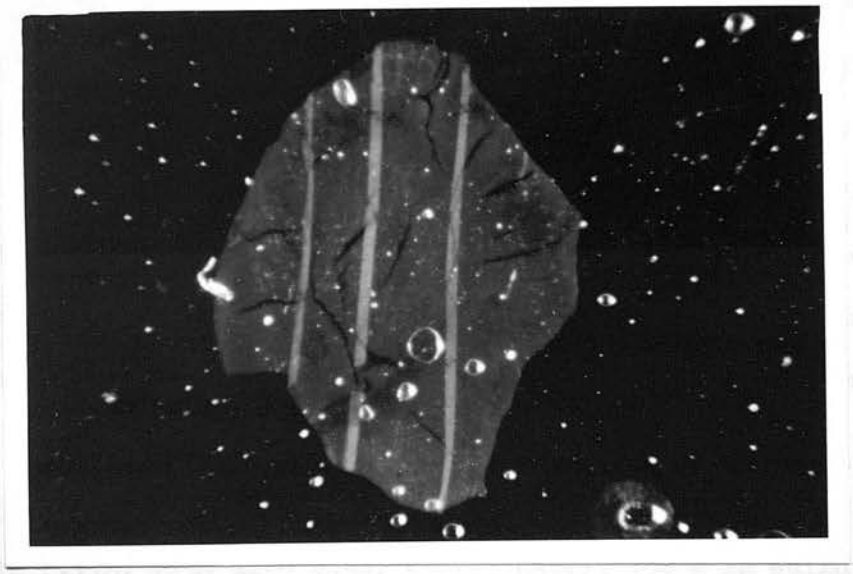


Figure 37. Mounted coronal section of rat cerebral hemisphere, obliquely illuminated by a filament lamp.

The outline of the section and the situation of the principal fibre bundles, e.g. corpus callosum, were observed, and the area to be examined was placed im the centre of the field. Thus when the X40 oil immersion objective was introduced, the region which was in the field of view had already been identified.

Results

Examination of diffuse parenchymal formaldehyde-induced fluorescence. (A-E)

A. Variations between experiments in the intensity of the formaldehyde-induced fluorescence in the neuropil of the rat nucleus caudatus-putamen, relative to autofluorescence in the neuropil of the cerebral cortex.

Introduction

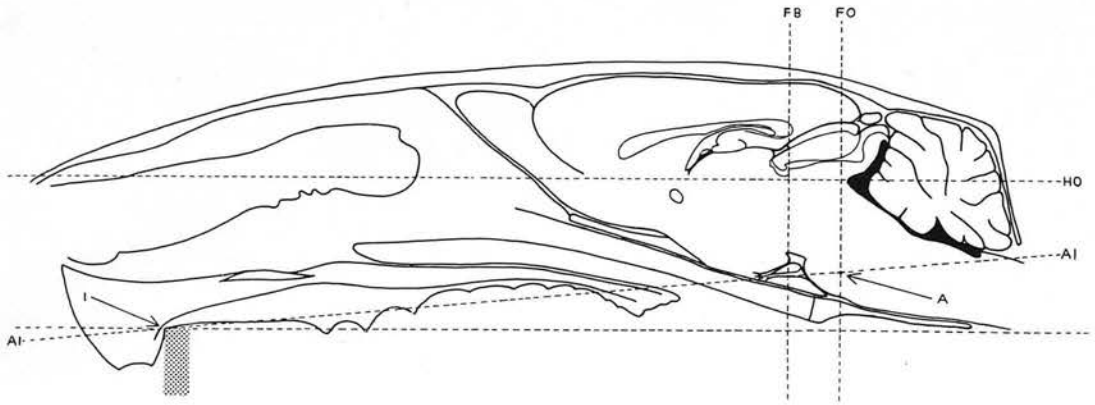
Previous reports have described the appearance of the rat nucleus caudatus-putamen shown by the formaldehyde-fluorescence method. (See General Introduction) Although individual DA-containing varicosities have been visualized under certain conditions, previous reports have usually described a diffuse formaldehyde-induced fluorescence, mainly derived from DA, throughout the neuropil. In the present study, microfluorimetric measurements were taken of the fluorescence in the neuropil of a region of the nucleus caudatus-putamen and were compared with measurements of non-specific (i.e. not derived from a Pictet-Spengler reaction involving formaldehyde) fluorescence in the neuropil of a region of the cerebral cortex. The ratio of the fluorescence intensity in the two regions of each rat was obtained in several sets of experimental conditions. The ratios were then compared, to investigate the effects of variables associated with different experiments on the intensity of formaldehyde-induced fluorescence and autofluorescence.

Materials and Methods

Male P.V.G. rats were used, approximately 150 g in weight. They were decapitated under chloroform anaesthesia and dissected samples were prepared for fluorescence microscopy as previously described.

In each experiment, a dissected sample (which included parts of the nucleus caudatus-putamen and cerebral cortex) was obtained from each of 3 rats and prepared for fluorescence microscopy. This was repeated 7 times so that a total of 24 rats were used and the effects of 8 examples of the experimental procedure were examined.

Each sample was a coronal slice of a cerebral hemisphere, approximately 3 mm in thickness. The anterior plane of dissection was approximately parallel with the frontal zero plane described by König and Klippel. The posterior plane of dissection was approximately 0.5 mm posterior to the posterior aspect of the anterior commissure in the mid-sagittal plane.



A paramedian sagittal section through the skull of a female rat (150 g.). The broken lines represent the reference planes of the coordinate system. HO, horizontal zero plane; FO, frontal zero plane; AI, plane through the interaural line (A) and the parallel through the rostral edge of the upper incisor bar (I); FB, frontal base plane.

Figure 38. From König and Klippel's Stereotaxic Atlas of the Rat Brain. (72)

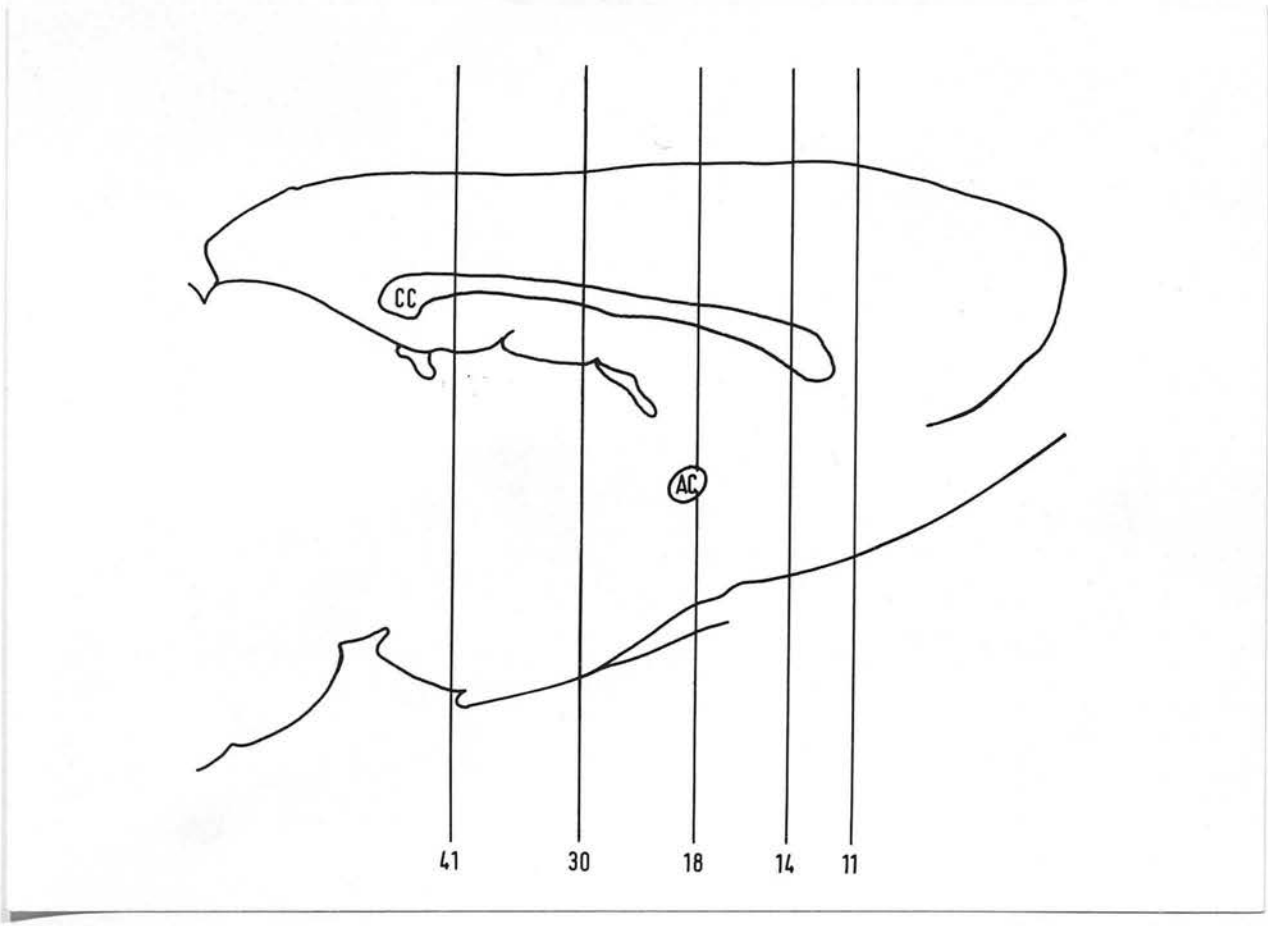


Figure 39. Mid-sagittal diagram showing several coronal planes parallel to the frontal zero plane shown in figure 38. They are numbered according to König and Klippel. (72) AC = Anterior commissure. CC = corpus callosum.

Representative sections from different parts of each tissue sample were stained with Luxol Fast Blue and Cresyl Violet, and the sections were identified which corresponded to the region between the planes 18 and 14 in figure 39. Three adjacent sections were selected for fluorescence microscopy from this part of each tissue sample. The possible variation in the antero-posterior level of the coronal plane of the selected sections was over approximately 1.5 mm, extending anteriorly from the anterior border of the median part of the anterior commissure. (This included the plane illustrated in figure 7, which corresponds approximately to plane number 15 of König and Klippel. (See figure 39))

The intensity of diffuse fluorescence was measured from circular areas, each with a diameter of 10 μm. The areas for fluorimetry were selected from the neuropil, i.e. regions which appeared to be devoid of cell bodies, of blood vessels and in the case of the nucleus caudatus-putamen, of fibre bundles of the internal capsule. The areas were arbitrarily chosen in each region and while the choice of a suitable area was being made, the intensity of the exciting light, controlled by an iris diaphragm, was much reduced to minimize photodecomposition.

Measurements of the intensity of diffuse fluorescence in the neuropil were taken from 6 areas of each selected region of each section. The mean of the 18 readings thus obtained from either the nucleus caudatus-putamen or the cerebral cortex from each tissue sample was taken as an estimate of fluorescence intensity. The concentration of fluorophore giving rise to an arbitrary unit of fluorescence intensity was not constant in the different experiments because of variations in the intensity of the light source between different periods of microscopy. (See Section A,G)

Measurements were restricted to the dorso-lateral part of the nucleus caudatus-putamen, lateral to an imaginary straight line at an angle of 45° to the vertical, dividing the nucleus caudatus-putamen of each tissue section into 2 parts with approximately equal areas. The measurements of the diffuse non-specific 'background' fluorescence of the cerebral cortex were taken from areas at a depth of approximately 350 μm, from parts of the cortex included in the coronal sections of the nucleus caudatus-putamen. These cortical regions consisted of the anterior part of the parietal region and the adjacent part of the frontal lobe, i.e. areas 1,2, 3 and parts of 4 and 6. (Kreig, 1946) (73)

Results

The neuropil of the nucleus caudatus-putamen exhibited a diffuse green fluorescence which appeared to be evenly distributed throughout the neuropil.

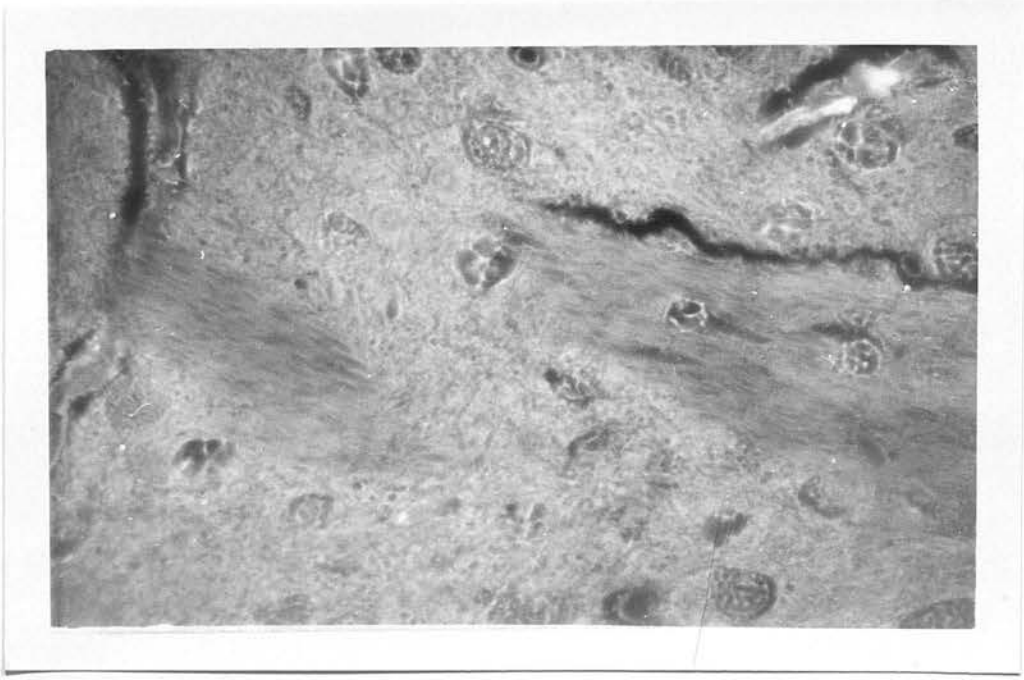


Figure 40. Diffuse fluorescence in the neuropil of the rat nucleus caudatus-putamen prepared by the formaldehyde-fluorescence method. X 640. Two bundles of fibres of the internal capsule can be seen as darker areas. Many cell bodies approximately 10 µm in diameter are also visible.

Figure 41. Diffuse fluorescence in the neuropil of the rat nucleus caudatus-putamen prepared by the formaldehyde-fluorescence method. X 640. Many cell bodies are seen.

The neuropil of the cerebral cortex exhibited a relatively faint diffuse green fluorescence which was almost entirely due to autofluorescence. (See Section A;B)

The day after embedding the samples in paraffin wax, 8 µm sections were cut and stored, in darkness, in plastic boxes. The sections were mounted in Entellan on the day before examination and the period of storage of the sections before mounting varied from 0-5 days.

In each experiment, the measurements of fluorescence intensity were taken during one continuous period of microscopy. As will be described

elsewhere, (Section A;G) variations in the intensity of the light source occur during a period of microscopy, but in these studies they were judged to be sufficiently small to be ignored. However, variations in the intensity of the exciting light between different periods of microscopy were judged to be sufficiently large to consider that an arbitrary unit of fluorescence intensity was associated with different amounts of fluorophore in different periods of microscopy. (Section A;G) The fluorescence in the nucleus caudatus-putamen was therefore compared with the autofluorescence in the neuropil of the cerebral cortex in each experiment, so that the effects of the 8 sets of experimental conditions could be compared. It should be noted that the selected amplification of the current from the photomultiplier often differed between different periods of microscopy so that the value of an arbitrary unit of fluorescence intensity varied between the experiments reported in table 3.

	Fluorescence intensity Arbitrary units		
	A	B	
Experiment	Nucleus caudatus-putamen	Cerebral cortex	Ratio $\frac{A}{B}$ for each rat
1	66	35	1.88 M = 1.91
	69	39	1.77
	72	33	2.18
2	58	52	1.12 M = 1.26
	61	54	1.12
	69	45	1.53
3	54	38	1.42 M = 1.43
	58	38	1.52
	48	36	1.33
4	55	38	1.44 M = 1.41
	64	42	1.52
	47	37	1.27
5	40	31	1.29 M = 1.28
	40	35	1.14
	43	31	1.38
6	62	47	1.32 M = 1.45
	64	41	1.56
	67	43	1.56
7	76	40	1.90 M = 1.74
	72	43	1.68
	76	45	1.69
8	56	33	1.70 M = 1.77
	66	35	1.88
	66	36	1.83

Table 3. The fluorescence intensity of the neuropil of the rat nucleus caudatus-putamen and cerebral cortex in sections prepared by the formaldehyde-

fluorescence method. M = Mean value. 3 rats were examined in each experiment and each value represents the mean of 18 readings from 1 rat. The value of an arbitrary unit of fluorescence intensity was only constant within each experiment, i.e. it varied between different experiments.

Discussion

The appearance of the formaldehyde-induced fluorescence in the neuropil of the nucleus caudatus-putamen corresponded to that in the majority of previously published reports. (See General Introduction) Thus the fluorescence was diffuse, and individual DA-containing varicosities of the terminal parts of the nigro-striatal neurones were not observed. It should be noted that with the optical system used, the smallest separation which two structural elements could have had in order for them to be seen as two separate elements, (if they emitted light at 480 nm), is 0.24 µm. (81) However the degree of extraneuronal diffusion of the fluorophore derived from DA was uncertain because of the following factors. (See General Introduction)

(i) In sections as thick as 8 µm, the outline of any varicosities still containing fluorophore (i.e. which had not diffused into the surrounding parenchyma) may have been obscured by surrounding varicosities or by fluorophore in an extraneuronal situation due to diffusion of DA, and/or the fluorophore derived from DA, from other varicosities.

(ii) The size of some DA-containing varicosities would have been below the limit of resolution of the microscope.

The degree of diffusion of the fluorophore derived from DA would have differed between the different experiments, as the reaction conditions of the formaldehyde-fluorescence method are difficult to reproduce.

The results in table 3 show significant variations, between experiments, in the ratio of the fluorescence intensity in the neuropil of the nucleus caudatus-putamen to the fluorescence intensity in the neuropil of the cerebral cortex, in each rat. As will be reported elsewhere, (Section A', B) the fluorescence of the neuropil of the nucleus caudatus-

putamen consists of a component due to formaldehyde-induced fluorescence derived almost entirely from DA, and a component due to non-formaldehyde-induced autofluorescence. The latter is of approximately equal intensity to that of the autofluorescence in the neuropil of the cerebral cortex.

The different sets of experimental conditions in the 8 experiments may have been associated with differences in the intensity of autofluorescence (produced by an exciting light of specified intensity) which were independent of differences in formaldehyde-induced fluorescence, as it has been reported that autofluorescence may be diminished during preparation by the formaldehyde-fluorescence method. (44) It should also be noted that when comparing the ratios between different experiments, an "inner-filter" quenching effect could have occurred (see General Introduction) in the nucleus caudatus-putamen but not in the cerebral cortex. Any such effect would have varied depending on the intensity of the exciting light, so that any "inner-filter" quenching could have affected the ratio of fluorescence in the nucleus caudatus-putamen to that in the cerebral cortex by different degrees in different experiments.

Some of the factors which may have contributed to the differences in the ratios, between experiments, are variations in the degree of freeze-drying of tissue and variations in the humidity of the air in the reaction vessel. However, variations in any step of the formaldehyde-fluorescence method may have contributed.

In these experiments the period of storage of the tissue sections, before mounting in Entellan, varied from 0-5 days. It was noted that the ratio of the fluorescence in the nucleus caudatus-putamen to that of the cerebral cortex was relatively small when the tissue sections had been mounted in Entellan on the same day as sectioning compared with

the results of a longer interval before mounting. The effects of storage of tissue sections before mounting were investigated further. (See Section, A;F)

Summary

The intensity of diffuse fluorescence in the neuropil of the nucleus caudatus-putamen and of the neuropil of the cerebral cortex was measured in sections of the rat brain prepared by the formaldehyde-fluorescence method. The appearance of the fluorescence of these two brain regions is also described. The diffuse fluorescence in the neuropil of the nucleus caudatus-putamen consisted of a mixture of formaldehyde-induced fluorescence, (almost entirely derived from DA in the terminal parts of nigro-striatal axons) and of tissue autofluorescence. The diffuse fluorescence in the neuropil of the cerebral cortex was almost entirely due to autofluorescence.

The ratio of fluorescence intensity in the two regions was compared, for each rat, in 8 different experiments and significant differences in the ratios between experiments are reported and discussed. One of the many factors which could have contributed to the differences was the period of storage of the tissue sections before they were mounted in Entellan. (The effects of storage of tissue sections before mounting was investigated further. See Section A;F))

B. Diffuse autofluorescence in the neuropil of 3 regions of the rat brain prepared by the formaldehyde-fluorescence method.

Introduction

When measuring the intensity of diffuse formaldehyde-induced fluorescence in the neuropil of a region of the brain, e.g. due to DA in the neuropil of the nucleus caudatus-putamen, or due to administered L-DOPA in any brain region, the 'background' autofluorescence has to be taken into account. Autofluorescence is a feature of specimens which have not been treated with formaldehyde and may be diminished by treatment with formaldehyde gas. (44)

In this study some characteristics of autofluorescence in the neuropil of 3 brain regions are reported and discussed.

Materials and Method

Experiment A. The fluorescence intensity of the neuropil of a region of the cerebral cortex was compared with that of the neuropil of the molecular layer of the cerebellum in samples from each of 4 rats, prepared by the formaldehyde-fluorescence method. Male P.V.G. rats were used, approximately 150 g in weight. They were decapitated under chloroform anaesthesia, and dissected samples were prepared for fluorescence microscopy and examined as previously described. The sections were stored for 2 days before being mounted in Entellan. The details of the sample containing a region of cerebral cortex have been described, (Section A;A) together with details of the region examined.

The samples of cerebellum were taken from the paravermal region and the plane of sectioning was not constant between samples.

The intensity of the diffuse fluorescence in the neuropil of both regions was measured from circular areas, each with a diameter of 10 μm. (See Section A;A) Measurements of the intensity were taken from 6 arbitrarily chosen areas from the selected part of each section. Three

adjacent sections from each tissue sample were chosen for fluorescence microscopy. The mean of the 18 readings from each of the 2 brain regions from each rat was taken as an estimate of fluorescence intensity.

Experiment B. Fuxe (47) has reported the effects of a dose of reserpine (10 mg/kg. intraperitoneally) on the diffuse formaldehyde-induced fluorescence in the neuropil of the rat nucleus caudatus-putamen. After 4 hrs. only a very weak fluorescence was observed.

In the present experiment the intensity of the remaining diffuse fluorescence in the neuropil of the nucleus caudatus-putamen 4 hrs. after reserpine 10 mg/kg i.p. was measured in 2 rats, and compared with the fluorescence in the neuropil of the region of the cerebral cortex. A sample containing nucleus caudatus-putamen and cerebral cortex from a rat which had not received reserpine was also examined.

Male P.V.G. rats were used, approximately 150 g in weight. They were decapitated under chloroform anaesthesia, and dissected samples were prepared for fluorescence microscopy and examined as previously described. The sections were stored for 2 days before being mounted in Entellan. The details of the sample containing the parts of the nucleus caudatus-putamen and cerebral cortex have been described (Section A;A) together with details of the regions examined.

Measurements of the fluorescence intensity of the neuropil from both regions were taken from circular areas, each with a diameter of 10 μm. Measurements were taken from 6 arbitrarily chosen areas of each selected part of each section. Three adjacent sections were selected for fluorescence microscopy from each tissue sample. The mean of the 18 readings from each of the 2 brain regions from each sample was taken as an estimate of fluorescence intensity.

Results

Experiment A.

The appearance of the cerebral cortex is shown in figure 42.

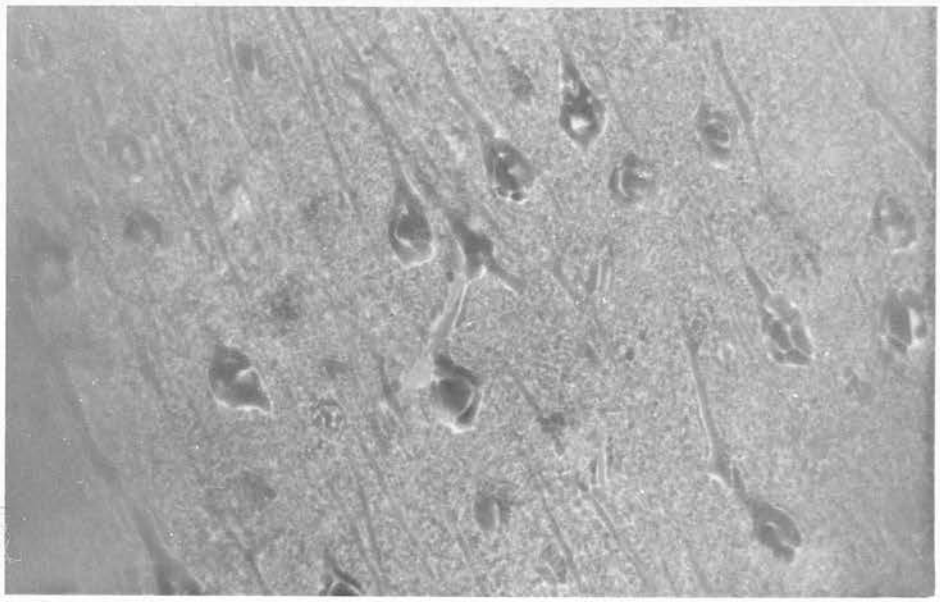


Figure 42. Rat cerebral cortex, prepared by the formaldehyde-fluorescence method. X640.

Rat	Fluorescence intensity (arbitrary units) Cerebral cortex		Cerebellum molecular layer	
1	47 (43-50)		47 (44-49)	
2	41 (38-42)	Mean	46 (42-40)	Mean
3	43 (41-45)	44	39 (36-42)	43
4	44 (42-47)		40 (37-41)	

Table 4. The fluorescence intensity of the neuropil of two regions of the rat brain in samples prepared by the formaldehyde-fluorescence method. (The measured fluorescence was almost entirely derived from autofluorescence). Each value represents 1 tissue sample and is the mean of 18 readings. The interquartile range is shown for each value.

Experiment B

Rat	Treatment	Fluorescence intensity (arbitrary units) Nucleus caudatus-putamen	Cerebral cortex
1	None	70	34
2	Reserpine 10 mg/kg	33	35
3	Reserpine 10 mg/kg	32	31

Table 5. The fluorescence intensity of the neuropil of two regions of the rat brain in samples prepared by the formaldehyde-fluorescence method. Each value is the mean of 18 readings from 1 tissue sample. Reserpine was given i.p. 4 hrs. before killing.

(It should be noted that the value of an arbitrary unit of fluorescence intensity varied between experiments A and B).

It can be seen that the measured intensity of the remaining fluorescence in the nucleus caudatus-putamen in the rats treated with reserpine was almost identical to that of the cerebral cortex in all 3 animals. This finding was confirmed in a subsequent experiment. In the reserpinized rats, the fibre bundles of the internal capsule appeared slightly darker than the surrounding neuropil.

Discussion

The results of experiment A indicate that the neuropil of the region of the cerebral cortex which was examined had a similar intensity of autofluorescence, compared with that in the molecular layer of the cerebellum. (This finding was confirmed in subsequent experiments.)

In experiment B, any effect of the administration of reserpine, 10 mg/kg i.p. 4 hrs. before killing, on the measured fluorescence in the cerebral cortex could not be detected. Although 5 HT, DA and NA have been found in the mammallian cerebral cortex (32,1), this suggests that the fluorescence in

this region was derived almost entirely from autofluorescence. The results also suggest that the autofluorescence in the neuropil of the nucleus caudatus-putamen had an almost identical intensity to that of the region of cerebral cortex examined. Thus it is likely that, in any experiment, the intensity of the fluorescence in the cerebral cortex or molecular layer of the cerebellum is almost identical to the contribution that autofluorescence makes to the fluorescence intensity of the neuropil of the nucleus caudatus-putamen, in rats not treated with reserpine.

Summary

The intensity of diffuse fluorescence of the neuropil of the nucleus caudatus-putamen, cerebral cortex and molecular layer of the cerebellum was measured in sections of the rat brain prepared by the formaldehyde-fluorescence method. (In 2 rats, the DA in the nucleus caudatus-putamen had been depleted by the administration of reserpine 10 mg/kg i.p. 4 hrs. before killing).

The results indicate that the autofluorescence (i.e. not formaldehyde-induced) of the neuropil was of similar intensity in the 3 brain regions. This was confirmed in subsequent experiments. This suggests that the intensity of fluorescence in the neuropil of the cerebral cortex or molecular layer of the cerebellum can be used to infer the approximate contribution of autofluorescence to the total fluorescence in the neuropil of the nucleus caudatus-putamen, in rats not pretreated with reserpine.

C. The fluorescence intensity in the neuropil of the nucleus caudatus-putamen and cerebral cortex in tissue samples not exposed to formaldehyde.

Introduction

The differentiation of specific, (i.e. formaldehyde-induced) fluorescence from autofluorescence can be made by contrasting tissue samples prepared by the formaldehyde-fluorescence method with samples prepared in the same way but without exposure to formaldehyde gas. (44) In this study the specificity of the diffuse fluorescence in the neuropil of the rat nucleus caudatus-putamen was examined.

Materials and Method

In each of 5 experiments, the fluorescence intensity of the neuropil of a region of the cerebral cortex was compared with that of the neuropil of the nucleus caudatus-putamen, in a sample from 1 rat which was prepared by the formaldehyde-fluorescence method except that the paraformaldehyde was omitted from the reaction vessel. In each experiment another tissue sample which was exposed to formaldehyde gas was also examined. During embedding of the samples in paraffin wax the non-formaldehyde-treated sample was embedded first to avoid possible infiltration of the sample by paraffin wax contaminated by formaldehyde derived from formaldehyde-treated samples.

Male P.V.G. rats were used, approximately 150 g in weight. The killing of the animals, preparation and examination of the samples, details of the regions examined and details of the measurement of fluorescence intensity have been previously described. (Section A; Introduction, A and B) The sections were stored for varying times in the 5 experiments, ranging from 1-5 days, before being mounted in Entellan. Three adjacent sections were selected from each tissue sample. The mean of the 18 readings from each of the 2 brain regions of each rat was taken as an estimate of fluorescence intensity.

Results

Figure 43 shows the rat cerebral cortex in a sample which had not been exposed to formaldehyde gas. A diffuse green fluorescence was observed.

Figure 43. Rat cerebral cortex, prepared by the formaldehyde-fluorescence method but omitting exposure to formaldehyde gas. (A blood vessel is included, whose wall contains yellow autofluorescence granules.) X530.

Experiment	Non-HCHO treated	HCHO treated
	Ratio $\frac{A}{B}$	Ratio $\frac{C}{D}$
1	1.03	1.74
2	0.92	1.24
3	1.08	1.86
4	1.06	1.70
5	1.06	2.36

Table 6. The ratio of the fluorescence intensity in the neuropil of the nucleus caudatus-putamen (A) to the fluorescence intensity in the neuropil of the cerebral cortex (B), in tissue samples prepared by the formaldehyde-fluorescence method but omitting exposure to formaldehyde gas. In addition, the ratio of the fluorescence intensity in the neuropil of the nucleus caudatus-putamen (C) to the fluorescence intensity in the neuropil of the cerebral cortex (D) in the tissue samples prepared by the formaldehyde-fluorescence method is also shown. Each ratio represents 1 rat. Each value of A B C or D was the mean of 18 readings, i.e. 6 from each of 3 sections from 1 tissue sample.

(Possible factors responsible for the differences in the ratios $\frac{C}{D}$ in the 5 experiments have been discussed previously. (Section A;A))

In 2 of the experiments B > D and in the other 3 experiments, D > B.

Discussion

The results in table 6 show that the ratios $\frac{C}{D}$ are significantly greater than the ratios $\frac{A}{B}$, at a level $p = 0.031$. (Sign test, 1-tailed) (84)

This confirms that part of the diffuse fluorescence in the neuropil of the nucleus caudatus-putamen in the samples prepared by the formaldehyde-induced fluorescence, was specific, i.e. resulting from a reaction with formaldehyde.

It has been reported (44) that autofluorescence is often diminished by treatment with formaldehyde gas. However, this was not a constant finding in the experiments in this study.

Summary

The fluorescence intensity of the neuropil of the nucleus caudatus-putamen and cerebral cortex was measured in sections of the rat brain prepared by the formaldehyde-fluorescence method and by this method omitting the exposure to formaldehyde gas.

The results confirm that only part of the fluorescence intensity in the neuropil of the nucleus caudatus-putamen in samples prepared by the formaldehyde-fluorescence method is specific, i.e. resulting from treatment with formaldehyde.

D. The emission spectra of autofluorescence and diffuse formaldehyde-induced fluorescence derived from L-DOPA and DA.

Introduction

As previously described (see General Introduction) an emission spectrum is recorded by scanning the intensity of the spectral distribution of the emitted fluorescence. The 'true' (i.e. undistorted) emission spectrum can be expressed as energy per unit wavelength interval, but the measured (uncorrected) spectrum differs from a 'true' specimen because of such factors as:-

(i) Varying spectral transmission of the optics, with wavelength.

(ii) Varying spectral response of the photomultiplier, with wavelength.

(iii) Photodecomposition during recording of the spectrum.

(iv) Any reabsorption of emitted light, e.g. occurring in the specimen.

In the General Introduction the reported emission maxima of the fluorophores derived from DA and L-DOPA have been reviewed. There are variations in the reported values which lie within the range 480-490 nm, following preparation by the standard formaldehyde-fluorescence procedure.

It has also been noted that when a high concentration of CA is present, side reactions can occur during the formaldehyde-fluorescence method which are associated with a shift of the emission maximum to a longer wavelength.

In this study the uncorrected emission spectra of diffuse fluorescence were examined in several situations:-

Experiment A

(i) In the neuropil of the nucleus caudatus-putamen in a rat which had received L-DOPA 400 mg/kg i.p. 1 hr. before killing. This dose led to an increase in the diffuse formaldehyde-induced striatal fluorescence which was probably derived from a mixture of L-DOPA and additional striatal DA. (32)

(ii) In the neuropil of the nucleus caudatus-putamen of a rat untreated by drugs. The fluorescence of this region consisted of a combination of diffuse formaldehyde-induced fluorescence mainly derived from DA (see General Introduction) and diffuse autofluorescence.

(iii) In the neuropil of the nucleus caudatus-putamen in a rat which had received reserpine 10 mg/kg i.p. 4 hr. before killing. The residual diffuse fluorescence was almost entirely derived from autofluorescence. (Section A;B)

Experiment B

(i) In the neuropil of the molecular layer of the cerebellum in a rat which had received L-DOPA 150 mg/kg i.p. 1 hr. before killing, and the decarboxylase inhibitor Ro 4-4602 50 mg/kg i.p. 1½ hr. before killing. This dose regimen of Ro4-4602 has been shown to reduce extracerebral decarboxylation of administered L-DOPA, thus increasing blood levels of L-DOPA for a given dose. (32) (The maximum dose of L-DOPA which can be administered without causing discomfort to a rat gives a relatively small increase in diffuse fluorescence in the cerebellum, compared with the level of background autofluorescence. Therefore, a combination of L-DOPA and Ro4-4602 was used to produce a marked intensity of formaldehyde-induced fluorescence relative to the intensity of the autofluorescence.)

The resulting formaldehyde-induced fluorescence was almost certainly mainly derived from L-DOPA, because of minimal dopa-decarboxylase activity in the mammallian cerebellum. (45)

(ii) In the neuropil of the molecular layer of the cerebellum in a rat untreated by drugs. The fluorescence was almost entirely due to autofluorescence.

Materials and Method

Experiment A

Male P.V.G. rats were used, approximately 150 g in weight. They were decapitated under chloroform anaesthesia and dissected samples of the nucleus

caudatus-putamen were prepared for fluorescence microscopy as previously described. The sections were stored for 2 days before being mounted in Entellan. The details of the sample have been described, (Section A; A) together with details of the region examined. A solution of L-3, 4-dihydroxyphenylalanine (Koch-Light) (L-DOPA) was prepared for injection by dissolving this substance in 0.08 N HCl at a concentration of approximately 10 mg/ml.

Three rats were used:- (i) One was treated with L-DOPA 400 mg/kg i.p. 1 hr. before killing, (ii) One was untreated by drugs, (iii) One was treated with reserpine 10 mg/kg i.p. 4 hr. before killing.

The spectral distribution of the intensity of fluorescence was measured from the neuropil of the nucleus caudatus-putamen in each rat. 4 adjacent tissue sections represented each tissue sample. One circular area (diameter 10 μm) was selected from each section and the intensity of the emitted fluorescence was measured using the Veril S 200 graduated interference filter, which was situated between the specimen and the photomultiplier. The barrier filter '47' had been removed from the light path. (See Section A; Introduction) Measurements were taken in the range 430-550 nm at intervals of 10 nm. The emission from each selected area with a diameter of 10 um was measured over the whole of the 430-550 nm range. The measurements from 2 of the 4 areas representing each rat were started at 430 nm and continued at intervals of 10 nm to 550 nm. The measurements from the remaining 2 areas were started at 550 nm and thus recorded in the reverse order. This minimized distortion of each spectrum due to photodecomposition during recording. The mean of the 4 measurements at each specified wavelength was taken as the value for the intensity of emitted fluorescence corresponding to that wavelength.

Experiment B

Male P.V.G. rats were used, approximately 130 g in weight. They were decapitated under chloroform anaesthesia and dissected samples of cerebellum

were prepared for fluorescence microscopy as previously described. The sections were stored for 2 days before being mounted in Entellan. The samples were taken from the paravermal region and the plane of sectioning was not constant. A solution of L-DOPA was prepared as described in experiment A.

Two rats were used:- (i) One was treated with L-DOPA 150 mg/kg i.p. 1 hr. before killing, and Ro4-4602 (N^1 - (DL-seryl) - N^2 - (2,3,4-trihydroxybenzyl) - hydrazine HCl) (La Roche) 50 mg/kg i.p. 1½ hr. before killing, (ii) One rat was untreated by drugs.

The spectral distribution of the intensity of fluorescence was measured from the neuropil of the molecular layer of the cerebellum in each rat. 4 adjacent tissue sections represented each tissue sample and emission spectra were recorded as described in experiment A.

Results

Experiment A

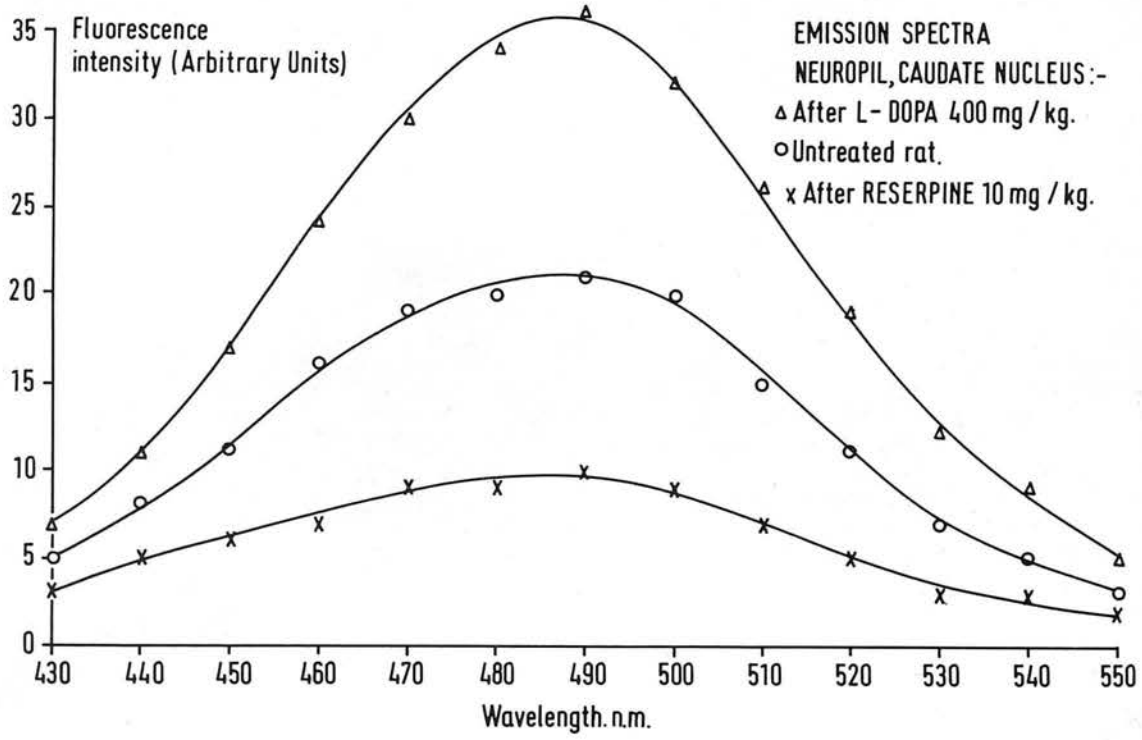


Figure 44. Uncorrected emission spectra of the neuropil of the rat nucleus caudatus-putamen, in tissue sections prepared by the formaldehyde-fluorescence method. A Veril S 200 graduated interference filter (Schott) was used. Each point is the mean of 4 measurements from 1 rat.

The 3 spectra can be compared at each specified wavelength. When the barrier filter '47' was in place, for routine measurements of fluorescence intensity, the region of the emission mainly above 470 nm was transmitted for observation and measurement and this part of the emission of the 3 spectra is described in table 7.

Fluorescence intensity (expressed as % of the corresponding value for the emission intensity obtained from the rat treated with L-DOPA, 400 mg/kg).

See Figure 44

Wavelength	Untreated rat	After reserpine 10 mg/kg
470 nm	63%	30%
480	61	29
490	59	28
500	62	28
510	60	27
520	59	27
530	58	29
540	61	36
550	60	40
Standard deviation	1.5	4.3
Mean	60	30

Table 7. A comparison of part of the 3 emission spectra shown in figure 44.

Experiment B

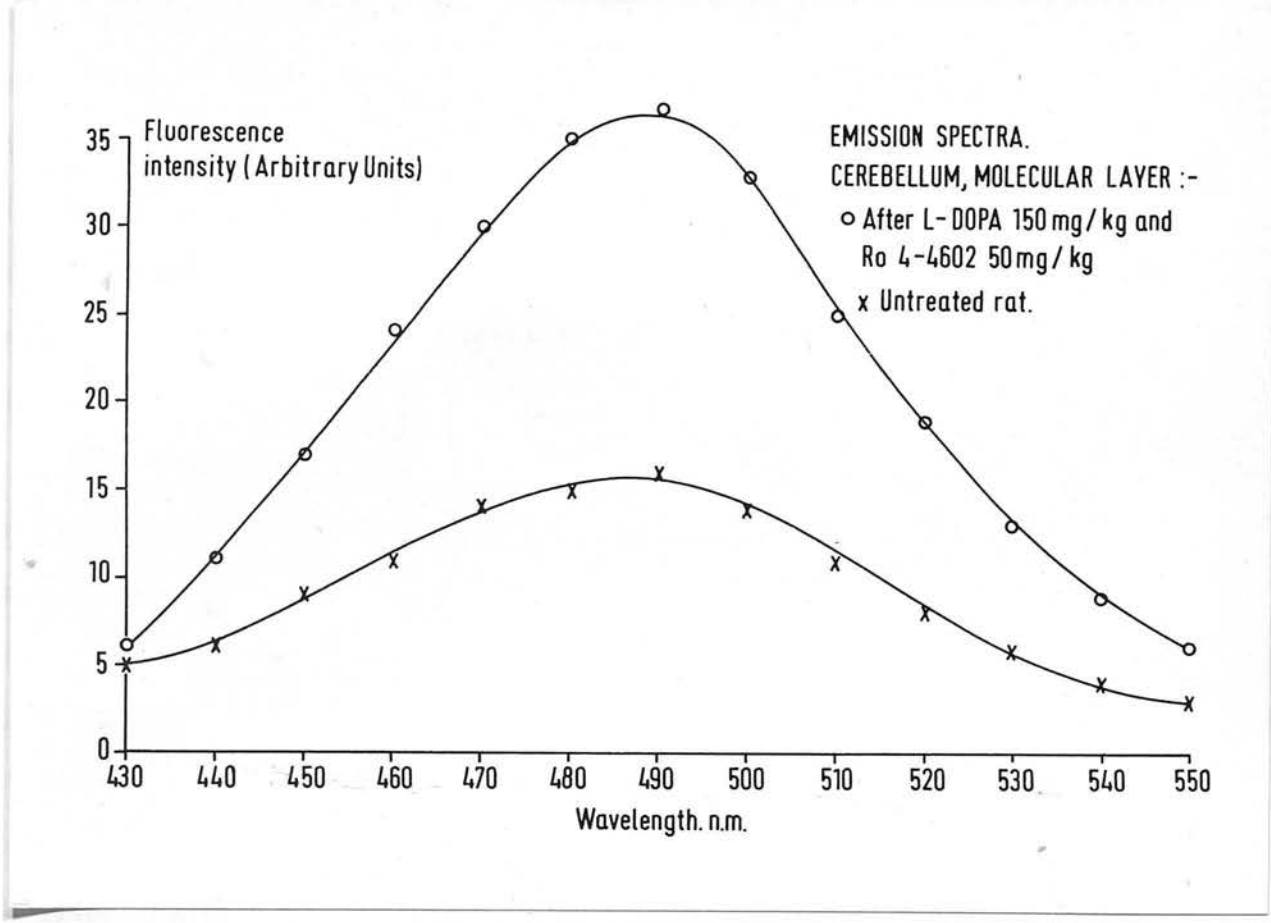


Figure 45. Uncorrected emission spectra of the neuropil of the rat cerebellum (molecular layer), in tissue sections prepared by the formaldehyde-fluorescence method. A Veril S 200 graduated interference filter (Schott) was used. Each point is the mean of 4 measurements from 1 rat.

In the emission range 470-550 nm, the mean of the values of the fluorescence intensity from the untreated rat, each value having been expressed as a percentage of the corresponding value for the emission intensity from the rat treated with L-DOPA and Ro4-4602, was 44% with a S.D. of 2.6.

Discussion

In each of the emission spectra the uncorrected emission maximum is at approximately 490 nm. It can be seen from figure 35 (which shows the spectral response of the photomultiplier) that although it can be deduced that the emission maxima in the green/yellow part of the spectra shown in figures 44 and 45 were not at a wavelength shorter than 490 nm, the declining spectral sensitivity of the photomultiplier from 400 to 600 nm means that each "true" emission maximum could have been at a wavelength longer than 490 nm.

When the barrier filter '47' is in place, i.e. for routine use, the region of the emission above 470 nm is transmitted for observation and measurement. Although the spectra in figures 44 and 45 are uncorrected, a comparison between the spectra was possible and was made in the range 470-550 nm.

In experiment A the results suggest (table 7) that there were no obvious differences within the range 470-550 nm between the emission spectra derived from:-

(i) A mixture of the fluorophores derived from DA and L-DOPA

(ii) The fluorophore derived from DA

(iii) Autofluorescence of the neuropil of the nucleus caudatus-putamen.

In experiment B, the results suggest that there was no obvious difference within the range 470-550 nm between the emission spectra derived from:-

(i) The fluorophore derived from L-DOPA

(ii) Autofluorescence of the neuropil of the molecular layer of the cerebellum.

In the range 470-550 nm the values of the fluorescence intensity from the cerebellar autofluorescence in the untreated rat (figure 45) each value having been expressed as a percentage of the corresponding value for the

emission intensity from the rat treated with L-DOPA 400 mg/kg (figure 44) was calculated at each given wavelength. The mean of all these values was 46%, S.D. 4.9. Thus there were no obvious differences between all the spectra shown in figures 44 and 45, in the range 470-550 nm.

Summary

This study reports and compares the uncorrected (i.e. distorted by the properties of the microscope and accessories) emission spectrum of autofluorescence and of the spectra of formaldehyde-induced fluorescence derived from DA and L-DOPA. It was deduced that the true (i.e. undistorted) emission maxima of these 3 spectra in the yellow/green region were not below 490 nm. Within the range 470-550 nm, which includes most of the part of an emission spectrum used for routine observation and measurement of fluorescence when the barrier filter '47' (Zeiss) is selected, there were no obvious differences between the emission spectra examined.

Methodological variables affecting the investigation of formaldehyde-induced fluorescence. (E-J)

E. The relation between diffuse fluorescence intensity and the microtome setting for section thickness.

Introduction

This study investigated the effects of variations in the microtome setting for section thickness (with the range of microtome setting from 6-16 μm), on the measured fluorescence intensity of the neuropil, in 4 rats. 2 rats were untreated by drugs and 2 received L-DOPA 100 mg/kg i.p. 1 hr. before killing and nialamide 200 mg/kg i.p. 3 hr. before killing. This combination of L-DOPA and nialamide produced marked increases in the diffuse formaldehyde-induced fluorescence in the neuropil of the nucleus caudatus-putamen and in the neuropil of the cerebral cortex. (The resulting increase in the nucleus caudatus-putamen was greater than the increase in the cerebral cortex).

Thus the relation between the microtome setting for section thickness and the intensity of diffuse fluorescence in the neuropil was investigated for each of 4 degrees of fluorophore concentration:-

(i) Nucleus caudatus-putamen in rats treated with L-DOPA and nialamide. (The fluorescence was almost certainly mainly derived from the fluorophores of DA and L-DOPA (38,32) together with tissue autofluorescence.)

(ii) Cerebral cortex in rats treated with L-DOPA and nialamide. (The fluorescence was probably mainly derived from the fluorophore of L-DOPA (32) together with tissue autofluorescence.)

(iii) Nucleus caudatus-putamen in rats untreated with drugs. (The fluorescence was almost entirely derived from the fluorophore of DA (see General Introduction) together with tissue autofluorescence.)

(iv) Cerebral cortex in rats untreated by drugs.

(The fluorescence was almost entirely derived from tissue autofluorescence.)

Materials and Methods

Male P.V.G. rats were used, approximately 130 g in weight. They were decapitated under chloroform anaesthesia and dissected samples were prepared for fluorescence microscopy and examined as previously described. (Section A; Introduction, A) The sections were stored for 2 days before being mounted in Entellan. The details of the sample containing parts of the nucleus caudatus-putamen and cerebral cortex have been described (Section A; A) together with details of the regions examined.

The intensity of fluorescence in the neuropil of the nucleus caudatus-putamen or cerebral cortex was measured from circular areas, each with a diameter of 10 μm. Measurements were taken from 6 arbitrarily chosen areas from each of the 2 regions to be investigated, in each section.

Sections from each tissue sample were cut at several microtome settings:- 6, 8, 10, 12, 14 and 16 μm. 2 adjacent sections represented each tissue sample at each thickness. Thus for each region, at a given thickness, 12 measurements were recorded fromeach sample. The mean was taken as the value for the fluorescence intensity.

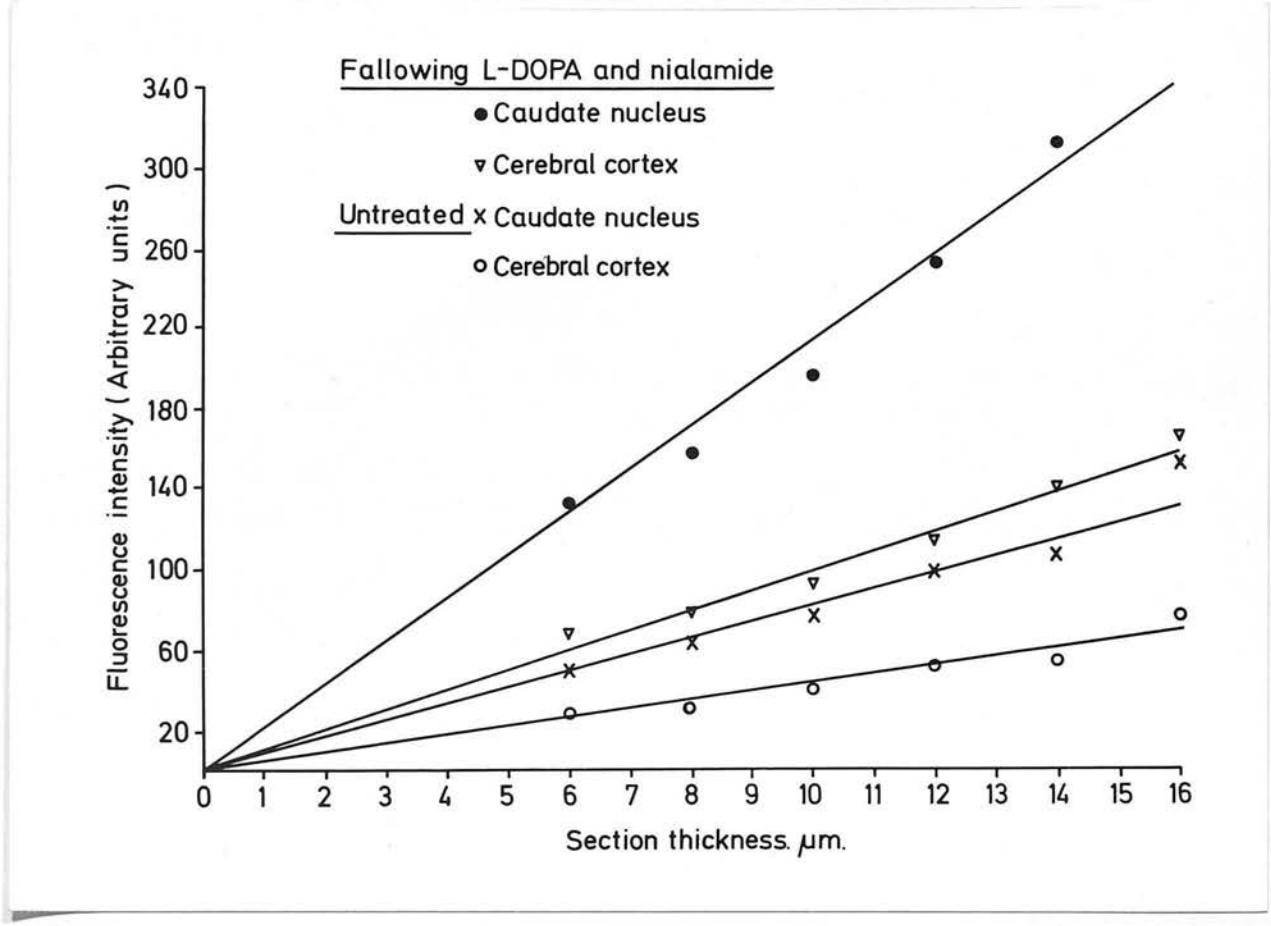


Figure 46. The relationship between the microtome setting for section thickness and intensity of diffuse fluorescence in the neuropil, in sections of the rat brain prepared by the formaldehyde-fluorescence method. Each point is the mean of 24 measurements of fluorescence intensity, representing 4 tissue sections, i.e. 2 tissue sections from each of 2 rats.

The relationship between the range of measured values and section thickness was investigated by an analysis of the measurements from 1 of the rats treated with L-DOPA and nialamide, and from 1 of the untreated rats. The interquartile range (i.e. the range of the middle 2 quarters of each

set of 12 ranked measurements) was calculated for each value of fluorescence intensity and expressed as a percentage of the corresponding mean value of fluorescence intensity.

	L-DOPA	Nialamide	Untreated		
	Nuc.caudatus -putamen	Cerebral cortex	Nuc.caudatus -putamen	Cerebral cortex	Mean value
6 µm	6	18	9	7	9%
8 µm	5	5	4	18	
14 µm	10	13	3	6	8.6%
16 µm	14	5	7	11	

Table 8. The interquartile range of measurements of fluorescence intensity, expressed as a percentage of the corresponding mean fluorescence intensity. Rat brain samples were prepared by the formaldehyde-fluorescence method. Each value is associated with the interquartile range of 12 readings from 2 tissue sections from 1 rat. L-DOPA 100 mg/kg i.p. was given 1 hr. before killing and 2 hr. following Nialamide 200 mg/kg i.p. The microtome settings for section thickness are shown.

Discussion

The results shown in figure 46 indicate that there was an approximately linear relationship between the microtome setting for section thickness and measured fluorescence intensity in the neuropil in the tissue samples examined. This suggests that, in the sections studied, the whole of the thickness of each section contributed to the fluorescence (measured by vertical illumination) i.e. that an "inner filter" quenching effect, due to inadequate excitation of the deeper parts of the section, was not demonstrated. (See General Introduction) (96) Since the reaction conditions and intensity of exciting light may differ between experiments, these results do not rule out the possibility that similar concentrations of fluorophore could be subject to demonstrable "inner filter" quenching in other

experiments. However it is unlikely that similar concentrations of fluorogenic compounds would have given rise to "inner filter" quenching in sections cut at a microtome setting of 8 μm, in any subsequent experiment using the same reaction conditions and optical arrangements.

The results of table 8 show that there is no demonstrable difference between the interquartile range, (expressed as a percentage of mean fluorescence intensity), of measurements from the sections cut at microtome settings of 6 μm and 8 μm, compared with the sections cut at microtome settings of 14 μm and 16 μm. This suggests that within the range 6-16 μm, the microtome setting did not affect the accuracy of measurements of fluorescence intensity.

It should be noted that minor variations in focussing did not make any demonstrable difference to the measured fluorescence intensity at any of the microtome settings used.

Summary

This study reports the relation between measured intensity of diffuse fluorescence in the neuropil of rat brain samples prepared by the formaldehyde-fluorescence method and the microtome setting for section thickness. This was investigated for each of 4 degrees of fluorophore concentration over a range of microtome settings of 6-16 μm.

There was an approximately linear relationship between microtome setting and fluorescence intensity for each of the 4 degrees of fluorophore concentration. This suggests that the whole of the thickness of the sections studied contributed to the measured fluorescence, i.e. that "inner filter" quenching, (caused by insufficient excitation of the deeper parts of each section by the vertical illumination), was not significant. Thus "inner filter" quenching is unlikely to have affected the measurement of similar concentrations of fluorophores, in subsequent experiments, in which the sections were cut at a routine microtome setting of 8 μm.

Within the range 6-16 μm, the microtome setting did not appear to affect the interquartile range, (obtained by discounting the highest 25% and the lowest 25% of the range), of a series of measurements of fluorescence intensity, when the interquartile range was expressed as a percentage of the corresponding mean fluorescence intensity.

F. Changes in the intensity of formaldehyde-induced fluorescence and autofluorescence during storage of tissue sections.

Introduction

Several factors have been shown to affect the intensity of the formaldehyde-induced fluorescence of the fluorophores derived from biogenic amines. (33) The present study reports the effects of other variables, i.e. the duration and conditions of storage of the tissue sections prior to fluorescence microscopy. Changes will be described in the intensity of the diffuse formaldehyde-induced fluorescence in the neuropil of the rat nucleus caudatus-putamen due to storage of tissue sections. Changes, during storage, in the intensity of autofluorescence in the neuropil of a region of the cerebral cortex were also investigated.

The formaldehyde-induced fluorescence in the neuropil of the rat nucleus caudatus-putamen is almost entirely due to the fluorophore derived from DA in the terminal parts of the nigro-striatal system of DA-containing neurones. (52) However, with or without formaldehyde treatment, the neuropil of the cerebral cortex shows a diffuse green fluorescence, which, after formaldehyde treatment, is only marginally affected by reserpine administration, 10 mg/kg i.p. 4 hrs. before killing (Section A;B) and is therefore almost entirely due to non-specific, i.e. not formaldehyde-induced, autofluorescence. (It should be noted that autofluorescence may be diminished by formaldehyde treatment) (44)

After depletion of striatal DA by reserpine, 10 mg/kg i.p. 4 hrs. before killing, it has been shown that the residual fluorescence of the striatal neuropil is of almost identical intensity to that of the cortical neuropil in each experiment (Section A;B) which indicates that both regions have a similar intensity of autofluorescence in the neuropil. Therefore the fluorescence in the neuropil of the nucleus caudatus-putamen after formaldehyde treatment is a combination of formaldehyde-induced fluorescence and autofluorescence. In this study the contribution of the autofluorescence to the total fluorescence of the striatal neuropil has been inferred from

measurements of the fluorescence in the neuropil of a region of the cerebral cortex.

Materials and Methods

Male P.V.G rats of approximately 150 g were used and were decapitated under chloroform anaesthesia. Dissected samples, each containing regions of the nucleus caudatus-putamen and cerebral cortex were prepared for fluorescence microscopy and examined as previously described. (Section A;Introduction,A) Sections at a microtome setting of 8 μm were cut on the day after paraffin wax embedding and the ribbons were then stored in darkness for varying periods, in slide trays contained in non-airtight plastic boxes, before being mounted in Entellan. The time between killing and freezing of the samples was almost identical for all the tissue in each experiment.

The intensity of fluorescence in a region of neuropil was measured from circular areas each with a diameter of 10 μm. The areas were arbitrarily chosen in each region and while a choice of an area was being made, the intensity of the exciting light, controllable by an iris diaphragm, was much reduced. 3 adjacent sections were taken to represent each tissue sample, and measurements of the intensity of fluorescence were taken from 6 areas from each selected region of each section. The mean of the 18 readings from either the nucleus caudatus-putamen or cerebral cortex of each tissue sample was taken as an estimate of fluorescence intensity.

Changes during long-term storage:- A tissue sample from each of 3 rats was prepared for fluorescence microscopy and sectioned on the day after embedding, the tissue sections were stored as ribbons for varying periods of up to 13 weeks, before being mounted in Entellan on the same day as examination.

Changes during short-term storage:- A tissue sample from each of 7 rats was prepared for fluorescence microscopy and sectioned on the day

after paraffin wax embedding. The sections were then examined 5 days after sectioning, to investigate the effects of 2 sets of storage conditions, Set A and Set B, during the 5 day period. In Set A, some of the tissue sections were mounted in Entellan within 2 hrs. of sectioning and then stored in darkness, for 5 days before examination. In Set B, the remaining tissue sections were stored as ribbons for 3 days before being mounted in Entellan and the mounted sections were then stored for a further 2 days, in darkness, before examination. Sections from both Set A and Set B were examined during the same period of microscopy.

Results

Changes during long-term storage:- The size of an arbitrary unit of fluorescence intensity varied between different periods of microscopy, e.g. because of changes in the intensity of the exciting light. (Section A; A,G) Thus it was necessary to avoid the expression of the changes in fluorescence in units of fluorescence intensity. The ratio of the fluorescence intensity of the neuropil of the nucleus caudatus-putamen (derived from a combination of autofluorescence and formaldehyde-induced fluorescence) to the fluorescence intensity of the neuropil of the cerebral cortex (derived almost entirely from autofluorescence) was calculated for each period of microscopy.

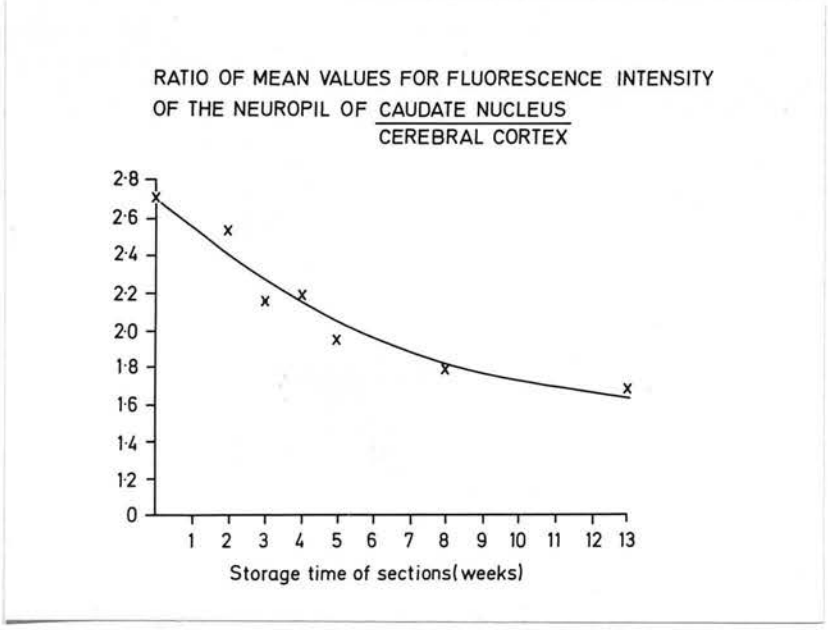


Figure 47. Changes in fluorescence intensity associated with storage of unmounted tissue sections prepared by the formaldehyde-fluorescence method. Each point represents 3 rats. For each rat, the mean of 18 measurements represented the value for the fluorescence intensity of each region.

The contrast in fluorescence intensity between the two regions became less marked during the period of storage and this change was accompanied by a gradual increase (> 150% after 13 weeks) in the intensity of the autofluorescence of the neuropil of the cerebral cortex. This increase was observed by comparing the stored sections with freshly prepared samples from new blocks of fresh tissue at each period of microscopy.

Changes during short-term storage:-

Fluorescence intensity. (Arbitrary units)

	Nucleus caudatus-putamen		Cerebral cortex	
Rat	Set A	Set B	Set A	Set B
1	59	71	38	34
2	49	63	30	30
3	47	60	31	31
4	52	64	36	37
5	48	59	35	30
6	55	70	34	31
7	49	59	34	32

Table 9. Changes in the fluorescence intensity of the neuropil of the nucleus caudatus-putamen during storage of tissue sections prepared by the formaldehyde-fluorescence method. The sections were stored under two sets of conditions, Set A and Set B, for 5 days. The values for the fluorescence of the neuropil of the cerebral cortex, derived almost entirely from autofluorescence, are also shown. Each value represents the mean of 18 readings of fluorescence intensity.

While the fluorescence intensity of the cortical neuropil did not show significant differences between the two sets of storage conditions, the values for the fluorescence intensity of the striatal neuropil were significantly higher in Set B, $P = 0.016$ (Sign test, 2-tailed).

Discussion

In the tissue which had been stored as ribbons over a period of 13 weeks, there was a marked increase in the autofluorescence of the neuropil of the cerebral cortex. If it is assumed that a similar increase occurred in the autofluorescence of the striatal neuropil, which would have had a similar initial fluorescence intensity to that of the cortical neuropil, the results

in figure 47 indicate that during the storage period the formaldehyde-induced component of the striatal fluorescence did not increase at the same rate as the autofluorescent component. The changes in figure 47 may have involved a decrease in the intensity of the formaldehyde-induced striatal fluorescence.

In table 9 the values for the autofluorescence of the neuropil of the cerebral cortex did not differ significantly between Set A and Set B, therefore the increase in striatal fluorescence in Set B was almost certainly due to an increase in the formaldehyde-induced component of the total fluorescence of the striatal neuropil.

This assumes that the intensity of the autofluorescent component of the striatal fluorescence can be inferred from the intensity of the autofluorescence of the cerebral cortex, and this assumption was confirmed in a subsequent experiment which examined tissue from rats whose striatal DA had been depleted by reserpine administration. On the same day as sectioning, the intensity of the residual fluorescence of the striatal neuropil after reserpine administration (10 mg/kg i.p. 4 hrs. before killing) was almost identical to that of the cortical neuropil, and there was no significant contrast between the regions after storage for 3 days as ribbons followed by 2 days storage mounted in Entellan.

The results in table 9 also suggest that the marked increase in the specific formaldehyde-induced fluorescence in Set B took place in the 3 days after sectioning, while the sections were being stored as ribbons, and that this increase was prevented by mounting the sections in Entellan. This was confirmed in subsequent experiments. If the autofluorescent component of the fluorescence of the striatal neuropil is assumed to have been identical to that of the cortical neuropil, then the mean increase in specific formaldehyde-induced fluorescence in the striatal neuropil of Set B, relative to Set A, was $> 80\%$.

The results show the importance of the conditions of storage of sections in quantitative fluorescence studies and indicate that a few days storage as ribbons can produce a considerable increase in the intensity of specific formaldehyde-induced fluorescence in the nucleus caudatus-putamen.

Summary

Changes are reported in the fluorescence intensity of formaldehyde-induced fluorescence and of autofluorescence during storage of tissue sections of the nucleus caudatus-putamen and cerebral cortex of the rat brain. Storage as 'ribbons' over 13 weeks was accompanied by a gradual increase in autofluorescence, while storage as 'ribbons' for 3 days after sectioning was associated with a marked increase in the formaldehyde-induced fluorescence of the neo-striatal neuropil. The latter was prevented by mounting the sections in Entellan.

G. Variations in fluorescence intensity due to changes in the intensity of the exciting light.

Introduction

Although the HBO 100 w/2 Osram mercury arc lamp is described as having an "extremely good arc stability" being "particularly well suited as a light source for photometric measurements of fluorescence" (80), variations in the intensity of exciting light occur within and between periods of microscopy.

Such variations can be monitored by the use of a fluorescence standard of uranyl glass (95). In the present study the Schott fluorescence standard GG 21, 3 mm in thickness, was used. The uncorrected emission spectrum of this standard was obtained using the Veril S 200 graduated interference filter, (Section A;Introduction) which was placed between the specimen and the photomultiplier after the barrier filter '47' had been removed from the light path.

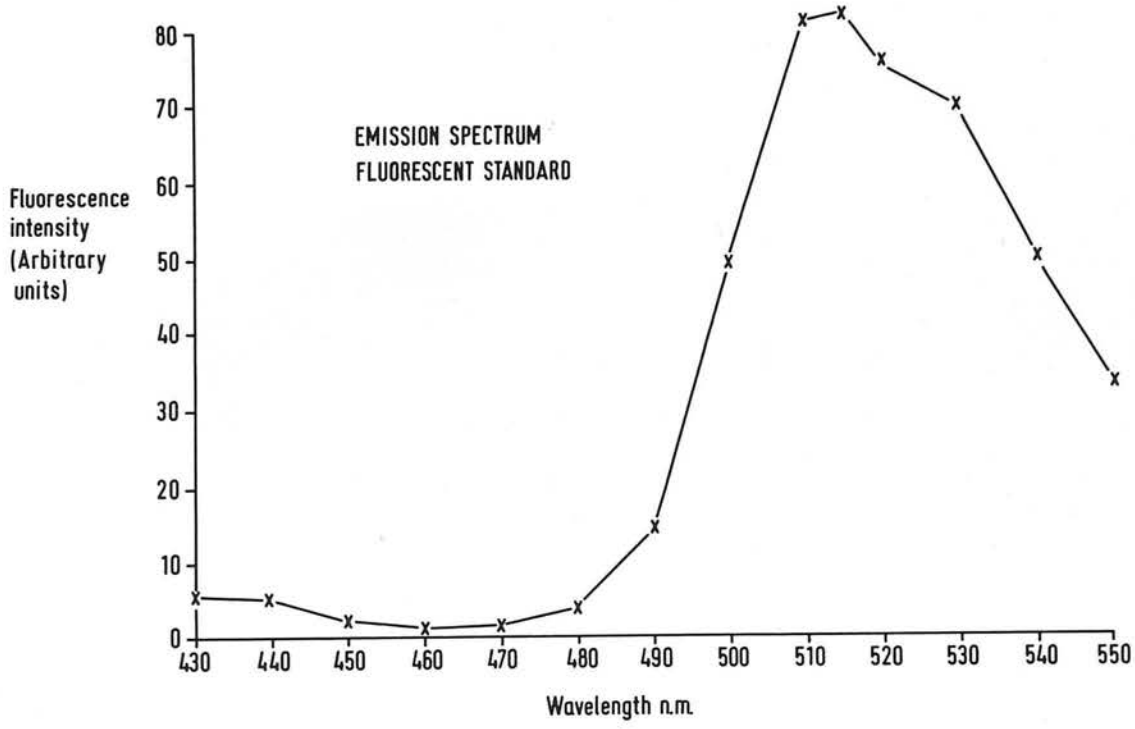


Figure 48. Uncorrected (See Section A;D) emission spectrum of Schott fluorescence standard GG 21, obtained with vertical illumination using a Veril S 200 graduated interference filter. A X40 Neofluor (Zeiss) objective was used (NA 0.75), which was placed in contact with the standard. Details of the fluorescence microscope have been described. (Section A;Introduction) Compare with figures 44 and 45.

It was found that a period of about 20 min was needed between switching on the HBO 100 lamp and taking measurements, as this initial period was associated with a gradual increase in the intensity of the emission of the lamp. In all the studies reported in this thesis a

single measurement of the fluorescence intensity of the standard was measured, using the X40 Neofluor objective as described, at intervals of about 15 min, throughout each period of microscopy. An individual period of microscopy could last up to 5 hrs. There was no consistent direction of change in the intensity of the exciting light, as detected by the standard, during a period of microscopy. Typically, the highest value for fluorescence of the standard did not exceed the lowest value by more than 10% of the latter during each period of microscopy. However, larger variations of up to 20% of the lowest value for the fluorescence of the standard were occasionally detected.

During the life time of an HBO 100 lamp, the intensity of the emitted light declines. Another factor which caused differences in the intensity of the exciting light between periods of microscopy was variation in the centring of the exciting light onto the specimen.

The emission spectrum of the standard differed from the spectra of the diffuse fluorescence shown in figures 44 and 45, and the standard was of a different thickness compared with the tissue sections. In addition, different objectives were used for examining the standard and tissue sections. Thus any change in the fluorescence of the standard, due to a change in the intensity of the exciting light, was expected to be different from the corresponding change in the fluorescence intensity of a tissue section.

The relationship between a change in fluorescence of the standard to a change in the fluorescence in tissue sections, resulting from a change in the intensity of exciting light, was investigated in this study.

Materials and Methods

2 male P.V.G. rats were used, approximately 140 g in weight. They were decapitated under chloroform anaesthesia and dissected samples were prepared for fluorescence microscopy and examined as previously described. (Section A;Introduction,A) The sections were stored for 2 days before

being mounted in Entellan. The details of the sample containing parts of the nucleus caudatus-putamen and cerebral cortex have been described, (Section A;A) together with details of the regions examined.

The intensity of diffuse fluorescence in the neuropil was measured from circular areas each with a diameter of 10 μm. Measurements were taken from 6 arbitrarily chosen areas from the selected part of each of the 2 regions of each section. 3 adjacent sections were selected for fluorescence microscopy for each tissue sample. The mean of the 18 readings from each of the 2 regions from each rat was taken as a value of fluorescence intensity.

The intensity of the exciting light was varied by inserting 5 different thicknesses of the UG5 exciter filter, ranging from 2-7 mm, into the light path.

Results

During the 3 hr. period of microscopy, the fluorescence intensity of the standard, associated with the routinely used UG5 filter, 2 mm in thickness, was measured every 15 minutes. These values, in arbitrary units, varied from 56 to 60. Variations within this range were in both directions and some occurred suddenly, during the measurement, accompanied by an observable change in the fluorescence intensity. Therefore these changes in the fluorescence intensity were almost certainly due to changes in the intensity of the light source.

15 adjacent sections represented each tissue sample and 3 were examined for each of the 5 different thicknesses of the UG5 exciter filter.

Fluorescence intensity in arbitrary units

Thickness of exciter filter UG5 (Schott)	Standard GG21 (Schott)		Nuc.caudatus -putamen (A)		Cerebral cortex (B)		Ratio of means $\frac{A}{B}$
2 mm	58	Rat 1	77		41		
				M.83		M.39	
		Rat 2	88		37		2.12
3 mm	42	Rat 1	63		34		
				M.65		M.32	
		Rat 2	67		30		2.03
4 mm	27	Rat 1	50		25		
				M.55		M.27	
		Rat 2	60		28		2.03
5 mm	22.5	Rat 1	48		26		
				M.53		M.27	
		Rat 2	58		28		1.96
7 mm	15	Rat 1	36		19		
				M.39		M.19	
		Rat 2	42		19		2.05

Table 10. Changes in the intensity of a fluorescent standard and changes in the fluorescence intensity of tissue sections, resulting from changes in the intensity of exciting light. The tissue sections were prepared by the formaldehyde-fluorescence method. The intensity of exciting light was varied by changing the thickness of the exciter filter. Each value representing the standard is a single measurement. Each value representing the tissue sections is the mean of 18 readings of fluorescence intensity. M = Mean.

In considering the relation of a change in the intensity of the fluorescence standard to a corresponding change in the fluorescence intensity of tissue sections, resulting from changes in the intensity of exciting light, there are 10 possible combinations of two different thicknesses of exciter filter. (Table 11)

The differences between 2 values of fluorescence intensity as a percentage of the lower value

Thicknesses of exciter filter	Fluorescent standard	Cerebral cortex	Nuc. caudatus-putamen
3 mm and 2 mm	38%	22%	28%
4 mm and 2 mm	114	44	51
5 mm and 2 mm	152	44	57
7 mm and 2 mm	285	105	113
4 mm and 3 mm	55	19	18
5 mm and 3 mm	87	19	23
7 mm and 3 mm	180	68	67
5 mm and 4 mm	20	0	4
7 mm and 4 mm	80	42	41
7 mm and 5 mm	50	42	36

Table 11. A comparison of the corresponding changes in fluorescence intensity, resulting from changes in the intensity of exciting light, in a fluorescent standard and in tissue sections prepared by the formaldehyde-fluorescence method. The thickness of the exciter filter determined the intensity of exciting light. The mean values shown in table 10, associated with each of the 10 possible pairings of exciter filter thickness, are compared by expressing the difference between each pair of mean values as a percentage of the lower value. These differences are shown graphically in figure 49.

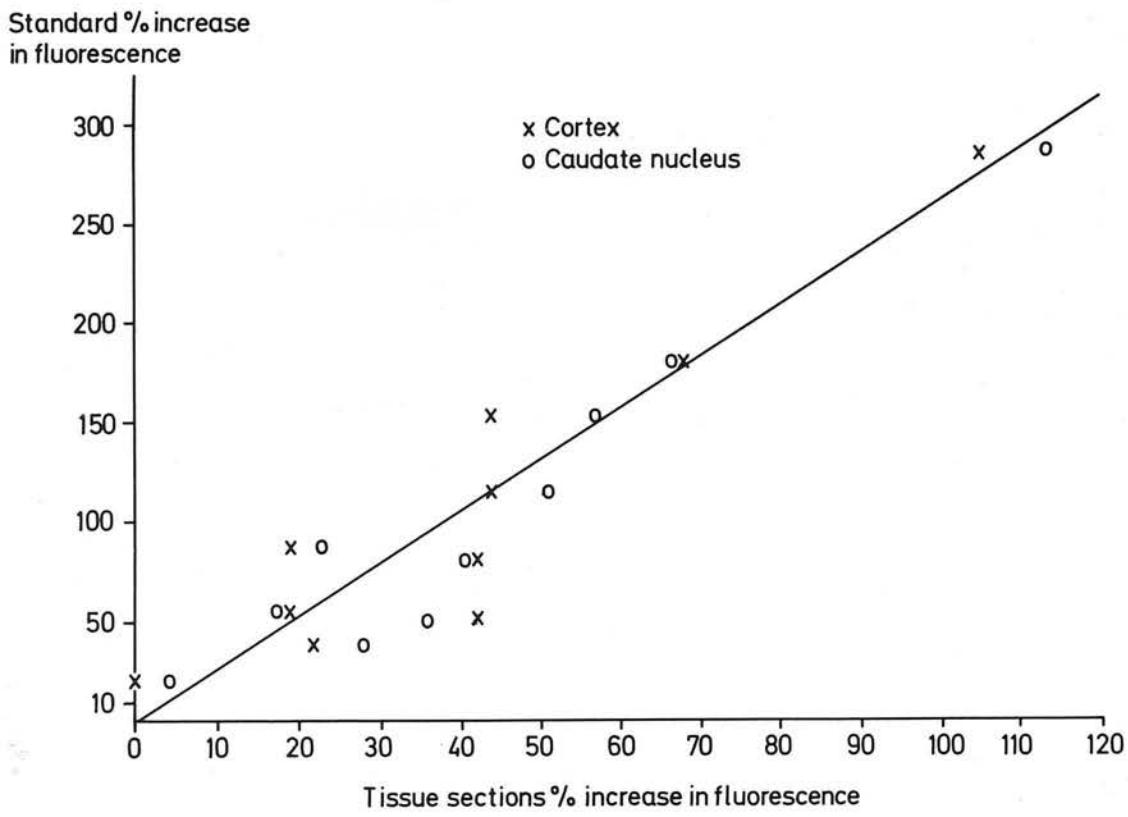


Figure 49. The relation between changes of fluorescence intensity shown by a fluorescent standard and corresponding changes shown by tissue sections, resulting from changes in the intensity of exciting light. See table 11. A change in intensity of fluorescence is expressed as a % of each lower value.

Discussion

In the studies reported in this thesis no attempt was made to compare fluorescence intensity between different periods of microscopy. This was because of variations between experiments in the yield of fluorophore and in the intensity of exciting light reaching the specimen.

This study investigated changes in the intensity of the exciting light during a period of microscopy. It was shown that the fluorescent standard GG 21 (Schott) 3 mm could be used to detect and monitor variations in the intensity of exciting light. For any 2 intensities of exciting light in the range of excitation intensity studied, a difference in the fluorescence intensity of tissue sections, expressed as a percentage of the lower value, was less than the corresponding difference shown by the fluorescent standard.

In this and all other studies, the excitation intensity of the HBO 100 lamp was monitored, at 15 min intervals throughout each period of microscopy, by the fluorescent standard. It was usually found that the highest value for the fluorescence of the standard did not exceed the lowest value by more than 10% of the latter during a period of microscopy. Thus, the results suggest that errors in the measurements of fluorescence in tissue sections in a typical experiment, due to changes in the intensity of exciting light, were less than 10% of the fluorescence intensity associated with excitation by the minimum intensity of exciting light during the period of microscopy.

In view of these results it was decided to routinely ignore variations in the intensity of exciting light during a period of microscopy, when measuring diffuse fluorescence in 8 μm sections. However on the rare occasions when a variation exceeding 10% of the lowest intensity of the standard was detected, the subsequent results were not compared with those taken before the change in the excitation intensity.

It should be noted that in table 10, the ratios of the fluorescence intensity of the nucleus caudatus-putamen to that of the cerebral cortex

showed only a small and inconsistent variation. This suggests that no significant "inner filter" quenching of the fluorescence (i.e. due to insufficient excitation of the deeper parts of the section) occurred in either region, associated with a decreasing intensity of the exciting light. (See General Introduction)

Summary

Using the HBO 100 Osram mercury lamp with the Zeiss large fluorescence microscope, variations in the intensity of the light source occur within and between periods of microscopy.

A fluorescent glass standard was used, in all studies reported in this thesis to detect and monitor (at 15 min intervals) variations in the intensity of the light source during each period of microscopy. This study investigated changes in the intensity of the fluorescent standard by comparing these with corresponding changes in the fluorescence intensity of the neuropil of tissue sections, which had resulted from changes in the intensity of the exciting light produced by varying the thickness of the excitor filter.

On the basis of the results it was decided that variations in the intensity of exciting light during a continuous period of microscopy (i.e. up to 5 hrs.) could be ignored when measuring areas of diffuse parenchymal fluorescence, 10 μm in diameter, in sections cut at 8 μm.

However, in this and a few other subsequent experiments, unusually large variations in the intensity of the light source during a period of microscopy, detected by use of the standard, were considered sufficient to prevent a comparison of subsequent readings with the readings taken before the change in excitation intensity.

H. The relationship between size of a dissected tissue sample and intensity of diffuse fluorescence in the neostriatal neuropil, after preparation by the formaldehyde-fluorescence method.

Introduction

The intensity of diffuse formaldehyde-induced fluorescence or autofluorescence may be affected by the following variables:- (See General Introduction)

(i) Size of tissue sample. (This may affect the degree of residual water in parts of the tissue sample after freeze-drying and the degree of exposure of the inner regions of a tissue sample to formaldehyde gas.)

(ii) The original position, within the tissue sample, of the tissue sections selected for microscopy, relative to the surfaces of the tissue sample.

The effect of size of tissue sample on fluorescence intensity was investigated in this study.

Materials and Methods

3 male P.V.G. rats were used, approximately 130 g in weight, and were decapitated under chloroform anaesthesia 1 hr after receiving L-DOPA 350 mg/kg i.p. Coronal slices, approximately 3 mm in thickness, of a cerebral hemisphere contained part of the nucleus caudatus-putamen and were prepared and examined as previously described. (Section A; Introduction, A) In addition, a smaller sample with dimensions of approximately 1 mm X 2 mm was dissected from the contralateral cerebral hemisphere of each rat, from the region under investigation, i.e. the dorso-lateral part of that region of the nucleus caudatus-putamen between the planes 18 and 14 shown in figure 39. This region was identified before dissection, with the aid of macroscopic reference points, as histological localization of the region could not be carried out due to the size of the sample.

The tissue sections were stored for 2 days before being mounted in Entellan. The solution of L-DOPA for injection was prepared in 0.08 NHCl at a concentration of 10 mg/ml.

The intensity of diffuse fluorescence in the neuropil of the nucleus caudatus-putamen was measured from circular areas, each with a diameter of 10 μm. Three adjacent 8 μm sections represented each tissue sample, and measurements were taken from 6 arbitrarily chosen areas from the selected region of each section. The mean of the 18 readings from each tissue sample was taken as the value for the fluorescence intensity.

L-DOPA was given to increase the intensity of formaldehyde-induced fluorescence in the nucleus caudatus-putamen (32) so that, assuming this region in both hemispheres would show a similar level of fluorescence intensity in each rat after L-DOPA administration, any difference between the two samples from each rat would be more readily detected.

Results

	Fluorescence intensity (Arbitrary units)	
Rat	Larger sample	Smaller sample
1	128	120
2	158	161
3	155	149

Table 12. The relationship between size of tissue sample and fluorescence intensity of the striatal neuropil in tissue prepared by the formaldehyde-fluorescence method. Each value is the mean of 18 measurements from 1 tissue sample. Each rat received L-DOPA 350 mg/kg i.p. 1 hr. before killing.

Discussion

The important variables in the freeze-drying process have been reviewed. (See General Introduction) Theoretically, the size of tissue sample can be an important variable affecting the residual water content of parts of a tissue sample. The size of a tissue sample may also affect the degree of exposure of the inner regions of the sample to formaldehyde gas. Although there was

variation between rats in the effect of L-DOPA on the intensity of diffuse fluorescence, this study did not demonstrate a marked or constant effect of the variation in size of the tissue samples on the yield of formaldehyde-induced fluorophore. Similar results were also found in subsequent experiment using rats untreated by drugs and rats which had received L-DOPA.

Summary

The relationship between size of a tissue sample and diffuse formaldehyde-induced fluorescence was examined in a region of the neuropil of the nucleus caudatus-putamen in rats pretreated with L-DOPA.

The size of a tissue sample may affect the degree of residual water in parts of a sample after freeze-drying, and the degree of exposure of the inner regions of a sample to formaldehyde gas.

The results did not demonstrate any effect of size of sample on the intensity of formaldehyde-induced fluorescence within the range of sizes used.

I. Histochemical differentiation of the formaldehyde-induced fluorophores derived from dopamine and L-DOPA.

Introduction

The fate of administered L-3, 4-dihydroxyphenylalanine (L-DOPA) has been investigated in animals by the formaldehyde-fluorescence method. (32) One difficulty in this type of study is that histochemical differentiation of L-DOPA from dopamine (DA) has been impossible as their fluorophores have similar properties. (66) In the present study a method of differentiating the fluorophores derived from L-DOPA and DA is reported, based on their rates of photodecomposition during irradiation with ultraviolet light. Fading of the fluorophores in a model system of dried protein droplets and in tissue sections is described.

Materials and Methods

A range of concentrations of L-3, 4-dihydroxyphenylalanine and dopamine hydrochloride (Koch-Light) were prepared by dissolving these substances in 0.04N HCl. Each solution was then added to an equal volume of 2% aqueous bovine serum albumin. (Merck) These final solutions contained 1% albumin and a range of 6 concentrations of L-DOPA from 0.062 to 3 mg/ml and a range of 9 concentrations of DA from 0.062 to 12 mg/ml. 1 µl droplets from each of these final solutions were placed on glass slides and air dried prior to exposure to gaseous formaldehyde, generated from paraformaldehyde stored at a relative humidity of 58%, for 1 hr at 80°C. The reaction vessel contained 5 g paraformaldehyde/litre. The droplets were then mounted in Entellan (Merck) and examined with a Zeiss large fluorescence microscope incorporating the EMI 6256B photomultiplier. The light source was a stabilized HBO 100 Osram mercury lamp and a combination of the exciter filters UG5 and BG38/2.5 mm (Schott) was selected together with the barrier filter 47 (Zeiss). The droplets were examined by vertical illumination using a X40 oil immersion objective with a numerical aperture of 1.0. The intensity of the exciting light was much reduced by an iris diaphragm during

the selection of the areas for fluorimetry, and the fluorescence intensity during photodecomposition was measured for 2 min, from circular areas each with a diameter of 10 μm. One area was chosen from the brightest region of fluorescence of each droplet to be examined.

In addition, the fading rates of the fluorophores derived from DA and L-DOPA were examined in tissue sections. L-DOPA and a dopa-decarboxylase inhibitor (Ro4-4602) were administered to rats intraperitoneally and the rates of photodecomposition of the increased diffuse formaldehyde-induced fluorescence in the neuropil of the neostriatum and of the molecular layer of the cerebellum were studied. The increased neostriatal fluorescence was almost certainly partly derived from an increase in neostriatal DA (as well as from L-DOPA) (see Section C) while the increase in the cerebellar fluorescence would have been almost entirely derived from L-DOPA (See Discussion).

8 male P.V.G. rats (mean wt. 140 g) received N^1-(DL-seryl) - N^2-(2, 3, 4-trihydroxybenzyl) - hydrazine (Ro4-4602 La Roche) 50 mg/kg i.p., followed 30 min later by L-DOPA 150 mg/kg i.p. These rats, together with 2 control animals (untreated by drugs) were decapitated under chloroform anaesthesia 1½ hours after the first injection. L-DOPA for injection was dissolved in 0.08 N HCl and Ro4-4602 was dissolved in distilled water, both at a concentration of 10 mg/ml. Dissected samples from the nucleus caudatus-putamen and cerebellum were then prepared for fluorescence microscopy and examined as previously described. (Section A; Introduction, A) The sections were stored for 2 days before being mounted in Entellan.

Two adjacent sections were examined from each tissue sample and the areas for fluorimetry were arbitrarily selected from the neuropil of the neostriatum or the molecular layer of the cerebellum, using a reduced intensity of exciting light. Measurements of the intensity of fluorescence during photodecomposition for 2 min were taken from 1 area of

the selected part of each section. The rate of photodecomposition for each sample was calculated from the means of the corresponding values of the two sets of readings from each tissue sample.

Measurements were taken from the dorso-lateral area of approximately coronal sections of the nucleus caudatus-putamen. The details of the sample of nucleus caudatus-putamen have been described (Section A;A) together with details of the region examined. The samples of cerebellum were obtained from the paravermal region and the plane of sectioning was not constant.

Results

The dried droplets had approximately circular outlines with diameters ranging from 3-4 mm. There was no consistent variation in droplet diameter in relation to DA or L-DOPA concentration in the droplet solution. The drying process led to the fluorophore being concentrated in a ring surrounding a central area which showed minimal fluorescence. The resulting fluorescent ring of each droplet showed differences in width, depending on the concentration of DA or L-DOPA in the droplet solution. This is illustrated in figure 50.

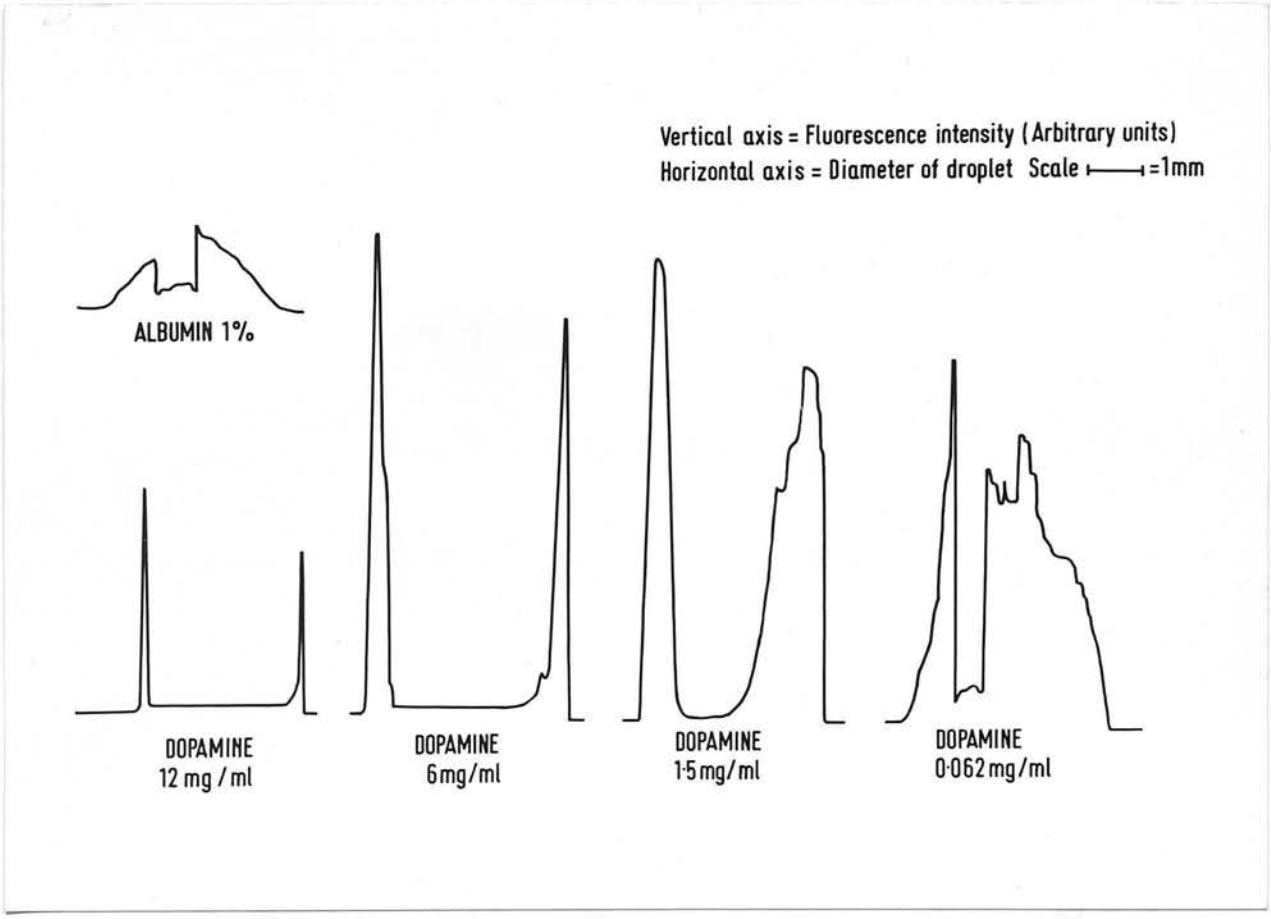


Figure 50. The distribution and intensity of formaldehyde-induced fluorescence in dried albumin-containing 1 µl droplets, in relation to the concentration of DA in the droplet solution. The fluorescence intensity was recorded graphically by a pen recorder from strips 10 µm in width, which were scanned at a constant speed across the diameters of four typical droplets. A 1 µl droplet of a 1% albumin solution was also examined. The concentration of DA in each droplet solution is shown.

The initial maximal intensity of fluorescence and the degree of fading of the fluorophores derived from the different concentrations of DA and L-DOPA in the droplet solutions are shown in table 13.

	Concentration in droplet solution. mg/ml.	Initial intensity of fluorescence. Arbitrary units.	Fluorescence intensity after 2 min of photo-decomposition. % of initial intensity.
DA	12	53	79
	9	65	77
	6	74	78
	3	75	79
	1.5	67	78
	0.75	55	78
	0.375	43	81
	0.125	38	82
	0.062	34	82
L-DOPA	3	47	68
	1.5	79	65
	0.75	76	62
	0.375	52	63
	0.125	39	67
	0.062	34	71
Control	Nil	12	83

Table 13. Photodecomposition of formaldehyde-induced fluorophores derived from L-DOPA and DA in a model system of dried albumin-containing 1 µl droplets. Each value of fluorescence intensity represents the mean of 3 readings, each from a separate droplet. The areas for fluorimetry were selected from the region of each droplet showing the most intense fluorescence. Values for control droplets, prepared from 1% albumin in 0.02N HCl are also shown.

Further droplets were examined and the results are shown in figure 51.

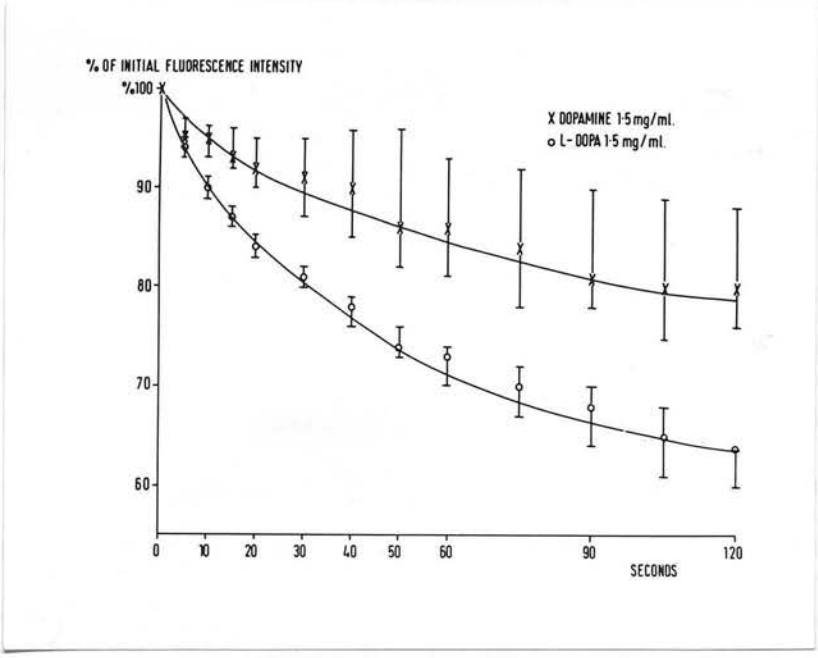


Figure 51. Rates of photodecomposition of formaldehyde-induced fluorophores derived from L-DOPA and DA in a model system of dried albumin-containing 1 µl droplets. A point represents the mean value of 5 readings, each from a different droplet, and the range of each mean value is given. The concentration of L-DOPA and DA in the droplet solutions is shown. The areas for fluorimetry were selected from the region of each droplet showing the most intense fluorescence.

In figure 51, the two sets of final values for the percentage of the initial intensity were significantly different at a level $P = 0.008$ (Mann-Whitney U test, 2-tailed). The mean values for the initial fluorescence intensity, in arbitrary units, were 80 (DA) and 77 (L-DOPA). As the preparations of DA and L-DOPA had similar molecular weights, the droplets examined for figure 51 were derived from 2 solutions of approximately equi-molar concentration. There was no obvious difference between the drying patterns of the droplets derived from the 2 solutions.

The rats treated with L-DOPA and Ro4-4602 showed a marked increase in diffuse parenchymal fluorescence in both regions studied. In the control animals (untreated by drugs) the neuropil of the molecular layer of the

cerebellum showed a diffuse non-specific autofluorescence, while the diffuse fluorescence in the neuropil of the neostriatum was derived from autofluorescence together with diffuse formaldehyde-induced fluorescence due to DA in the terminal parts of nigro-striatal neurones. (38) Values for the fluorescence intensity and rate of photodecomposition in the neuropil of the neostriatum and cerebellum of the control animals were subtracted from the values obtained from the rats treated with L-DOPA and Ro4-4602. The differences thus obtained represented the fluorescence intensity and photodecomposition of the increases in the formaldehyde-induced fluorescence in these regions, resulting from L-DOPA and Ro4-4602 administration.

In 7 of the 8 rats, the increased diffuse fluorescence in the cerebellum showed a greater degree of fading, compared with the increased diffuse fluorescence in the neostriatum. These differences were significant at a level $P = 0.02$ (Wilcoxon matched-pairs signed-ranks test, 2-tailed). After irradiation for 2 min the neostriatal increased fluorescence had faded to a mean level of 55% of the initial intensity, while the increased fluorescence of the cerebellum had fallen to a mean level of 45% of the initial intensity.

Discussion

The fluorophore resulting from the condensation of a catecholamine with formaldehyde is a 3,4-dihydroisoquinoline which is in a pH-dependent equilibrium with the corresponding tautomeric quinoidal form. The latter exhibits fluorescence with a maximum at approximately 480 nm, while the 3,4-dihydroisoquinoline has a less intense fluorescence with a maximum at 510 nm. (33) It should be noted that the reaction conditions for fluorophore formation in the model system of albumin-containing droplets may have differed from those associated with tissue sections, for example with respect to the pH which has been shown to affect the yield of fluorophore derived from DA in a similar model system. (66)

In figure 50, the maximal intensity of fluorescence which was measured from the droplet derived from the solution of DA 12 mg/ml was less than the maximal values obtained from the other droplets which were derived from more dilute solutions of DA. This suggests that quenching of the fluorophore had taken place and table 13 shows a similar finding with regard to the droplets derived from L-DOPA. The fluorescence of any substance will undergo quenching if the concentration is over a threshold level and this "true" concentration quenching should be distinguished from an "inner filter" effect caused by incomplete excitation of the specimen. (96) It is possible that "true" concentration quenching and an "inner filter" effect occurred in this study. Differences in the concentration of fluorophore in the fluorescent rings of the droplets derived from different concentrations of DA or L-DOPA would have been partly due to the changes in the drying pattern of the droplets, as well as to the concentrations of DA and L-DOPA in the droplet solutions. At equivalent concentrations up to 1.5 mg/ml in the droplet solutions, both L-DOPA and DA produced a similar drying pattern of the droplets with respect to the width of the fluorescent ring, but the width of the ring of a typical droplet derived from the solution of L-DOPA 3 mg/ml was less than that of a droplet derived from DA 3 mg/ml. This may explain the difference in the degree of apparent quenching of the fluorescence, between the fluorophores from DA and L-DOPA, associated with a concentration of 3 mg/ml in the droplet solutions (see table 13). Differences in the fading rates of the fluorophores derived from L-DOPA and DA were maintained despite the different concentrations of DA and L-DOPA in the droplet solutions and the different degrees of apparent quenching. (Table 13)

If Ro4-4602 is administered to the rat in combination with L-DOPA, there is a marked increase in the formaldehyde-induced diffuse fluorescence in all brain regions which is greater than that produced by the same dose of L-DOPA alone. (32) Although a sufficient dose of L-DOPA

alone gives rise to a marked increase in diffuse neostriatal fluorescence in the rat, large doses of L-DOPA only give rise to relatively small increases in the diffuse fluorescence of the cerebellum. (38) An additional effect of a combination of L-DOPA and Ro4-4602 is to produce a similar degree of increase in the intensity of the diffuse fluorescence in these regions. (See Section C) Ro4-4602 reduces the extracerebral metabolism of L-DOPA which leads to increased blood levels of L-DOPA together with an increased accumulation of L-DOPA in the CNS. (14,32) A combines biochemical-histochemical study in the rat (32) has indicated that the increase in diffuse fluorescence in the neostriatal parenchyma produced by administered L-DOPA following Ro4-4602 50 mg/kg i.p. 1½ hrs. before killing, was associated with a marked increase of DA in the neostriatal parenchyma, in contrast to regions such as the cerebellum and cerebral cortex where the increased fluorescence was mainly derived from L-DOPA. This can be explained by the high concentration of dopa-decarboxylase in the neostriatal parenchyma, compared with the other brain regions. (64) Ro4-4602 at this dose was found to have produced selective inhibition of extracerebral dopa-decarboxylase (and of the decarboxylase in the capillary walls of the CNS) while the enzyme remained relatively active in the brain parenchyma.

In the present study, the increased fluorescence resulting from L-DOPA and Ro4-4602 administration showed faster photodecomposition in the cerebellum than in the neostriatum. This suggests that a difference in the fading rates of the fluorophores derived from L-DOPA and DA, provides a method for distinguishing the fluorophores derived from these substances in tissue sections.

Summary

A method of differentiating the formaldehyde-induced fluorophores derived from L-DOPA and dopamine (DA) is reported, based on their rates of photodecomposition during irradiation with ultraviolet light.

In a model system of 1 µl droplets containing 1% bovine serum albumin, the photodecomposition of the fluorophores derived from a range of concentrations of L-DOPA and DA in the droplet solutions was examined. After irradiation for 2 min the fluorescence derived from L-DOPA was found to have shown a greater degree of fading compared with the fluorescence derived from DA, and a similar contrast was maintained irrespective of different concentrations of L-DOPA or DA in the droplet solutions.

In addition, photodecomposition of the fluorophores derived from DA and L-DOPA was examined in tissue sections of the rat brain. The results suggest that the findings in the model system can provide a method for distinguishing the fluorophores derived from DA and L-DOPA in tissue sections.

Section B

Studies of the nigro-striatal system of dopamine-containing neurones

Studies of diffuse formaldehyde-induced fluorescence in the neuropil of the rat nucleus caudatus-putamen. (A,B)

Functional activity of nigro-striatal dopamine-containing neurones. (C)

Results

A. Investigation of intersample variables affecting the intensity of fluorescence in a region of the nucleus caudatus-putamen prepared by the formaldehyde-induced fluorescence method.

Introduction

Studies of the intensity of formaldehyde-induced diffuse fluorescence in the neuropil of the rat nucleus caudatus-putamen have been described. (Section A;A) In those experiments each dissected sample was a coronal slice of a cerebral hemisphere, approximately 3 mm in thickness, the plane of dissection being approximately parallel with the frontal zero plane of König and Klippel. (See figure 38) The posterior plane of dissection was about 0.5 mm posterior to the posterior aspect of the anterior commissure in the mid-sagittal plane.

After preparation and sectioning, representative sections from different parts of each tissue sample were stained with Luxol Fast Blue and Cresyl Violet so that the region of each sample which corresponded to that part between the planes 14 and 18, shown in figure 39, could be identified. The possible variation in the antero-posterior level of the coronal plane of the sections selected for microscopy (between the planes 14 and 18) was over approximately 1.5 mm, extending anteriorly from the anterior border of the median part of the anterior commissure.

In the present study, dissected samples of this region of the nucleus caudatus-putamen were examined from both cerebral hemispheres from each of 7 rats to investigate the effects of intersample variables on the fluorescence intensity of the striatal neuropil.

Materials and Methods

Seven Female Campbell rats were used ranging from 120 to 170 g in weight. They were decapitated under chloroform anaesthesia and dissected samples from both hemispheres in each rat were prepared for fluorescence microscopy

and examined as previously described. (Section A;Introduction,A,G) The sections were stored for 2 days before being mounted in Entellan. The details of each sample have been described, together with details of the region examined. (Section A;A)

The intensity of diffuse fluorescence was measured from circular areas each with a diameter of 10 μm. Measurements were taken from 6 arbitrarily chosen areas from the selected part of each section. 3 adjacent sections were selected for microscopy from each tissue sample. The mean of the 18 readings from each tissue sample was taken as an estimate of fluorescence intensity.

The 14 samples were processed together and examined during one continuous period of microscopy.

Results

Rat	Fluorescence intensity. (Arbitrary units) Nuc.caudatus -putamen Right side	Rank of values	Nuc.caudatus -putamen Left side	Rank of values
1	76	4.5	72	1.5
2	76	4.5	77	4
3	67	1	71	1
4	84	7	79	7
5	80	6	78	5.5
6	73	2.5	72	1.5
7	73	2.5	78	5.5
Mean	75		75	

Table 14. Fluorescence intensity of a region of the neuropil of the nucleus caudatus-putamen in tissue sections prepared by the formaldehyde-fluorescence method. Each value represents the mean of 18 measurements from 1 tissue sample.

The mean of the differences in the fluorescence intensity between the two sides of each rat was approximately 3 arbitrary units, which is equivalent to 4% of the mean of the values from the 14 samples. In this experiment the mean contribution of the autofluorescence of the neuropil was approximately 55% of the total fluorescence. (See Section A;B)

The Spearman rank correlation coefficient (84), with respect to the correlation between right and left sides, is 0.64. Thus although there was a correlation between the 2 sides, it is not significant, even as a 1-tailed test, at the 0.05 level.

Discussion

If it is assumed that the nucleus caudatus-putamen in each animal had equal DA concentrations in corresponding regions of both hemispheres, the differences in fluorescence intensity between right and left sides in each animal indicated the effects of such variables as:-

(i) Variations in the region of nucleus caudatus-putamen examined, within the limits described

(ii) Variations in the degree of freeze-drying, or in the degree of exposure to formaldehyde gas

(iii) Variations resulting from the many other methodological variables previously described. (See General Introduction)

Differences between samples from different rats would be the result of the variables described above together with any differences in the neostriatal DA concentration between animals.

The intersample differences between the 2 samples from each rat were not sufficiently smaller than the intersample differences between different animals to show a significant correlation between paired samples from right and left hemispheres.

Summary

This study investigated variations between tissue samples in the intensity of diffuse formaldehyde-induced fluorescence and autofluorescence in the same region of the neuropil of the nucleus caudatus-putamen on the right and left sides of 7 rats.

The mean difference in the fluorescence intensity between the 2 sides of each rat was about 4% of the mean of the values from the 14 samples. (The mean contribution of the autofluorescence to the total fluorescence of the neuropil was approximately 55% of the total fluorescence.)

The differences between each pair of samples (i.e. between the right and left side of each rat) were sufficiently similar to the differences between samples from different animals so that no significant correlation between the fluorescence intensity of paired samples from right and left hemispheres could be demonstrated. (Spearman rank correlation coefficient test.)

B. Comparisons of the intensity of formaldehyde-induced fluorescence and autofluorescence between different regions of the neuropil of the nucleus caudatus-putamen.

Introduction

Studies of the intensity of formaldehyde-induced diffuse fluorescence in a region of the neuropil of the rat nucleus caudatus-putamen have been previously described. (Section A;B, Section B;A) In those experiments each dissected sample was a coronal slice of a cerebral hemisphere, approximately 3 mm in thickness, with planes of dissection approximately parallel with the frontal zero plane of König and Klippel. (See figure 38) The posterior plane of dissection was about 0.5 mm posterior to the anterior commissure in the mid-sagittal plane. After preparation and sectioning, representative sections from different parts of each tissue sample were stained with Luxol Fast Blue and Cresyl Violet to identify the region of each sample between the planes 14 and 18 shown in figure 39.

The intensity of the diffuse fluorescence in the neuropil of the nucleus caudatus-putamen was measured from circular areas each with a diameter of 10 μm. These areas were arbitrarily selected from the dorso-lateral part of the nucleus caudatus-putamen, i.e. dorso-lateral to an imaginary straight line at an angle of 45° to the vertical, dividing the nucleus caudatus-putamen of each tissue section into 2 parts with approximately equal areas.

The possible variation in the antero-posterior level of the coronal plane of the sections selected for microscopy was over approximately 1.5mm. extending anteriorly from the anterior border of the median part of the anterior commissure, i.e. corresponding to the region between the planes 14-18 of König and Klippel. (See figure 39) Any consistent differences in the intensity of fluorescence between sections from different coronal planes within the range 14-18 would have affected the results. The present study

investigated the intensity of fluorescence in different parts of the neostriatal neuropil between the planes 14-18 and in other parts of the nucleus caudatus-putamen.

Materials and Methods

Experiment A

Six female Campbell rats, 120-170 g in weight, were decapitated under chloroform anaesthesia and dissected samples from one hemisphere of each rat were prepared for fluorescence microscopy and examined as previously described. (Section A;Introduction,A,G) The sections were stored for 2 days before being mounted in Entellan.

Representative sections from different parts of each tissue sample were stained with Luxol Fast Blue and Cresyl Violet and the sections were identified which corresponded to:-

(i) The region of and between the planes 14 and 15 as described by König and Klippel (43, figure 39) i.e. Region A.

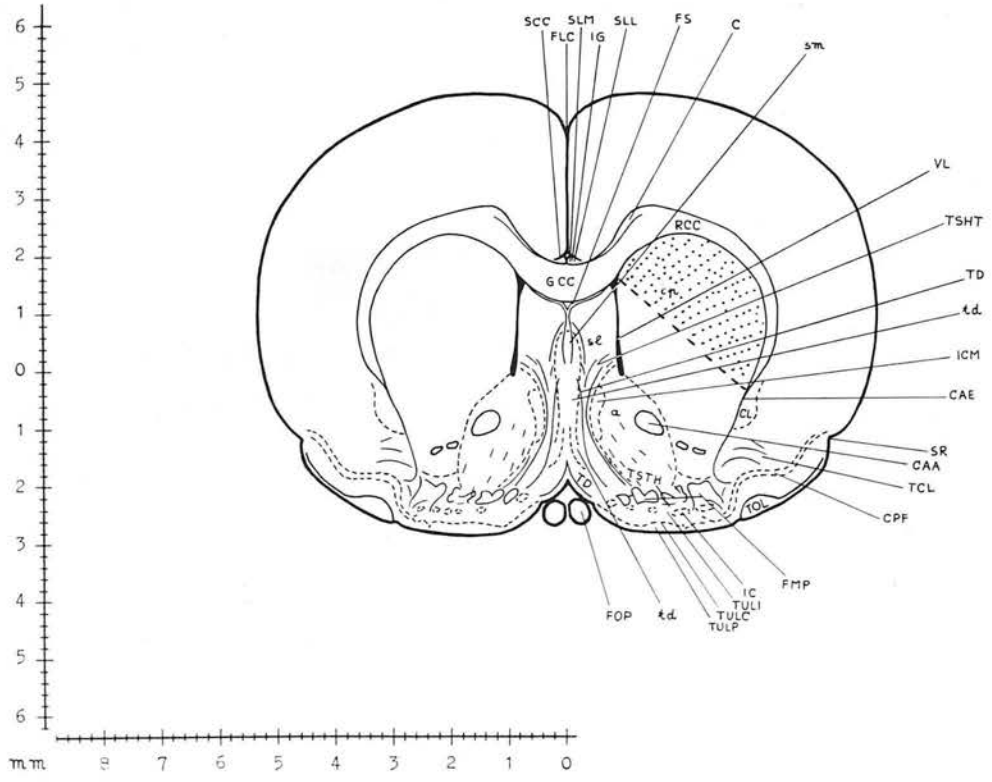


Fig. 14b

A 8620 μ

Figure 52. Coronal section of rat brain from König and Klippel's Stereotaxic Atlas of the Rat Brain. (72) This corresponds to plane 14. Measurements of fluorescence intensity were restricted to the stippled area. cp = Nucleus caudatus-putamen.

(ii) The region of and between the planes 17-18 as described by König and Klippel (72, figure 39) i.e. Region B.

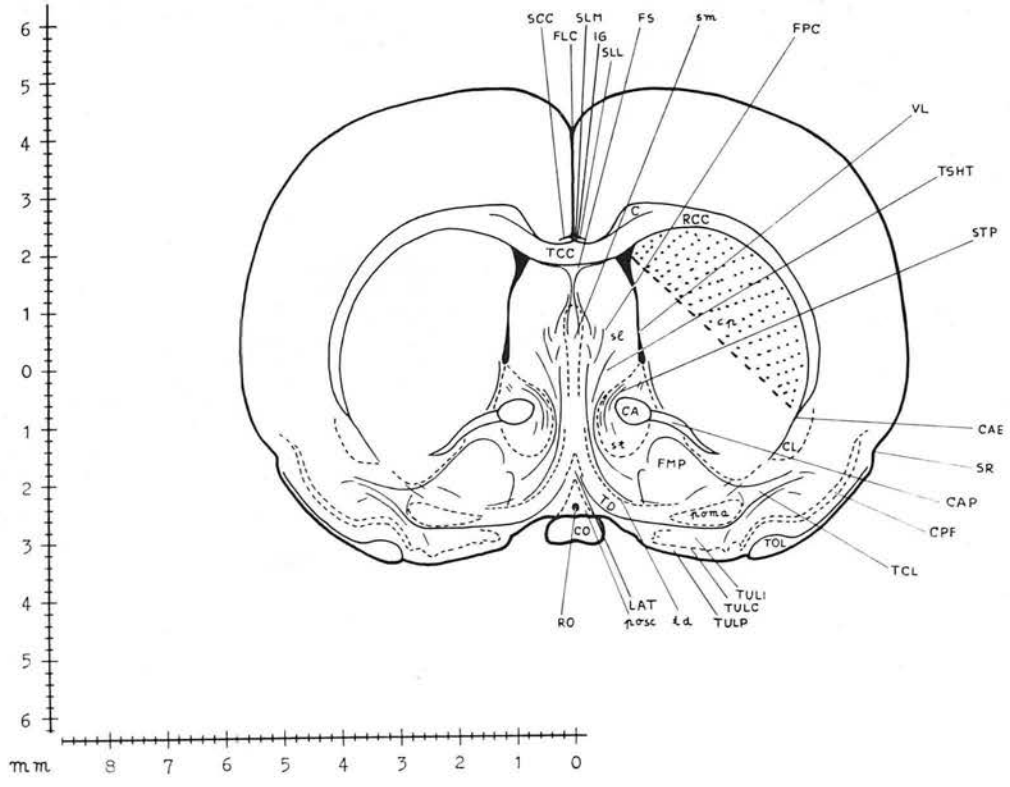


Fig. 17b
A 7470 μ

Figure 53. Coronal section of rat brain from König and Klippel's Stereotaxic Atlas of the Rat Brain. (72) This corresponds to plane 17. Measurements of fluorescence intensity were restricted to the stippled area. cp = Nucleus caudatus-putamen.

The intensity of the diffuse fluorescence in the neuropil of the nucleus caudatus-putamen was measured from circular areas each with a diameter of 10 μm. These areas were arbitrarily selected from the dorso-lateral part of the nucleus caudatus-putamen, i.e. dorso-lateral to an imaginary straight line at an angle of 45° to the vertical, dividing the nucleus caudatus-putamen of each tissue section into 2 parts with approximately equal areas. Measurements were taken from 6 arbitrarily chosen areas from the selected part of each section. 3 adjacent sections were selected for microscopy from

each of the 2 regions to be investigated from each tissue sample. The mean of the 18 readings from each of the 2 regions from each tissue sample was taken as an estimate of fluorescence intensity. All measurements were taken within a continuous period of microscopy.

Experiment B

Seven female P.V.G. rats, 150-180 g in weight, were decapitated under chloroform anaesthesia and 2 dissected samples from one hemisphere of each rat were prepared for fluorescence microscopy and examined as previously described. (Section A;Introduction,A,G) The sections were stored for 2 days before being mounted in Entellan. 1 sample from each rat consisted of a coronal slice approximately 3 mm thick, consisting of that part extending anteriorally from the anterior commissure in the mid-sagittal plane. The other sample consisted of a coronal slice approximately 3 mm thick which was that part extending posteriorally from the anterior commissure in the mid-sagittal plane.

Representative sections from different parts of each tissue sample were stained with Luxol Fast Blue and Cresyl Violet and the sections were identified which corresponded to:-

(i) The region of and between planes 11 and 12 as described by König and Klippel. (72, figure 39) i.e. Region C.

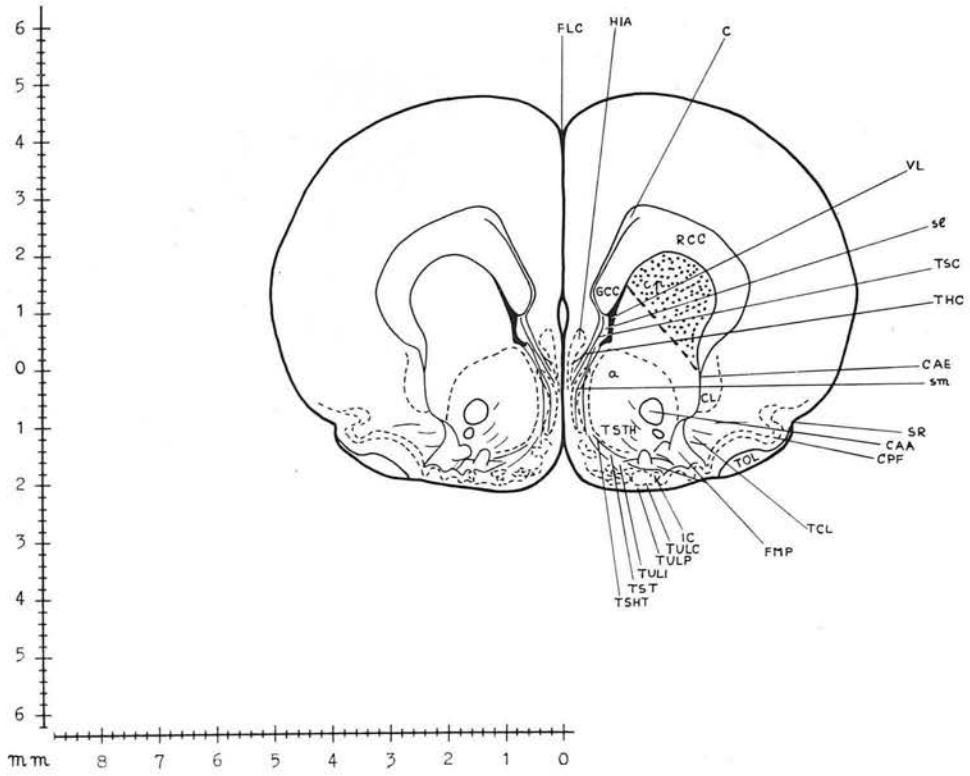


Fig. 11b
A 9650 μ

Figure 54. Coronal section of rat brain from König and Klippel's Stereotaxic Atlas of the Rat Brain. (72) This corresponds to plane 11. Measurements of fluorescence intensity were restricted to the stippled area. cp = Nucleus caudatus-putamen.

(ii) The region of and between planes 16 and 17 as described by König and Klippel. (72, figure 39) i.e. Region D.

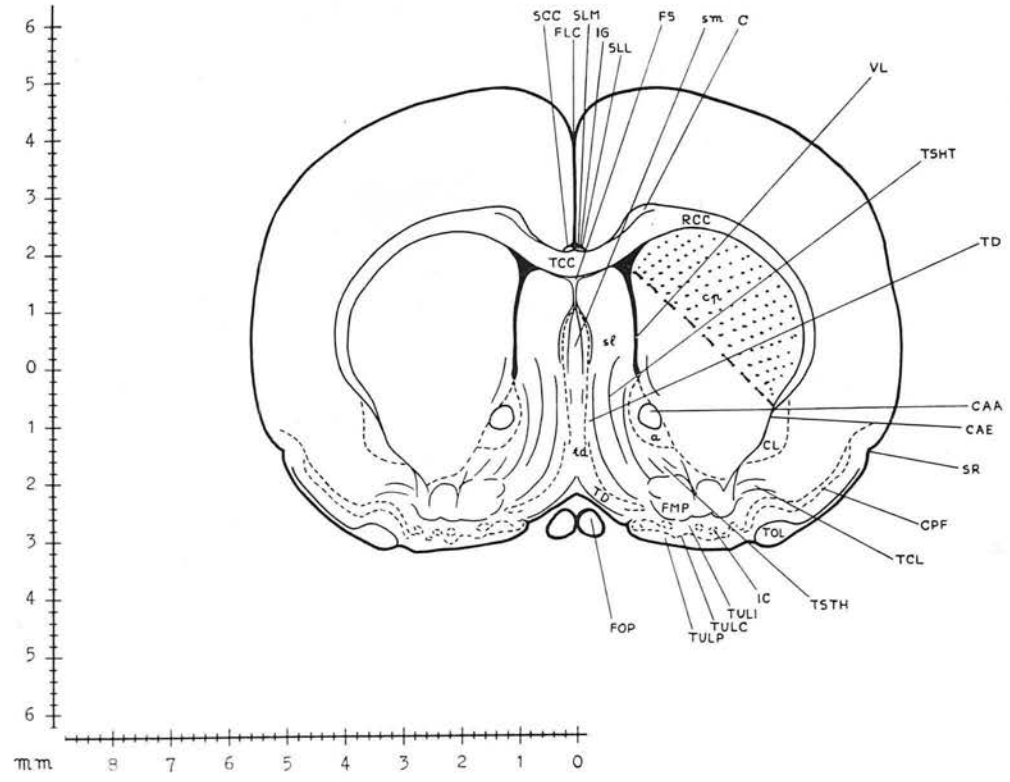


Fig. 16b

A 7890 μ

Figure 55. Coronal section of rat brain from König and Klippel's Stereotaxic Atlas of the Rat Brain. (72) This corresponds to plane 16. Measurements of fluorescence intensity were restricted to the stippled area. cp = Nucleus caudatus-putamen.

(iii) The region of and between planes 26 and 30 as described by König and Klippel. (72, figure 39) i.e. Region E.

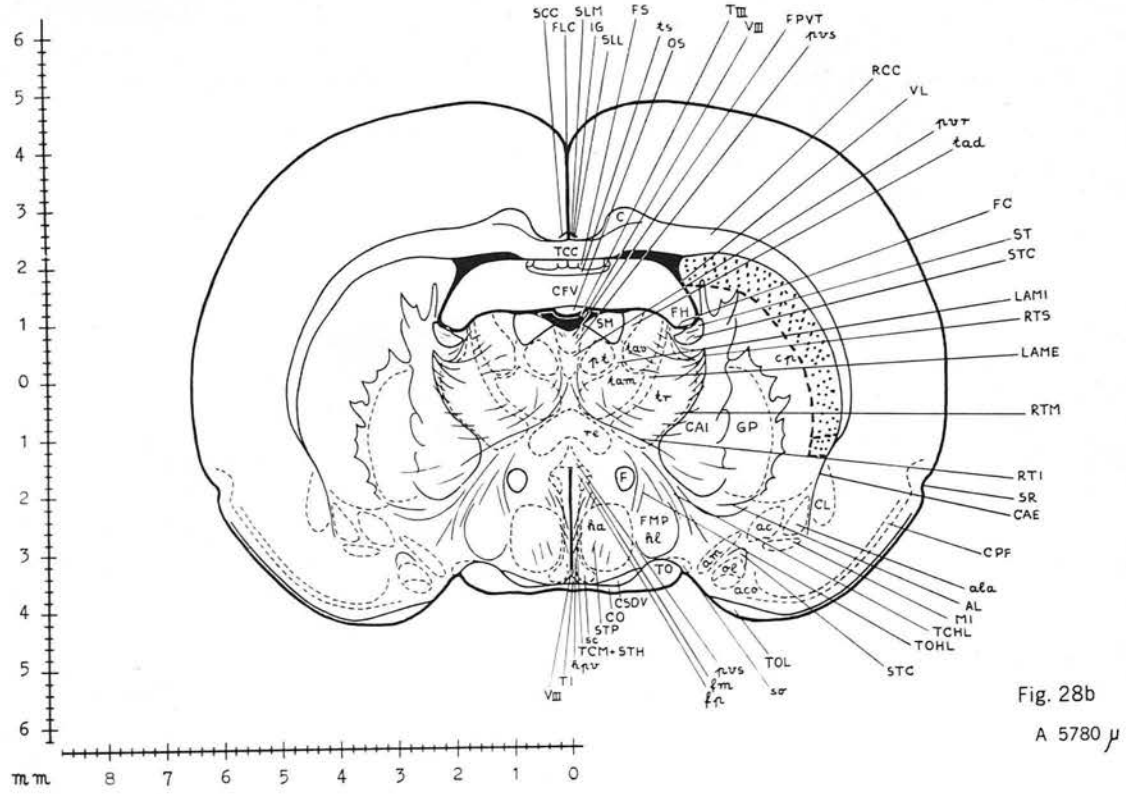


Figure 56. Coronal section of rat brain from König and Klippel's Stereotaxic Atlas of the Rat Brain. (72) This corresponds to plane 28. Measurements of fluorescence intensity were restricted to the stippled area. cp = Nucleus caudatus-putamen. F = columna fornicis.

The intensity of the diffuse fluorescence in the neuropil of the nucleus caudatus-putamen was measured from circular areas each with a diameter of 10 μm. In regions C and D the areas were arbitrarily selected from the dorso-lateral part of the nucleus caudatus-putamen, i.e. dorso-lateral to an imaginary straight line at an angle of 45° to the vertical, dividing the nucleus caudatus-putamen of each tissue section into 2 parts with approximately equal areas. In region E measurements were restricted to the dorso-lateral part of the nucleus caudatus-putamen, dorso-lateral to an imaginary curved line (remaining approximately equidistant from the corpus

callosum) which divided the nucleus caudatus-putamen of each tissue section into 2 parts with approximately equal areas. (See figure 56) Measurements were not taken from the lower extremity of the nucleus caudatus-putamen on each section, i.e. from that part below a horizontal plane through the superior part of the columna fornicis. (See figure 56)

Measurements were taken from 6 arbitrarily chosen areas from the selected part of each section. 3 adjacent sections were selected for microscopy from each of the 3 regions of each rat brain. The mean of the 18 readings from each of the 3 regions of each rat brain was taken as an estimate of fluorescence intensity. All measurements were taken within the same continuous period of microscopy.

Results

Experiment A

	Region A	Region B
Rat 1	70	77
2	81	78
3	81	77
4	87	75
5	74	80
6	78	73
Mean	79	77

Table 15. Fluorescence intensity of 2 regions of the neuropil of the rat nucleus caudatus-putamen, in tissue sections prepared by the formaldehyde-fluorescence method.

Each value is the mean of 18 measurements of fluorescence intensity.

The difference between the 2 regions are not significant at the level $p = 0.05$ (Wilcoxon Matched-Pairs Signed-Ranks Test. (84)

In this experiment, measurements of the fluorescence intensity of the neuropil of the cerebral cortex indicated that the non-specific autofluorescence of the neuropil of the nucleus caudatus-putamen contributed a mean of approximately 57% of the measured fluorescence. (See Section A;B)

Experiment B

Region	C	D	E
Rat 1	59	58	50
2	53	67	48
3	54	62	50
4	45	64	60
5	64	66	57
6	58	61	50
7	60	60	44
Mean	56	63	51

Table 16. Fluorescence intensity of 3 regions of the neuropil of the rat nucleus caudatus-putamen, in tissue sections prepared by the formaldehyde-fluorescence method.

Each value is the mean of 18 measurements of fluorescence intensity.

A Friedman Two-Way Analysis of Variance (84) of this data gives a value of $p = 0.0084$. Thus the samples of C, D and E can be assumed not to have been drawn from the same population. ($p < 0.02$)

The application of the Wilcoxon Matched-Pairs Signed-Ranks Test (84) indicates that:-

(i) The differences between the regions C and D are not significant at the level $p = 0.05$. (two-tailed)

(ii) The differences between the regions C and E are not significant at the level $p = 0.05$. (two-tailed)

(iii) The differences between the regions D and E are significant at a level $p = 0.02$. (two-tailed)

In this experiment measurements of the fluorescence intensity of the neuropil of the cerebral cortex indicated that the non-specific autofluorescence of the neuropil of the nucleus caudatus-putamen contributed

means of approximately 68% (region C), 60% (region D) and 74% (region E) of the total measured fluorescence of these regions, assuming that there was a similar level of autofluorescence intensity in all the regions studied. (See Section A;B)

Discussion

In previous studies, measurements were taken from the dorso-lateral part of coronal sections of the neuropil of the nucleus caudatus-putamen at and between the coronal planes 14-18 of König and Klippel. (See figure 39) In the present study, the results of experiment A did not indicate any significant difference between the anterior and posterior parts of this region.

The results of experiment B indicate that a region of the nucleus caudatus-putamen posterior to anterior commissure exhibited less intense fluorescence in the neuropil compared with another region studied, after preparation by the formaldehyde-fluorescence method. If it is assumed that there were no regional differences in the level of autofluorescence in the neuropil of the nucleus caudatus-putamen, then these results suggest that region E exhibited less formaldehyde-induced fluorescence than region D, probably due to a difference in the concentration of DA between the 2 regions.

Summary

A comparison was made between the intensity of fluorescence in several regions of the neuropil of the nucleus caudatus-putamen, in tissue sections of rat brains prepared by the formaldehyde-fluorescence method.

A significant difference was found between 2 of the regions studied (Wilcoxon Matched-Pairs Signed-Ranks Test, $p = 0.02$, two-tailed).

C. Quantitative histochemical studies of neostriatal DA depletion following D-L-α-methyl-p-tyrosine administration.

Introduction

Andén, Corrodi, Dahlström, Fuxe and Hökfelt (3), using the histochemical formaldehyde-fluorescence method (33) reported a reduced intensity of formaldehyde-induced fluorescence in the neuropil of the rat nucleus caudatus-putamen following the administration of α-methyl-p-tyrosine (α-MT). This compound inhibits tyrosine hydroxylase (3) thus limiting the biosynthesis of dopamine (DA). The diffuse formaldehyde-induced fluorescence of the neuropil of the rat nucleus caudatus-putamen is almost entirely derived from the fluorophore formed from dopamine in the terminal parts of the axons of the nigro-striatal neurones (52,32), therefore a reduction of striatal fluorescence following α-MT administration indicates failure of synthesis to replace dopamine breakdown to non-fluorogenic products.

In this study, the depletion of formaldehyde-induced fluorescence in a region of the rat striatal neuropil has been measured using a microfluorimetric histochemical method, following the administration of DL-α-methyl-p-tyrosine methylester hydrochloride (Sigma). The degree of depletion after α-MT administration was compared during several behavioral situations:-

a) During a period in an exercise drum, involving muscular co-ordination and activity.

b) During an initial training session for establishing a conditional avoidance response (CAR), which involved delivering electric shocks to the floor of a modified Levine shuttle box.

c) During a period of CAR performance in trained rats.

The results give information on the functional activity of the nigro-striatal neurones.

Recent ideas concerning the functional activity of the nigro-striatal DA-containing neurones have been reviewed by Fuxe, Hökfelt and Ungerstedt. (54)

They noted that these neurones are probably of great importance for normal movements and postures, as rigidity and tremor are associated with degeneration of this system in Parkinsonism. There is evidence that increased DA neurotransmission in the rat neostriatum is associated with increased locomotor activity, mainly composed of repetitive movements. A parallel has been suggested between such movements in animals and stereotyped behaviour in schizophrenia and it is possible that increased activity in the nigro-neostriatal DA neurones may be a feature of some psychotic states.

Evidence to support this suggestion is the association of the therapeutic effect of phenothiazines or butyrophenones, in the treatment of schizophrenia or mania, with their blocking action at DA receptor sites. However the antipsychotic effect of these drugs may be due to other actions.

Additional reports have suggested that DA-containing neurones are concerned in thermoregulation, normal eating and drinking behaviour and central vasomotor mechanisms.

Materials and Methods

Male albino Wistar rats were used weighing an average of 130 g and were killed by decapitation under chloroform anaesthesia.

Experiment A

Group 1. Four rats, untreated by α-MT, served as controls.

Group 2. Four rats were injected intraperitoneally (i.p.) with α-MT 300 mg/kg (10 mg/ml in distilled water) 3 hrs. 40 min before killing, and remained in their cage during the 3 hrs. 40 min period.

Group 3. Four rats received α-MT, 300 mg/kg i.p. 3 hrs. 40 min before killing. During the 40 min prior to killing, each rat was placed in an exercise drum 25 cms in diameter, consisting of two vertical discs and a strip of metal sheeting 7 cms wide, which formed the circumference. Each drum revolved around a rotating horizontal axis consisting of a brass rod 2 cms in diameter. The rats remained on the axis rod, which revolved at a speed of X10 min., and they faced against the direction of movement. The maintenance of this position involved continuous muscular co-ordination and activity.

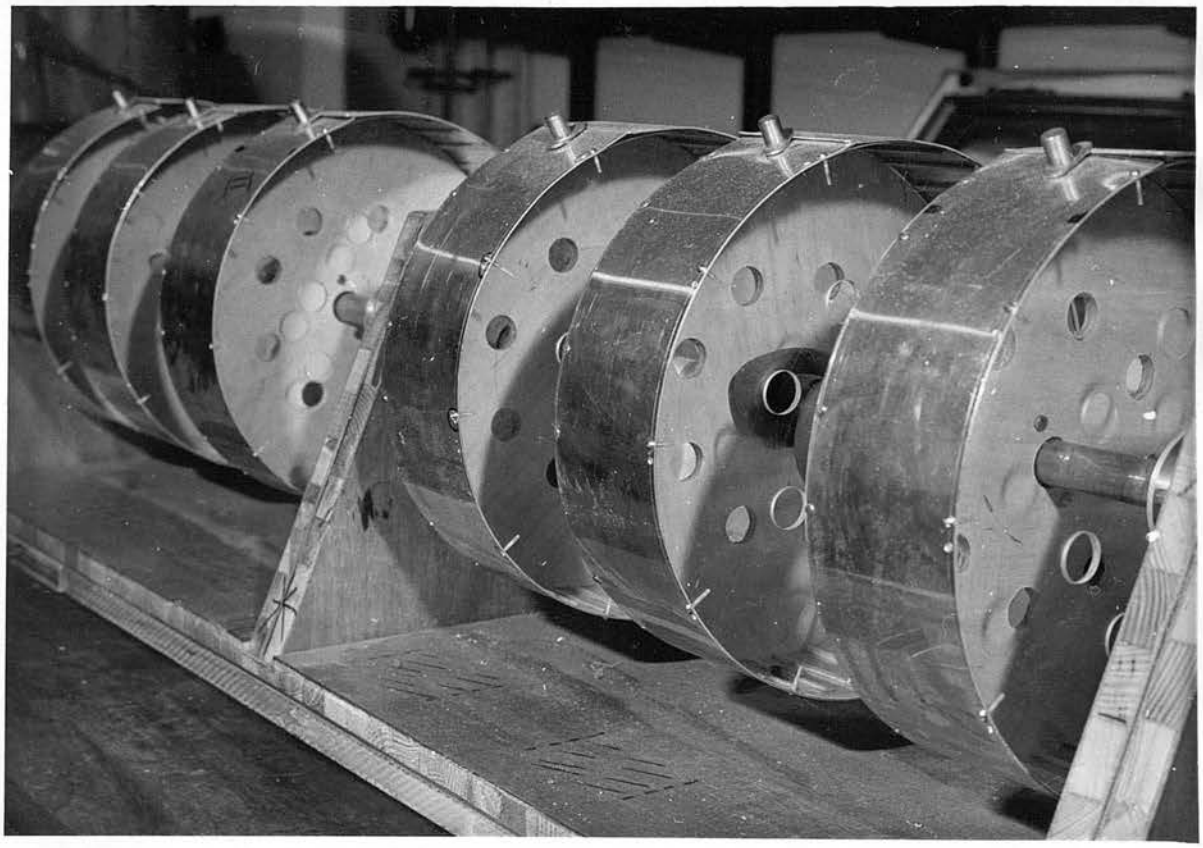


Figure 57. Exercise drums. The rats remained on the revolving axis rod.

Group 4. Four untrained rats were injected with α-MT, 300 mg/kg i.p. 3 hrs. 40 min before killing, and received an initial training session for establishing a CAR, during the 40 min before killing. A modified Levine shuttle box was used (99) consisting of two chambers 25.5 cms x 25.5 cms x 23.0 cms deep, separated by a metal sheet, with a doorway 11.5 cms by 10.0 cms in the centre. The floor of each chamber consisted of a grid of parallel 0.75 cm diameter chromium plated brass rods placed 1.8 cms apart. The walls were of zinc sheet and the lids of clear perspex. A buzzer provided the stimulus to be conditioned (CS) which sounded for 5 seconds. If the

animal did not cross from one compartment to the other within that time, an electric shock of 1.0 mA (unconditioned stimulus or US) was received from the grid. Both the US and CS were terminated after 4 seconds or previously by the animal crossing. The rats in this group were exposed to 200 such trials randomly spaced. The intershock interval ranged from 5-15 seconds. The interval between onset of shock and crossing decreased during training, although no avoidance responses were made. The mean shock duration was approximately 2 seconds.

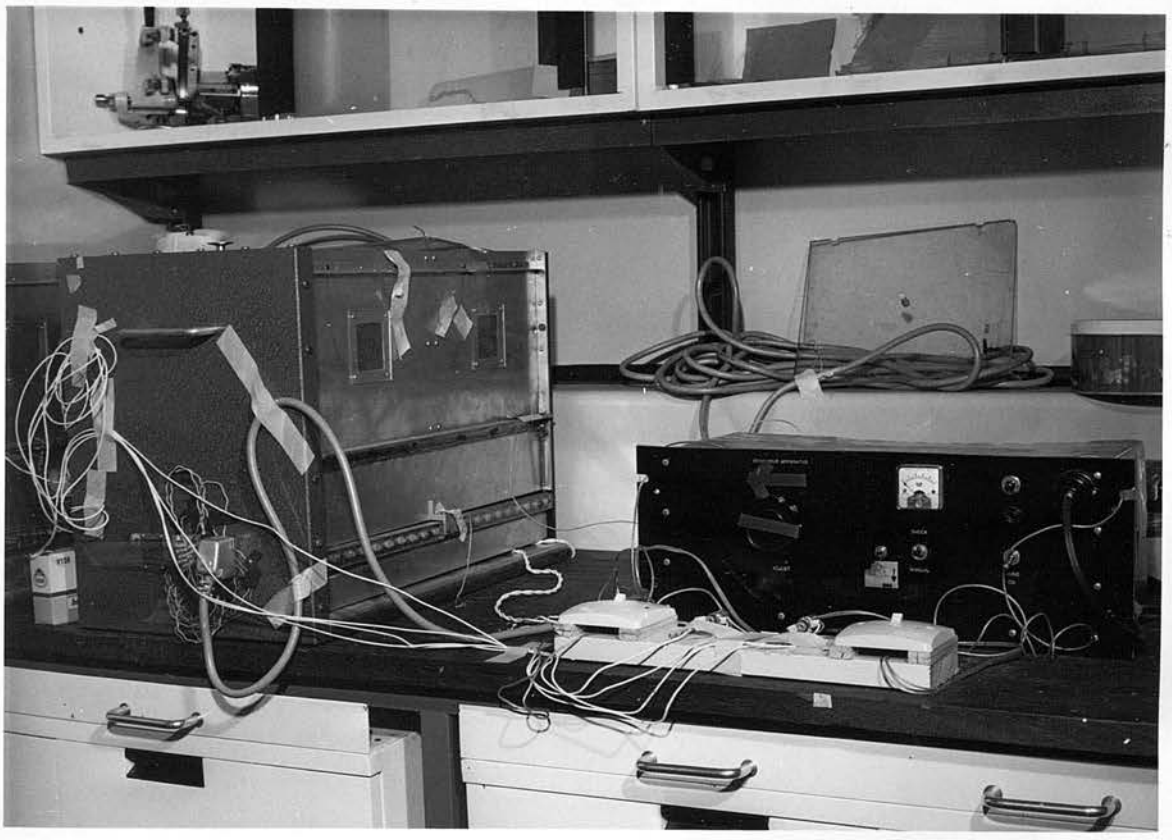


Figure 58. Modified Levine shuttle box.

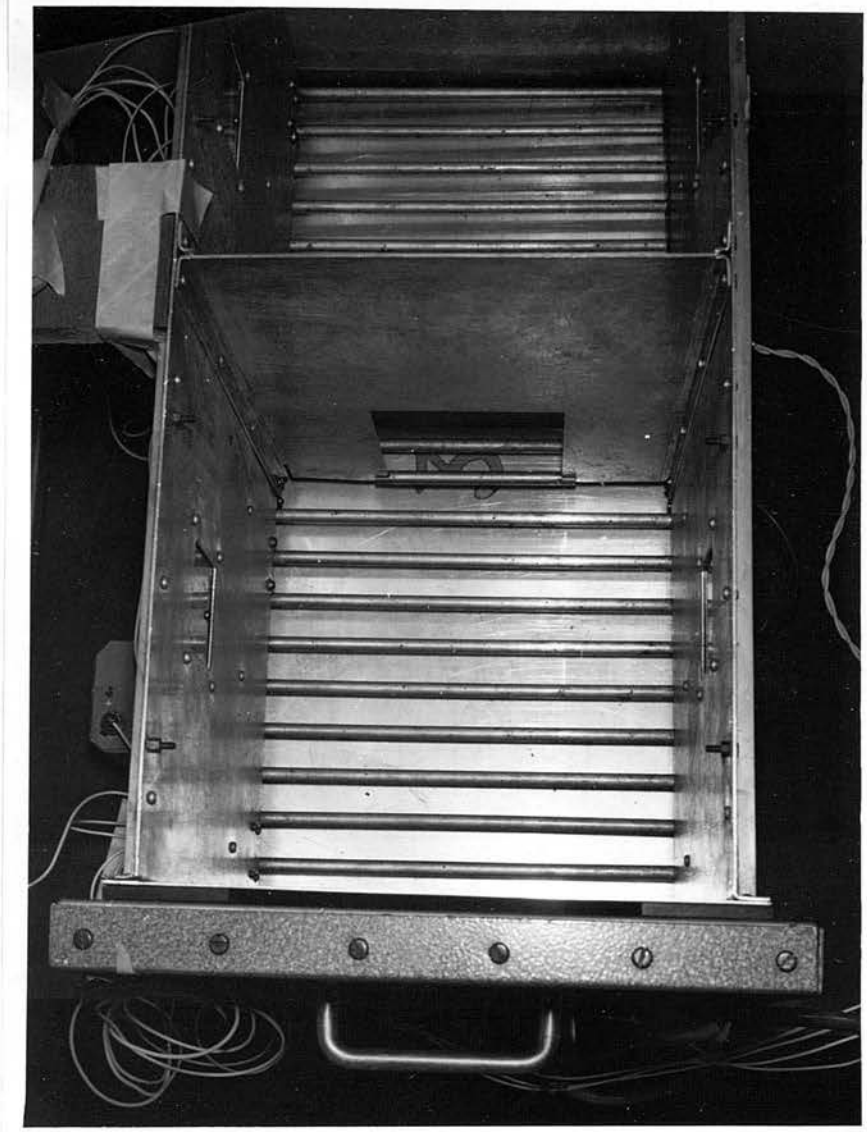


Figure 59. Modified Levine shuttle box, from above.

The four groups of rats were injected with α-MT and killed at intervals so that the dissected tissue samples of nucleus caudatus-putamen from each group could be frozen in isopentane, cooled by liquid nitrogen, at a constant time after killing. For each four groups of rats, the time between killing and freezing of the specimens was approximately 20 min. The samples were then immersed in liquid nitrogen prior to preparation for fluorescence microscopy and examination as previously described. (Section A; Introduction,A,G) The sections were stored for 2 days before being mounted in Entellan. The details of the sample and of the region examined have also

been described. (Section A;A)

The intensity of diffuse parenchymal fluorescence was measured from circular areas, each with a diameter of 10 μm. In each section, the areas for fluorimetry were selected from the neuropil of the nucleus caudatus-putamen, i.e. areas which appeared to be devoid of cell bodies, of blood vessels and of fibres of the internal capsule.

Three adjacent sections were taken to represent each tissue sample and measurements of the intensity of the diffuse fluorescence were taken from six areas of the selected part of each section. The mean of the 18 readings obtained from each tissue sample was taken as the estimate of the fluorescence intensity of the region.

Experiment B

Six rats received CAR training in a modified Levine shuttle box on nine consecutive days prior to the experiment. Each training session consisted of 300 trials. In the final training session an average of 81% of the shocks in the first 200 trials were avoided by the rats.

On the day of the experiment, α-MT 300 mg/kg i.p. was given 3hrs. 40 min before killing, and 200 CAR trials were carried out during the 40 min before killing. Two untrained rats served as controls for each trained rat, and also received α-MT 300 mg/kg i.p. 3hrs. 40 min before killing. Each pair of untrained rats was placed in a second shuttle box and received, in parallel, the same number and duration of shocks as their corresponding trained rat. Each trained rat with its two untrained controls were injected with α-MT and killed at intervals, so that the time between killing and freezing in liquid nitrogen could be constant (approximately 20 min) for each set of three rats. The tissue samples were then prepared for fluorescence microscopy and examined as described above.

Results

Experiment A

Group	1	2	3	4
Number of rats	4	4	4	4
α-MT treatment	None	300mg/kg	300mg/kg	300mg/kg
Activity during 40 min prior to killing	Remained in cage	Remained in cage	Muscular activity	CAR training
Fluorescence intensity (arbitrary units)	51 55	35 32	34 30	32 36
	45 47	35 35	38 32	43 38
	(Mean 49)	(Mean 35)	(Mean 35)	(Mean 37)

Table 17. Depletion of diffuse formaldehyde-induced fluorescence in the neuropil of the rat caudate nucleus after α-methyl-p-tyrosine methylester HCl (α-MT) 300 mg/kg i.p. 3 hrs. 40 min before killing.

An individual value represents one tissue sample from one rat, and is the mean of 18 readings of the fluorescence intensity of the striatal neuropil.

In Group 1, measurements of fluorescence intensity were also taken from the neuropil of the cerebral cortex, at a depth of approximately 350 μm, from parts of the cortex included on each of the three coronal sections which were examined from each sample of nucleus caudatus-putamen. Six measurements were taken from each section. The region of cortex examined consisted of the anterior part of the parietal region (areas 1, 2 and 3; Kreig, (73)), and the adjacent part of the frontal lobes (parts of areas 4 and 6; Kreig. (73)). In Group 1, the values for the fluorescence of the cortical neuropil, expressed in the same arbitrary units as in the table, were 21, 25, 22 and 23 (Mean 23) each value being the mean of 18 readings from each tissue sample. The

diffuse green fluorescence of the cortical neuropil was almost entirely due to non-specific autofluorescence, and after the depletion, by reserpine administration, (10 mg/kg i.p. 4 hrs before killing) of the formaldehyde-induced fluorescence derived from striatal dopamine, the intensity of 'background' fluorescence of the striatal neuropil has been shown to be almost identical to that of the above region of cortical neuropil. (Section A;B)

It follows that the mean value for the fluorescence intensity of the striatal neuropil in Group 1, minus the mean value for the fluorescence intensity of the cortical neuropil, indicates the degree to which the striatal fluorescence was formaldehyde-induced, i.e. almost entirely derived from DA. These values suggest that in Group 1 approximately 53% of the total fluorescence in the striatal neuropil was derived from DA, the rest being due to 'background' autofluorescence. The value for the intensity of background fluorescence in Group 1 was taken to represent the intensity of background fluorescence in Groups 2, 3 and 4, as the tissue samples in all the groups were processed together, in the same experiment. The amount of depletion of specific formaldehyde-induced striatal fluorescence in Groups 2, 3 and 4, expressed as a percentage of that part of the striatal fluorescence derived from dopamine, is as follows:-

Group 2: 54%, Group 3: 54% and Group 4: 46%.

The values of Group 1 are significantly higher than those of any of the three other groups, $P = 0.028$ (Mann-Whitney U test, two-tailed). However, the differences between Groups 2, 3 and 4 are not significant.

Experiment B

α-MT administration caused a reduction in the number of shocks avoided in all six trained rats, compared with the CAR performance on the final day of training. The mean number of the 200 shocks which were avoided by the trained rats was reduced from 81% to 20%. This effect of α-MT on CAR performance is significant at a level $P = 0.025$ (Wilcoxon Matched-Pairs Signed-Ranks Test, one-tailed). There was no significant difference between the degree of depletion in trained and untrained rats. The mean value for the depletion of

fluorescence in the striatal neuropil for the 18 rats was approximately 50% of that part of the fluorescence derived from striatal dopamine (see experiment A).

Discussion

There is evidence that the rate of reduction of striatal DA after α-MT administration is associated with the nervous impulse flow of the nigro-striatal neurones, i.e. the greater the nervous impulse activity, the greater will be the degree of dopamine depletion. (3,50) However, as breakdown of monoamines to non-fluorogenic products can also result from spontaneous leakage of DA through the axon membrane or spontaneous loss from the storage granules into the axoplasm, the depletion of amine following α-MT administration will not entirely be due to nervous impulse flow.

In experiment A (groups 2, 3 and 4), the degree of depletion (approximately 50%) of the fluorescence derived from striatal DA, suggests that at least half the DA in the region of nucleus caudatus-putamen studied was metabolized to non-fluorogenic products in the 3 hrs. 40 min between α-MT injection and killing. The degree of DA depletion in the region studied could have been greater than the degree of depletion of the fluorescence intensity, if there was any significant quenching of the fluorescence. (96) Rech, Borys and Moore (94) used a biochemical method to estimate rat whole-brain DA depletion after α-methyl-p-tyrosine 200 mg/kg i.p. 4 hrs before killing, when they found an approximately 60% reduction. It should be noted that an histochemical method for estimation of brain amines has the advantage of more accurate localization of the findings, compared with a biochemical study. As tyrosine hydroxylase would not have been completely inhibited during at least the initial part of that period, some of the DA which was metabolised following α-MT would have been replaced. Therefore, the turnover rate during this period was almost certainly greater than the degree of depletion suggests.

The lack of significant differences in the degrees of fluorescence depletion in groups 2, 3 and 4 of experiment A, suggests that any differences

in nervous impulse flow in the three behavioural situations were not large enough to make detectable differences in the degree of DA depletion. The results shown by groups 2 and 4 of experiment A confirm a finding by FUXE and HANSON (50) who also reported that electric shocks delivered to untrained rats did not apparently alter striatal DA depletion after α-MT administration.

In experiment B, another of FUXE and HANSON'S findings was confirmed, namely that α-MT disrupts CAR performance. However, in the present study no difference was found between the estimated degree of striatal DA depletion in trained and untrained rats following α-MT administration. This is in contrast to FUXE and HANSON'S report of a similar schedule of CAR performance having a marked effect on the fluorescence of the striatal neuropil after α-MT administration by increasing the depletion of fluorescence.

Summary

This study investigated the use of a microfluorimetric histochemical method for the measurement of the depletion of DA in the rat nucleus caudatus-putamen following α-methyl-p-tyrosine (α-MT) administration. The depletion in three behavioural situations was compared with that of a control group which remained in a cage.

The results of the control group indicate that there had been a reduction of approximately 50% in the intensity of the formaldehyde-induced fluorescence derived from DA in a region of the neuropil of the nucleus caudatus-putamen, during the interval (3 hrs 40 min) between the administration of α-MT 300 mg/kg i.p. and killing. Disruption of conditioned avoidance response (CAR) performance after α-MT administration was confirmed, but in this study the exposure of previously trained rats to CAR performance after α-MT administration did not have a significant effect on the depletion of the formaldehyde-induced fluorescence of the neostriatal neuropil. The levels of α-MT-induced depletion of formaldehyde-induced

fluorescence in the striatal neuropil following a period of muscular co-ordination/activity and following a period of CAR training were also not significantly different from those shown by a control group.

Section C

Studies of the fate of administered L-DOPA, which is used for the treatment of Parkinson's disease, in the mammalian CNS.

Effects of administration of L-DOPA on the rat and cat brains (A,B)

Investigation of factors which may affect regional and species differences in, and degree of, the intensity of diffuse formaldehyde-induced parenchymal fluorescence, resulting from L-DOPA administration to the rat and cat. (C,D)

The therapeutic use of L-DOPA. (E,F)

Results

A. A histochemical and biochemical study of the effects of L-DOPA administration.

Introduction

The therapeutic activity of L-DOPA in Parkinsonism may be mainly due to its conversion to DA by dopa-decarboxylase in the terminal parts of the remaining nigro-striatal DA-containing neurones. (30) The association between Parkinsonism and degeneration of these nigro-striatal neurones has been previously described. (See General Introduction) The administration of L-DOPA in Parkinsonism could supply the surviving nigro-striatal axons (or cell bodies) with excess L-DOPA thus leading to an increased rate of formation and release of DA in the neostriatum.

In the rat and mouse part of a dose of L-DOPA is decarboxylated to DA in the capillary walls of most brain regions by dopa-decarboxylase in the endothelial cells and pericytes, (15) so that an increase in neostriatal DA concentration shown biochemically does not necessarily indicate an increase in the concentration of DA in the neostriatal parenchyma. However it is possible that any DA formed in capillary walls, or by dopa-decarboxylase in non-dopaminergic neurones, could diffuse into the surrounding parenchyma.

It has been reported (32,91) that in both the rat and cat administered L-DOPA can lead to an increase in neostriatal DA, estimated biochemically. In the rat, it was concluded that the decarboxylase inhibitor Ro4-4602, 50 mg/kg 1½ hrs before killing, inhibited the decarboxylase in the capillary walls while having a minimal effect on the decarboxylase in the neostriatal parenchyma. The effects of the combination of Ro4-4602 50 mg/kg 1½ hrs before killing and L-DOPA, which led to an increase in neostriatal DA estimated biochemically, indicated that the administration of L-DOPA had produced an increase in DA in the neostriata l parenchyma (i.e. excluding capillary walls). In the cat, the effect of unilateral lesions of the

nigro-striatal pathway on the increase in neostriatal DA concentration after L-DOPA administration, indicated that L-DOPA induced a rise in DA concentration which was mainly dependent on the integrity of the nigro-striatal neurones.

The aim of the present biochemical-histochemical study, was to confirm the previous report (32) which indicated that administered L-DOPA can lead to increased DA concentration in the neostriatal parenchyma (i.e. excluding capillary walls) of the rat.

The DA concentration of the nucleus caudatus-putamen and of the cerebellum was estimated biochemically, and the intensity of fluorescence in the neuropil of both these regions was measured in untreated rats and in rats pretreated with:-

(i) L-DOPA

(ii) The dopa-decarboxylase inhibitor Ro4-4602 (N^1-(DL-seryl)-N^2-(2, 3, 4-trihydroxybenzyl)-hydrazine) and L-DOPA

(iii) The monoamine oxidase inhibitor nialamide and L-DOPA.

The dose of Ro4-4602 which was used has been reported to inhibit the enzyme in the capillary walls while having a minimal effect on the enzyme in the parenchyma. (32) The dose of nialamide used was designed to inhibit the monoamine oxidase in both the capillary walls and the parenchyma, thus inhibiting the metabolism of newly-formed DA to non-fluorogenic products.

The neuropil of the rat nucleus caudatus-putamen exhibits a diffuse formaldehyde-induced fluorescence, almost entirely derived from DA in the terminal parts of the axons of the nigro-striatal DA-containing neurones, (6) and a sufficient dose of administered L-DOPA leads to increased diffuse formaldehyde-induced fluorescence in the neuropil of all brain regions. The formaldehyde-fluorescence method also demonstrates, in the rat and mouse, an increase in capillary wall fluorescence in both the nucleus caudatus-putamen and cerebellum after the administration of a sufficient dose of L-DOPA, due

to the accumulation of DA. In the present study, the effects of L-DOPA administration on the formaldehyde-induced capillary wall fluorescence of both regions were also noted, to investigate the contribution of DA formed in the capillary walls to the total increase in the concentration of neostriatal DA.

Materials and Methods

Male P.V.G. rats were used, approximately 140 g in weight, and were decapitated under chloroform anaesthesia. 4 rats were untreated by drugs, 2 rats received L-DOPA 200 mg/kg i.p. 1 hr. before killing, 2 rats received nialamide 200 mg/kg i.p. 3 hrs. before killing and L-DOPA 200 mg/kg i.p. 1 hr. before killing, and 2 rats received Ro4-4602 50 mg/kg i.p. 1½ hrs. before killing and L-DOPA 200 mg/kg i.p. 1 hr. before killing. The solutions of L-DOPA and nialamide for injection were prepared in 0.08 N HCl at a concentration of 10 mg/ml. The solution of Ro4-4602 for injection was prepared in distilled water at a concentration of 10 mg/ml.

Histochemical study

A dissected sample from each rat, containing a specified part of the nucleus caudatus-putamen, was prepared for fluorescence microscopy as previously described. (Section A;Introduction,A) A sample of cerebellar hemisphere (approximately 3mm x 3mm x 3mm) was also obtained from each rat. The plane of sectioning was not constant for the cerebellar samples. The sections were stored for 2 days before being mounted in Entellan and were examined as previously described. (Section A;Introduction,A,G)

The intensity of diffuse fluorescence in the neuropil of the nucleus caudatus-putamen and molecular layer of the cerebellum was measured from circular areas, each with a diameter of 10 µm. Measurements were taken from 6 arbitrarily chosen areas from the selected part of each section. 3 adjacent sections were selected for fluorescence microscopy from each tissue sample. The mean of the 18 readings from each of the 2 brain regions of each rat was taken as an estimate of fluorescence intensity.

In addition, observations by eye were made of the intensity of capillary wall fluorescence which was compared with the intensity of the diffuse fluorescence of the surrounding neuropil.

Biochemical study

2 additional samples were dissected from each rat for biochemical analysis.

(i) One sample contained that part of the nucleus caudatus-putamen of one hemisphere, anterior to a coronal plane (parallel to the frontal zero plane of König and Klippel, see figure 38) approximately 0.5 mm posterior to the anterior commissure in the mid-sagittal plane. Each sample weighed approximately 60 mg.

(ii) The other sample consisted of one cerebellar hemisphere. Each sample weighed approximately 70 mg.

The estimations of DA concentration in these samples were carried out by Dr I.A. Pullar (M.R.C. Brain Metabolism Unit).

The samples were each homogenised in 2.0 ml 0.1N HCl containing 50 mg % ascorbic acid and 100 mg % EDTA. The homogeniser was washed twice with 1 ml volumes of the 0.1N HCl solution and the protein was then precipitated from the combined homogenate and washings by the addition of 0.2 ml 40 % $ZnSO_4$ and 0.15 ml 20 % NaOH. After centrifugation at 3000 g for 5 min the supernatant was transferred to a 30 ml glass-stoppered test tube. The precipitate was washed with 1 ml of distilled water and the dopamine in the combined supernatant and washings was acetylated by the addition of 0.25 ml acetic anhydride and 0.5g $NaHCO_3$. The acetylation was allowed to proceed for 10 min and the acetyl dopamine was then extracted into an organic phase by shaking it with 10 ml of methylene dichloride. After centrifugation at 3000 g for 5 min the lower organic layer was transferred to a 100 ml round-bottomed flask and the extraction was repeated with a further 10 ml of methylene dichloride. After further centrifugation at 3000 g for 5 min, the organic layer from the second extraction was pooled with that from the first and was

evaporated to dryness at room temperature under a stream of nitrogen. The residue was dissolved in 1.5 ml distilled water, 1.0 ml of which was transferred to a 15 ml test tube. To this was added 0.5 ml of an ethylene diamine: ammonium chloride mixture (Ethylenediamine: 4M NH_4Cl - 3: 3.9 v/v). After heating at 60° for 20 min, the mixture was cooled to room temperature before being saturated with NaCl. The fluorophore was then extracted into 1.5 ml isobutanol. The fluorophore was estimated on a Zeiss spectrophotofluorimeter from an excitation scan from 350 nm to 500 nm, the emission monochromator being set at the emission maximum for the dopamine fluorophore, i.e. 520 nm. (The excitation maximum was 420 nm).

L-DOPA was not detected by this method which would, however, have detected noradrenaline and the glycol metabolites of noradrenaline.

The acetylation was carried out because the molar fluorescence of the fluorophore from acetyl dopamine is greater (X3 approximately) than that formed from DA itself. (34)

Results

Group	1	2	3	4
Treatment	Untreated	L-DOPA 200 mg/kg	L-DOPA 200 mg/kg and nialamide 200 mg/kg	L-DOPA 200 mg/kg and Ro4-4602 50 mg/kg
Number of rats	4	2	2	2
Nuc.caudatus-putamen				
Mean concentration DA µg/g	6	12	74	53
Mean fluorescence intensity of neuropil	42	61	110	108
Cerebellum				
Mean concentration DA µg/g	0	4	42	3
Mean fluorescence intensity of neuropil (molecular layer)	30	43	78	107

Table 18. The effects of administered L-DOPA on the dopamine (DA) concentration of the neostriatum and cerebellum and on the diffuse formaldehyde-induced fluorescence intensity in the neuropil of these regions.

The value for the fluorescence intensity of each region from each rat was the mean of 18 readings of fluorescence intensity. The mean of the values for each group of rats is shown, in arbitrary units.

In the untreated rats and in those treated with L-DOPA and Ro4-4602, the capillary walls did not appear to fluoresce more intensely than the surrounding parenchyma. However, in groups 2 and 3, the walls of the majority of the capillaries were observed to show a more intense fluorescence compared with the intensity of the diffuse fluorescence in the surrounding parenchyma. This contrast was greater in the rats treated with L-DOPA and nialamide.

Figure 60. Formaldehyde-induced fluorescence in the neuropil and capillary walls of the nucleus caudatus-putamen in a rat treated with L-DOPA 200 mg/kg i.p. 1 hr. before killing and nialamide 200 mg/kg i.p. 3 hrs. before killing. X590.

The walls of the capillaries show an intense fluorescence compared with the intensity of the fluorescence in the surrounding parenchyma.

Discussion

The results indicate that administered L-DOPA, alone or in combination with nialamide or Ro4-4602, led to an increase in the concentration of DA in the part of the nucleus caudatus-putamen studied, and therefore confirms previously reported findings. (32) This assumes that NA, glycols derived from NA and certain alcohols derived from DA, which would have been detected by the method used for DA estimation, did not make a significant contribution to the value obtained. Constantinidis, Bartholini, Tissot and Pletscher, (32) estimated neostriatal NA after L-DOPA 75 mg i.p. 1 hr. before killing, alone and in combination with doses (similar to those used in this study) of Ro4-4602 and nialamide, and reported concentrations below 1 µg/g.

Diffuse autofluorescence, in the neuropil of both regions studied, contributed to the measured fluorescence and would have been almost entirely responsible for the measured fluorescence in the neuropil of the molecular layer of the cerebellum in the untreated rats, i.e. 30 arbitrary units of fluorescence. The autofluorescence in both regions studied would have been of similar intensity. (Section A;B)

Administered L-DOPA, alone or in combination with nialamide or Ro4-4602, led to an increase in diffuse formaldehyde-induced fluorescence in the neuropil of both the regions studied. There is evidence that the mammallian neostriatum has a much higher (X42) concentration of dopa-decarboxylase than the cerebellum. (45) This may be mainly due to dopa-decarboxylase in the terminal parts of the nigro-striatal neurones. It is probable that in the neuropil of the nucleus caudatus-putamen of the rats treated with L-DOPA (alone or in combination), the fluorophores from both L-DOPA and additional DA (formed from administered L-DOPA) made significant contributions to the total fluorescence, together with the fluorophore derived from the original neostriatal DA content. In the cerebellum, the diffuse formaldehyde-induced fluorescence in the neuropil may have been mainly derived from the fluorophore from L-DOPA, due to the relatively low concentration of dopa-decarboxylase in this region. (45)

The rats which received L-DOPA, or L-DOPA and nialamide, showed increases in DA concentration in both brain regions. However, as the capillary walls were observed to show an increase in fluorescence, almost certainly mainly due to DA accumulation, it is not certain if a significant part of the increased neostriatal DA concentrations were due to additional DA in the neuropil.

The rats which received L-DOPA and nialamide showed a large increase in DA concentration in both regions and the capillary walls in both regions showed a marked accumulation of fluorophore. (Mialamide would have inhibited the monoamine oxidase in the capillary walls, thus reducing the metabolism of

DA to non-fluorogenic products.) The large increase in DA concentration in the cerebellum indicates that despite the evidence (45) that there is a relatively low concentration of dopa-decarboxylase in the cerebellum, compared with the concentration in the neostriatum, large amounts of cerebellar DA can be formed from administered L-DOPA, probably due to the dopa-decarboxylase in the capillary walls.

The rats which received L-DOPA and Ro4-4602 showed a large increase in DA concentration of the neostriatum, compared with a minimal increase in the cerebellum. The capillary walls were not obviously more fluorescent than the surrounding parenchyma in both regions, and the neuropil in both regions showed a similar intensity of diffuse fluorescence. (The intensity of capillary wall fluorescence was estimated by comparing it with the intensity of the surrounding parenchyma.) However, the capillary walls may have contained some accumulated fluorophore, which was insufficient to be detected. The dose regime of Ro4-4602 which was used has been reported (32) to inhibit the enzyme in the walls of rat brain capillaries while having a minimal effect on the enzyme in the brain parenchyma. (Another effect of Ro4-4602 is to reduce extracerebral metabolism of L-DOPA thus increasing blood levels after a given dose. (32)) In the rats which received L-DOPA and Ro4-4602 the contrast between the DA concentrations of the neostriatum and cerebellum suggests that most of the additional neostriatal DA was located in the neostriatal parenchyma and not in the neostriatal capillary walls. This conclusion assumes that any DA formed in the neostriatal capillary walls made a contribution to the total neostriatal DA concentration which did not exceed the increased DA concentration in the cerebellum, resulting from the administration of L-DOPA and Ro4-4602.

The values for measured fluorescence intensity indicate regional differences in the effects of administered L-DOPA on the intensity of increased formaldehyde-induced fluorescence, which were affected by combining L-DOPA with

Ro4-4602. These regional differences were investigated further. (Section C;B,D)

Summary

The aim of this biochemical-histochemical study was to confirm previous evidence which had indicated that administered L-DOPA can lead to increased DA concentration in the parenchyma (i.e. excluding capillary walls) of the rat nucleus caudatus-putamen. (The therapeutic action of L-DOPA in Parkinsonism may depend on the presence of additional DA, derived from administered L-DOPA, in the neostriatal neuropil.)

The results indicate that L-DOPA (200 mg/kg i.p. 1 hr. before killing) alone or in combination with the monoamine oxidase inhibitor nialamide or the decarboxylase inhibitor Ro4-4602, led to an increase in the concentration of DA (estimated biochemically) in the region of the nucleus caudatus-putamen studied.

In the rat, administration of L-DOPA leads to the accumulation of DA in the capillary walls of most brain regions. However, the biochemical and histochemical results also suggest that the increased neostriatal DA concentration was associated with an increase in the concentration of DA in the neostriatal parenchyma.

B. Regional and species differences in the effects of L-DOPA on the CNS.

Introduction

The therapeutic effect of L-DOPA in Parkinsonism may be due to a compensatory increase in the lowered concentration of neostriatal DA, resulting from the conversion of administered L-DOPA to DA in the neostriatum, or in the cell bodies or ascending axons of the nigro-striatal DA-containing neurones. (30)

The histochemical formaldehyde-fluorescence method can be used to investigate the action of L-DOPA on the CNS, as the fluorophores derived from both L-DOPA and DA are visualized by this method.

The neuropil of the rat nucleus caudatus-putamen shows a diffuse formaldehyde-induced fluorescence, derived almost entirely from the fluorophore formed from DA in the terminal parts of the nigro-striatal DA-containing neurones (see General Introduction) and Constantinidis, Bartholini, Tissot and Pletscher (32) reported an increase in the diffuse parenchymal fluorescence of the nucleus caudatus-putamen following intraperitoneally administered L-DOPA. (The contribution of autofluorescence to the total measured fluorescence in the neostriatal neuropil has been discussed. (Section A;B)

As L-DOPA and DA give rise to fluorophores with very similar characteristics (Section A;I), an increased fluorescence in the parenchyma does not demonstrate that L-DOPA can lead to an increased concentration of DA in the rat neostriatum. Additional information can be obtained from parallel biochemical estimations of DA, but the interpretation of such results is made difficult because it has been shown that in the rat and mouse there is an enzymic blood-brain barrier to administered L-DOPA, which is due to the presence of dopa-decarboxylase and monoamine oxidase in the capillary walls of most brain regions. (15) As L-DOPA can lead to the formation and accumulation of DA in the capillary walls, (associated with an increase in formaldehyde-induced capillary wall fluorescence) an increase in the

concentration of neostriatal DA shown biochemically does not necessarily indicate an increase in the DA concentration of the parenchyma. (i.e. excluding capillary walls.)

However, there is other evidence which does suggest that L-DOPA can produce an increased concentration of DA in the parenchyma of the rat nucleus caudatus-putamen. Constantinidis et al. (32) gave L-DOPA to rats pretreated with a dopa-decarboxylase inhibitor which appeared to inhibit the enzyme in the capillary walls without significantly interfering with the enzyme in the neostriatal parenchyma. Their biochemical-histochemical study showed that there was a marked increase in the concentration of neostriatal DA which was accompanied by an increase in diffuse parenchymal fluorescence but by no obvious accumulation of fluorophore in the capillary walls.

In the present study, the effects of administered L-DOPA on the CNS has been investigated further by a microfluorimetric histochemical method for the measurement of diffuse formaldehyde-induced fluorescence in the neuropil of 3 brain regions of 2 commonly used animal models for investigating the actions of drugs, the rat and cat.

Materials and Methods

Male albino Wistar rats (120-240 g) and cats of both sexes (2-2.6 kg) were used. The rats were decapitated under chloroform anaesthesia and the cats were also decapitated, having been anaesthetized with pentobarbitone sodium 60 mg/kg i.p. Solutions of L-DOPA and nialamide for injection were prepared in 0.08 N HCl at a concentration of 10 mg/ml.

Dissected samples from the nucleus caudatus-putamen, cerebral cortex and cerebellum were prepared for fluorescence microscopy and examined as previously described. (Section A;Introduction,A,G) The sections were stored for 2 days before being mounted in Entellan.

The details of the sample containing a region of rat nucleus caudatus-putamen and cerebral cortex have been described, (Section A;A) together with

details of the region examined. The samples of cat nucleus caudatus-putamen were dissected from the dorso-lateral aspect of the anterior part of each nucleus and the plane of sectioning was not constant between samples.

The molecular layer of the rat cerebellar samples was examined. The samples were dissected from the paravermal region and the plane of sectioning was not constant between samples.

The intensity of diffuse parenchymal fluorescence was measured from circular areas each with a diameter of 10 μm. In each section the areas for fluorimetry were selected from the neuropil and were arbitrarily chosen in each region.

Three adjacent sections were taken to represent each tissue sample, and measurements of the intensity of fluorescence were taken from 6 areas from the region or regions to be examined in each section. The mean of the 18 readings obtained from each brain region of each animal was taken as the value for the fluorescence intensity. An indication of the spread of individual readings is given by the interquartile range. The concentration of fluorophore corresponding to an arbitrary unit of fluorescence intensity varied between experiments. (Section A; Introduction, G)

Results

Experiment A

The relation between dose of L-DOPA and fluorescence intensity of the neuropil of the rat nucleus caudatus-putamen was investigated.

In order to show that effects of the administration of L-DOPA on the intensity of diffuse parenchymal formaldehyde-induced fluorescence could be demonstrated by the microfluorimetric method, measurements were taken of the fluorescence intensity of the neuropil of the nucleus caudatus-putamen in untreated rats and in rats treated with increasing doses of L-DOPA. The treated animals had also received a constant dose of the monoamine oxidase inhibitor nialamide, and one rat was given nialamide alone. In

preliminary experiments it had been found that the increase in diffuse parenchymal fluorescence after a dose of L-DOPA was greater in all the regions studied (nucleus caudatus-putamen, cerebral cortex and molecular layer of cerebellum) if the animal had been pretreated with nialamide. L-DOPA was combined with nialamide so that the differences in the effects of the various doses of L-DOPA would be magnified and would, therefore, be more easily detected.

The values, in arbitrary units, for the intensity of the formaldehyde-induced fluorescence in the neuropil of the nucleus caudatus-putamen from five rats which had not been treated with nialamide, or with a combination of L-DOPA and nialamide, were: 40 (37-40), 40 (39-42), 43 (41-46), 44 (43-46) and 42 (42-44). Each of these values represents the mean of 18 readings from one tissue sample and the interquartile range is shown for each value. The values for the fluorescence intensity of the neuropil of the nucleus caudatus-putamen associated with varying doses of L-DOPA, each given after a constant dose of nialamide, are illustrated by figure 61, which also shows the value for a rat which received nialamide alone.

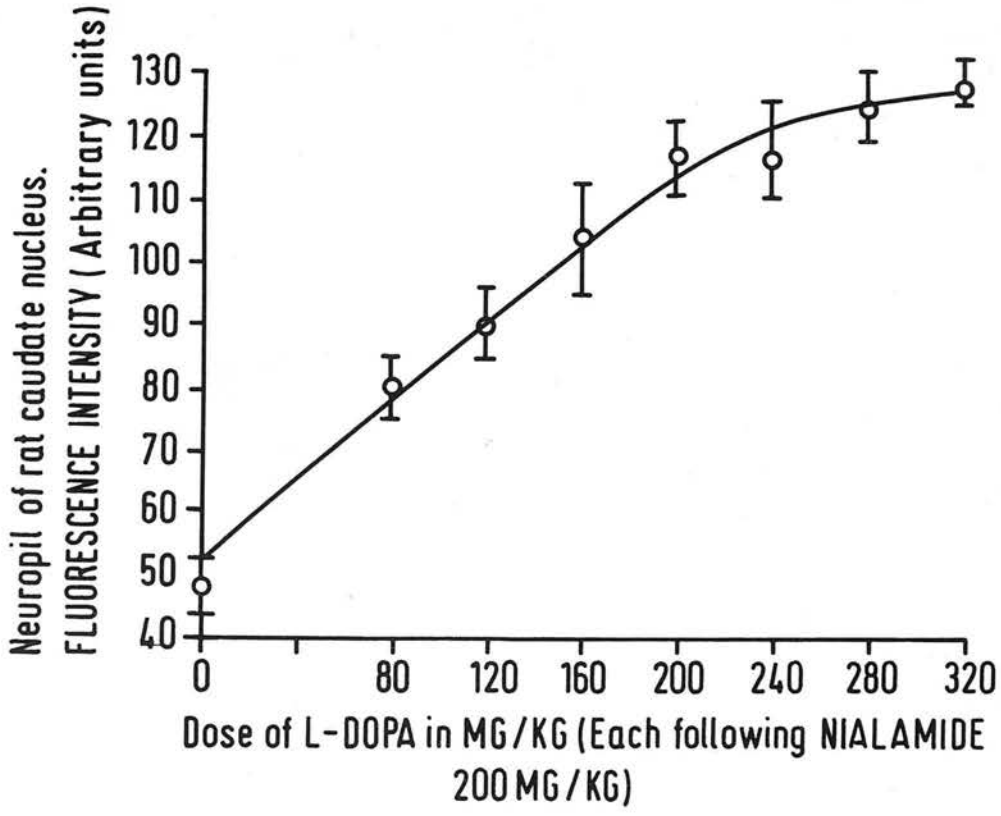


Figure 61. The relation between the fluorescence intensity of the neuropil of the rat nucleus caudatus-putamen and dose of L-DOPA, in tissue sections prepared by the formaldehyde-fluorescence method. L-DOPA was given intraperitoneally 1 hr. before killing and 2 hrs. after pretreatment with nialamide, 200 mg/kg i.p. One rat received nialamide alone. An individual value represents the mean of 18 readings from one rat and the interquartile range is shown for each mean value.

There was an approximately linear relationship between dose of L-DOPA and fluorescence intensity, up to about 200 mg/kg. Above this dose, the relationship was no longer linear, which may have been due to concentration-dependent quenching of the fluorescence, (96) although other factors could

have been involved, such as the rate of transport of L-DOPA from the lumen of the capillaries into the parenchyma.

Experiment B

In experiment A only one rat represented each dose of L-DOPA, and subsequently an attempt was made to find out the significance of differences in fluorescence intensity produced by the increment in the dose of L-DOPA which had been used, 40 mg/kg, within the approximately linear part of the relationship between dose and fluorescence intensity. Each of two dose levels of L-DOPA differing by 40 mg/kg (approximately 7 mg/animal) was given to a group of three rats, both groups having been pretreated with a constant dose of nialamide. The groups were matched with respect to body weight.

The results (table 19) show that the three members of the group which had received the larger dose of L-DOPA all had higher mean values for the fluorescence intensity of the neostriatal neuropil compared with the members of the other group. These differences between the groups have a level of significance, as calculated by a one-tailed Mann-Whitney U-test, of $P = 0.05$. (84)

Treatment	Fluorescence intensity (arbitrary units)
L-DOPA 120 mg/kg after nialamide 200 mg/kg (three rats)	65 (61-70) 64 (59-68) 64 (62-66)
L-DOPA 160 mg/kg after nialamide 200 mg/kg (three rats)	77 (69-80) 87 (81-94) 71 (67-75)

Table 19. The fluorescence intensity of the neuropil of the rat nucleus caudatus-putamen, following administration of L-DOPA and nialamide, in tissue sections prepared by the formaldehyde-fluorescence method.

L-DOPA was given intraperitoneally 1 hr. before killing and 2 hrs. after pretreatment with nialamide, i.p. An individual value represents the mean of 18 readings from one rat and the interquartile range is shown for each mean value.

Experiment C

Diffuse parenchymal fluorescence in the rat brain was measured following L-DOPA administration. The fluorescence intensity of the neuropil of the nucleus caudatus-putamen, cerebral cortex and molecular layer of the cerebellum was measured after different doses of L-DOPA, to investigate the threshold dose level above which an increase in diffuse parenchymal fluorescence could be detected. In addition, the relative intensities of parenchymal fluorescence in these regions were of interest because of previous reports (15,32) of regional differences in the intensity of parenchymal fluorescence after L-DOPA administration. In experiments C and D, L-DOPA was given alone, i.e. not following pretreatment with nialamide.

Treatment	Fluorescence intensity (arbitrary units)		
	Nuc. caudatus-putamen	Cerebral cortex	Cerebellum, molecular layer
None	62 (55-67)	47 (43-50)	47 (44-49)
None	64 (61-67)	41 (38-42)	46 (44-49)
None	67 (62-73)	43 (41-45)	39 (36-42)
None	64 (62-66)	44 (42-47)	40 (37-41)
L-DOPA 100 mg/kg	67 (64-71)	46 (44-47)	44 (40-49)
L-DOPA 200 mg/kg	64 (62-68)	46 (43-49)	49 (45-53)
L-DOPA 400 mg/kg	102 (98-106)	52 (47-56)	62 (60-65)

Table 20. The effect of L-DOPA administration on the fluorescence intensity of the neuropil of three brain regions in the rat, in tissue sections prepared by the formaldehyde-fluorescence method.

L-DOPA was given intraperitoneally, 1 hr. before killing. An individual value represents one tissue sample from one rat and is the mean of 18 readings of the fluorescence intensity. The interquartile range is shown for each mean value.

The results (table 20) suggest that there was a larger increase in parenchymal fluorescence of the nucleus caudatus-putamen as a result of L-DOPA 400 mg/kg, while the cortical and cerebellar parenchyma of the same animal showed much smaller increases. There is no suggestion that the lower doses were associated with increases in fluorescence, so that the threshold dose was $>$ 200 mg/kg on this occasion, although in subsequent experiments, values of $<$ 200 mg/kg were also obtained for the threshold dose. One of the many factors which could have been responsible for changes in the threshold dose is the variation which can occur between different sets of the reaction conditions for fluorophore production. However, in spite of this variation, a larger absolute increase of fluorescence intensity in the nucleus caudatus-putamen, compared with the increase in the cerebral cortex or cerebellum, was a constant finding. The difference in this and in the subsequent experiments, using doses of L-DOPA 150-400 mg/kg before killing, between the absolute increases in the fluorescence intensity of the nucleus caudatus-putamen and cerebral cortex, or between the absolute increases in the nucleus caudatus-putamen and cerebellum, each has a level of significance of $P = 0.038$ (Sign test, two-tailed).

Experiment D

Diffuse parenchymal fluorescence was compared in the rat and cat brains following L-DOPA administration. Biochemical estimation of dopamine levels in the nucleus caudatus-putamen after L-DOPA administration have indicated that certain doses of L-DOPA have very different effects on the rat and cat. (32,91) Marked increases in dopamine in the nucleus caudatus-putamen of the cat were reported, contrasting with smaller increases in this region of the rat, and in the present study this apparent specied difference was investigated histochemically. The results (table 21) suggest that L-DOPA 100 mg/kg produced a marked increase in the parenchymal fluorescence of the cat nucleus caudatus-putamen although this dose had no apparent effect on the rat brain sample. Thus, in addition to the previously reported biochemical

evidence of a species difference in the effect of a given dose of L-DOPA kg/body wt. on the DA concentration of the nucleus caudatus-putamen, the present results also suggest that there is a species difference in the effect of L-DOPA kg/body wt. on the resulting formaldehyde-induced fluorescence of this region. A subsequent experiment examined regions of the cerebral cortex and molecular layer of the cerebellum, in addition to the nucleus caudatus-putamen and in all 3 regions this species difference was confirmed.

Animal	Treatment	Fluorescence intensity (arbitrary units) Nucleus caudatus-putamen
Rat	None	58 (52-61)
Rat	None	61 (57-66)
Rat	None	69 (62-76)
Rat	None	62 (58-66)
Rat	L-DOPA 50 mg/kg	56 (58-62)
Rat	L-DOPA 100 mg/kg	56 (54-58)
Rat	L-DOPA 200 mg/kg	73 (63-80)
Rat	L-DOPA 300 mg/kg	73 (60-78)
Rat	L-DOPA 400 mg/kg	82 (77-87)
Cat	None	53 (49-55)
Cat	L-DOPA 50 mg/kg	83 (80-87)
Cat	L-DOPA 100 mg/kg	174 (146-196)

Table 21. The effect of L-DOPA administration of the fluorescence intensity of the neuropil of the nucleus caudatus-putamen in the rat and cat, in tissue sections prepared by the formaldehyde-fluorescence method.

L-DOPA was given intraperitoneally, 1 hr. before killing. An individual value represents one tissue sample from one animal and is the mean of 18 readings of the fluorescence intensity. The interquartile range is shown for each mean value.

Discussion

A microfluorimetric method is described for the measurement of diffuse parenchymal fluorescence in sections of brain tissue and has been used to investigate the action of L-DOPA on the CNS.

The fluorophores from L-DOPA and dopamine have almost identical spectral characteristics and in experiments with dried protein droplets (67) it has been shown that L-DOPA and dopamine give almost identical yields of fluorophore. However, as there is evidence that a dose of L-DOPA can produce an increased

level of dopamine in the parenchyma of the rat neostriatum, an increased intensity of diffuse fluorescence in the neuropil of the nucleus caudatus-putamen after L-DOPA administration is almost certainly partly due to increased levels of parenchymal dopamine and not solely to parenchymal L-DOPA.

RITZÉN (96) has reported that the fluorescence intensity of the fluorophore derived from dopamine is proportional to the concentration of the amine up to 8000 µg/g wet wt. of tissue; i.e. concentration-dependent quenching of the fluorescence occurs above this level. ANDÉN, FUXE HAMBERGER AND HÖKFELT (6) have calculated that the average dopamine-containing nerve terminal in the rat neostriatum has a concentration at this level, therefore, if additional dopamine in the parenchyma of the neostriatum is concentrated in the terminals, then concentration-dependent quenching of the fluorescence derived from the additional dopamine could occur. The degree of such quenching would depend on the degree of diffusion of DA, or the fluorophore derived from DA, from and within the terminals. There is evidence that many of the dopamine-containing terminals, which are distributed throughout the striatal neuropil, are too fine to be seen individually by a light microscope (52) however, previous studies have visualized some of the larger individual nerve terminals following 'perfect' reaction conditions, which presumably prevented diffusion of dopamine from these terminals. In all the experiments in this study, a diffuse, apparently non-localized fluorescence was seen in the neostriatal parenchyma, which may mean that there had been diffusion of dopamine from at least some of the terminals into the surrounding parenchyma.

The degree of diffusion from the terminals and within the terminals may have been an important factor in determining the degree of quenching of the fluorescence derived from an increase of neostriatal dopamine following L-DOPA administration. Any degree of diffusion from and within the terminals in

each experiment would probably have reduced any quenching of the fluorescence derived from increases of dopamine. (68)

Table 20 shows the results of an experiment to investigate the threshold dose of L-DOPA in the rat, above which an increase in diffuse fluorescence could be observed in the nucleus caudatus-putamen, cerebral cortex and molecular layer of the cerebellum. Although the estimate of the threshold dose determined in this and in subsequent experiments showed some variation, each dose of L-DOPA which gave rise to an increase in parenchymal fluorescence produced a larger absolute increase in the nucleus caudatus-putamen compared with the increase in the cortex or cerebellum. This may be explained by many factors, such as regional differences in rates of metabolism and transport of L-DOPA, in rates of blood flow and in the effectiveness of the enzymic blood-brain barrier to L-DOPA. In addition, some of the L-DOPA may have been taken up by the cell bodies of the nigro-striatal system of neurons, leading to the formation of additional dopamine which was then transported to the neostriatum.

Species differences were found between the rat and cat in the effects of L-DOPA administration on the intensity of diffuse neostriatal fluorescence. (Table 21) L-DOPA , 100 mg/kg, produced a marked increase in diffuse fluorescence of the cat brain sample, but had no apparent effect on the rat brain sample. This may have involved many factors, such as species differences in the rates of metabolism and transport of parenchymal L-DOPA, in the rate of extracerebral metabolism of L-DOPA, or in the rate of absorption of L-DOPA from the peritoneal cavity. However, a subsequent experiment showed that a cat and 2 rats, pretreated with L-DOPA 100 mg/kg i.p. 1 hr. before killing, had serum L-DOPA concentrations of 6.0, 4.1 and 7.8 µg/ml respectively, in samples of venous blood taken immediately after killing. (Serum L-DOPA estimations were carried out by Dr I.A. Pullar, M.R.C. Brain Metabolism Unit, University of Edinburgh). In addition, rates of cerebral blood flow may vary in the two species and there may have been a species

difference in the effectiveness of the blood-brain barrier in the neostriatum, i.e. the cat may have a 'weak' barrier compared with a 'strong' barrier in the rat. Although L-DOPA 100 mg/kg did not produce an obvious accumulation of fluorophore in the capillary walls of both species, the intensity of capillary wall fluorescence is not necessarily an indication of the activity of the dopa-decarboxylase in the capillary walls.

It is hoped that further investigation of the factors affecting the fluorescence intensity of the neuropil of the neostriatum following L-DOPA administration will give more information on the therapeutic activity of a dose of L-DOPA used for the treatment of Parkinson's disease.

Summary

A microfluorimetric method was used for quantitative histochemical study of the diffuse formaldehyde-induced fluorescence in the neuropil of various brain regions and of the effects of L-DOPA in the rat and cat. Regional and species differences in the effects of L-DOPA on the CNS were reported and discussed.

In the rat, suitable doses of L-DOPA led to increases in the intensity of fluorescence in the neuropil of the samples of nucleus caudatus-putamen which were greater than the increases in the neuropil of the other regions studied. Marked increases in the intensity of fluorescence in the neuropil of the cat brain samples were produced by doses of L-DOPA/kg body wt. which were too low to produce detectable increases in the fluorescence of the rat brain samples.

It is hoped that further investigation of the factors affecting the fluorescence intensity of the neostriatal neuropil following L-DOPA administration will give more information on the therapeutic activity of a dose of L-DOPA used for the treatment of Parkinsonism.

Appendix: A method for the estimation of serum L-DOPA

The serum, 0.1 ml, was diluted in a 1.5 ml polystyrene test tube to 1.0 ml with distilled water. The diluted serum was then cooled to $0^{\circ}C$ in an ice bath and protein was precipitated by the addition of 0.1 ml 4N perchloric acid

which was at 4°C. After a further 15 min at 0°C the sample was centrifuged at 12,000 *g* for 2 min and 0.7 ml of the supernatant was transferred to a clean 1.5 ml polystyrene test tube and was adjusted to pH 4.0 (using a glass electrode) with 0.1N and 1.0N KOH. The sample remained at 4°C for 30 min and the precipitate of potassium perchlorate was removed by centrifugation at 12,000 *g* for 2 min. An aliquot, 0.5 ml, of the supernatant was transferred to a third 1.5 ml polystyrene test tube and was heated at 56° for 30 min with 0.2 ml of an ethylene diamine/ammonium chloride mixture (ethylene diamine : 4M ammonium chloride 1:1.3 v/v). After cooling to room temperature the fluorescence of the sample was determined from an activation spectrum, the emission monochromator being set at the emission maximum for the fluorophore, 460 nm (uncorrected value). The activation maximum was found to be 350 nm (uncorrected value).

The recovery through the method was determined by substituting a solution of 1 µg L-DOPA in 1.0 ml for the original diluted serum sample. The fluorescence due to the reagents was evaluated by taking 1.0 ml distilled water through the method.

C. Animal models for an enzymic blood-brain barrier mechanism for therapeutically administered L-DOPA.

Introduction

In the rat and mouse there is an enzymic blood-brain barrier to administered L-DOPA due to dopa-decarboxylase and monoamine oxidase in the capillary walls of most brain regions. (15) The histochemical formaldehyde-fluorescence method, which visualizes both L-DOPA and DA, demonstrates an increase in capillary wall fluorescence in most brain regions after intraperitoneal L-DOPA administration, and this has been reported to be associated with the accumulation of DA in the endothelial cells and pericytes. (15) (Capillary wall structure has been reviewed by Maynard, Schultz and Pease. (78)) It should be noted that regions of autofluorescence, which may appear more intense than the surrounding parenchyma, are present in the walls of rat capillaries. However, these regions of autofluorescence appeared yellow, compared with green capillary-wall fluorescence resulting from L-DOPA administration.

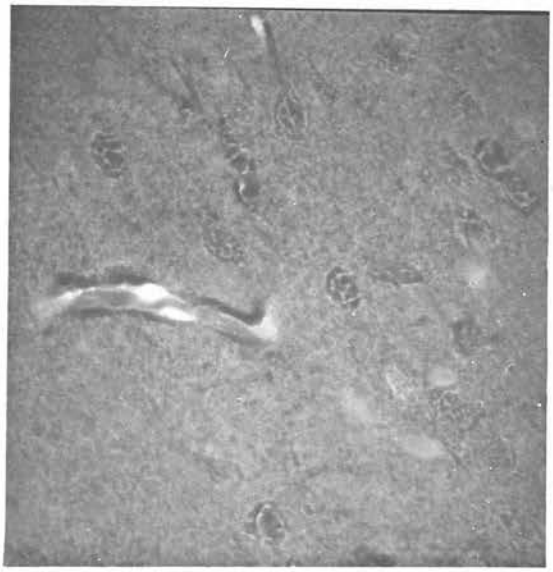


Figure 62. Autofluorescence of rat cerebral cortex in a tissue section prepared by the formaldehyde-fluorescence method but omitting exposure to formaldehyde gas. X530. The examination was carried out as previously described. (Section A;Introduction) The wall of the capillary shown contained regions of yellow autofluorescence which appeared more intense than the autofluorescence of the surrounding parenchyma. Marked autofluorescence was associated with a minority (approximately 10-20%) of the segments of capillaries seen in each tissue section.

The effects of L-DOPA 400 mg/kg i.p. 1 hr. before killing on the diffuse formaldehyde-fluorescence of the neuropil of 3 brain regions of the rat brain have been reported. (Section C;B) In that study, the majority of the blood vessels with a diameter of less than 10 μm showed an intense formaldehyde-induced fluorescence, relative to the intensity of the surrounding parenchyma. (See figure 63)

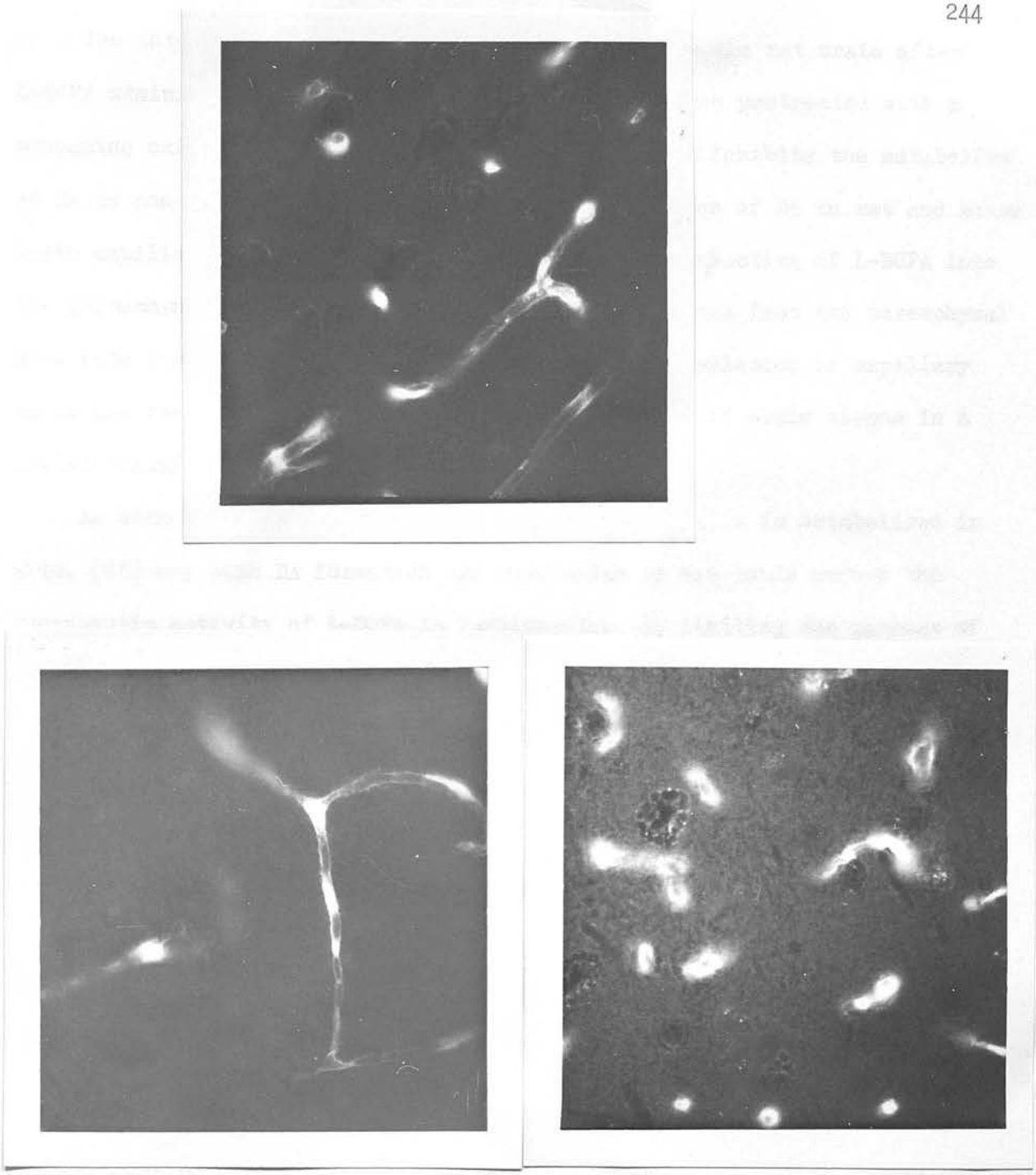


Figure 63. Formaldehyde-induced capillary wall fluorescence in the cerebral cortex of a rat pretreated with L-DOPA 400 mg/kg i.p. 1 hr. before killing. Cross sections of capillaries show non-fluorescent lumens completely surrounded by fluorescent capillary wall. X530. (Any blood vessel with a diameter of less than 10 μm was considered to be a capillary.)

The intensity of capillary wall fluorescence in the rat brain after L-DOPA administration is increased if the rat has been pretreated with a monoamine oxidase inhibitor such as nialamide, which inhibits the metabolism of DA to non-fluorogenic products. (15) Accumulation of DA in rat and mouse brain capillaries has also been demonstrated after injection of L-DOPA into the parenchyma, (15,16) indicating that L-DOPA can pass from the parenchymal side into the capillary wall. In addition, DA accumulation in capillary walls has been demonstrated in mice after incubation of brain tissue in a medium containing L-DOPA. (86)

As some or all the DA formed in rat capillary walls is metabolized in situ, (86) any such DA formation and metabolism in man could reduce the therapeutic activity of L-DOPA in Parkinsonism, by limiting the passage of L-DOPA into the brain. Although the rat has often been assumed to be a model for the fate of L-DOPA in Parkinsonism, the present study reports a difference in the nature of the blood-brain barrier to L-DOPA between the rat and another commonly used animal model, the cat.

Species differences have been described, between the rat and cat, in the effect of administered L-DOPA on the increases in diffuse formaldehyde-induced fluorescence (derived mainly from L-DOPA or L-DOPA and DA), in the neuropil of three brain regions. (Section C;B) Marked increases in the intensity of the diffuse fluorescence in the regions of cat brain were produced by doses of L-DOPA/kg body wt. which were too low to produce detectable increases in the fluorescence of rat brain samples. One of the many possible reasons for this may have been a species difference in the effectiveness of a blood-brain barrier to L-DOPA, i.e. the cat may have a 'weak' barrier compared with a 'strong' barrier in the rat.

In the present study the blood-brain barrier to L-DOPA in the rat has been compared with that of the cat by incubating tissue from three brain regions in a medium containing L-DOPA and nialamide. The intensity of capillary wall

fluorescence in each region was compared, by eye, with the intensity of the surrounding increased diffuse formaldehyde-induced fluorescence, which was measured by a microfluorimetric method.

Materials and Methods

Male P.V.G. rats (approximately 150 g) and cats of both sexes (approximately 2.3 kg) were decapitated under chloroform anaesthesia. Dissected samples containing parts of the nucleus caudatus-putamen, cerebral cortex and cerebellum were obtained, consisting of slices approximately 1.5 mm in thickness. Within 15 minutes of killing, the slices were placed in an incubation medium, which was then continually shaken for 40 minutes at 37°C. The medium was a Krebs-Ringer bicarbonate buffer, saturated with 95% O_2 and 5% CO_2, containing ascorbic acid 20 mg/100 ml, EDTA 5 mg/100 ml, glucose 120 mg/100 ml, L-DOPA 20 mg/100 ml and nialamide 20 mg/100 ml. (59) Five 50 ml stoppered conical flasks each contained two tissue slices and 5 ml of incubation medium. The samples were then prepared for fluorescence microscopy and examined as previously described. (Section A; Introduction, A, G) The sections were stored for 2 days before being mounted in Entellan on the day of examination.

The intensity of the diffuse fluorescence in the neuropil was measured from circular areas each with a diameter of 10 μm, in the three regions studied. The areas were arbitrarily chosen in each region and while the choice of an area was being made, the intensity of the exciting light was much reduced.

Measurements of parenchymal fluorescence in the rat nucleus caudatus-putamen together with observations of capillary wall fluorescence, were taken from the dorso-lateral area of approximately coronal sections of the anterior part of the neostriatum. The variation in the antero-posterior level of this coronal plane was over approximately 1.5 mm, extending anteriorly from the anterior border of the median part of the anterior

commissure. The measurements and observations of capillaries in the rat cerebral cortex were taken from parts of the cortex included on the coronal sections of each nucleus caudatus-putamen, at an approximate depth of 350 μm. Measurements and observations were obtained from the molecular layer of the samples of rat and cat cerebellar hemisphere and the plane of sectioning was not constant. The samples of cat nucleus caudatus-putamen were dissected from the dorso-lateral aspect of the anterior part of each nucleus and the samples of cat cerebral cortex were taken from the anterior part of the parietal region.

Three adjacent sections were taken to represent each tissue sample, and measurements of the intensity of diffuse fluorescence in the neuropil were taken from six areas of the selected part or parts of each section. The mean of the eighteen readings from each region or regions of each tissue sample was taken as an estimate of fluorescence intensity.

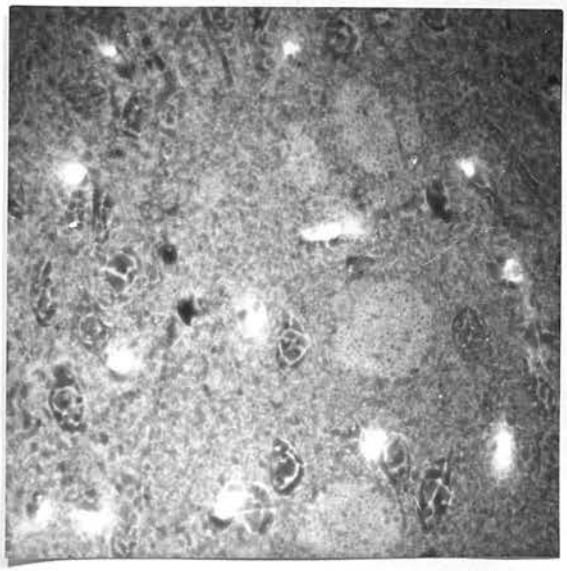
Samples were taken from two cats and two rats. Two tissue slices were examined from each rat. One contained the parts of the neostriatum and cerebral cortex, and the other was taken from the cerebellum. Three tissue slices were taken from each cat, one from each of the three brain regions studied.

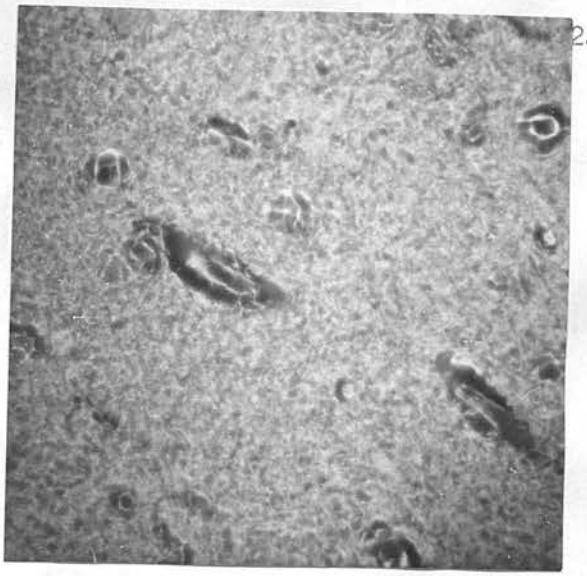
Results

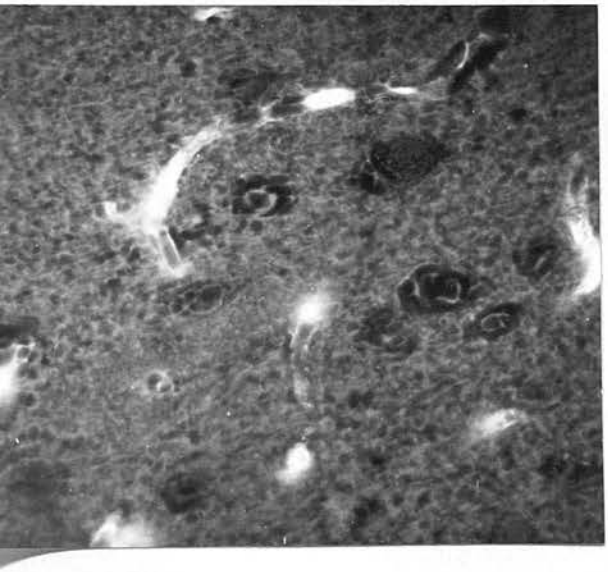
After incubation, all the sections examined contained a region, approximately 200 μm in depth, corresponding to the region adjacent to the surface of each tissue sample, which showed intense diffuse parenchymal fluorescence compared with the rest of the sample. Measurements and observations were not taken from this intensely fluorescent region, as any increase in capillary wall fluorescence could not be estimated due to the fluorescence intensity of the surrounding parenchyma.

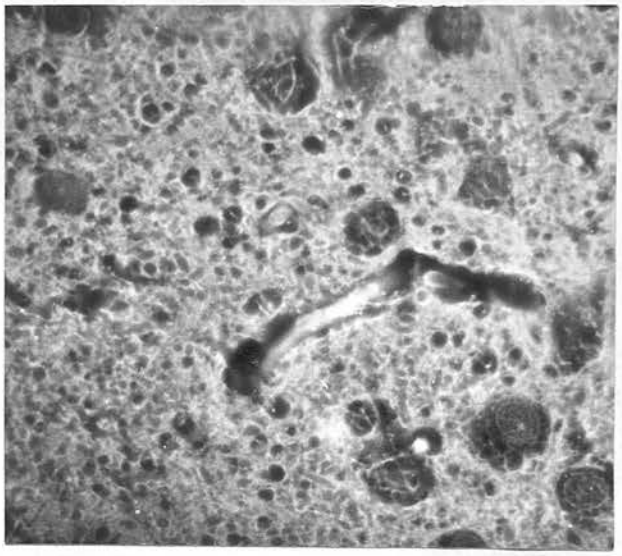
All the four tissue slices from the rat brain showed a marked increase in capillary wall fluorescence in the majority of the capillaries in all three regions studied. The fluorescence of a typical capillary wall was

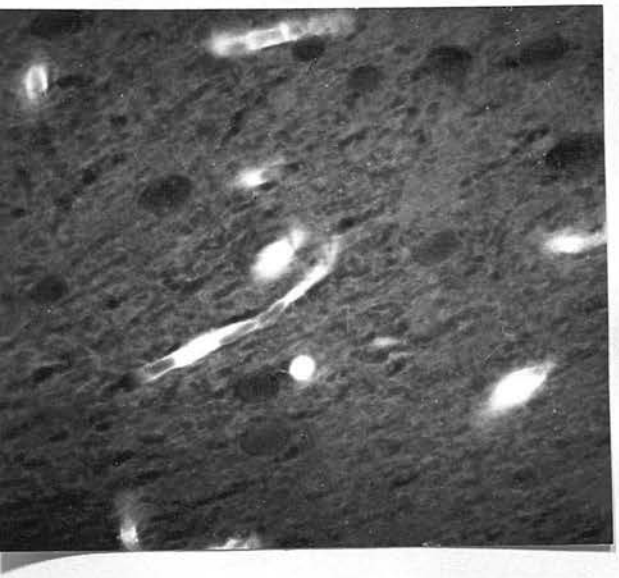
clearly observed to be more intense than the 'background' level of diffuse fluorescence in the surrounding neuropil. This was in contrast to the six cat brain slices in which the capillary walls did not appear to fluoresce more intensely than the surrounding neuropil.











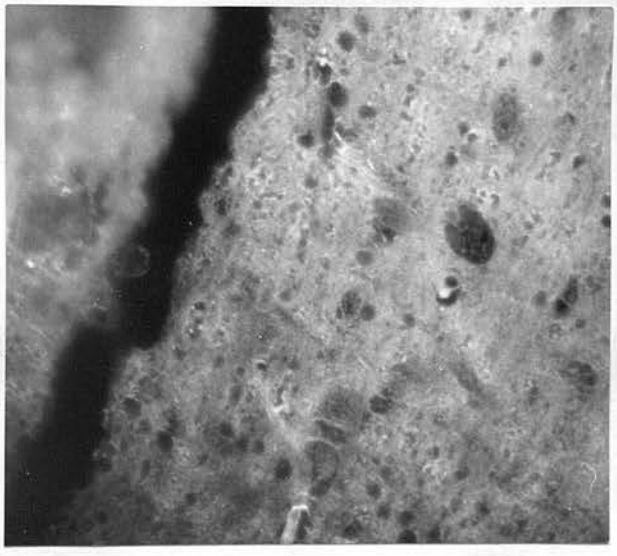


Figure 64. Typical appearance of capillary walls relative to the intensity

of fluorescence in the surrounding parenchyma, after incubation of rat and cat brain tissue in a medium containing L-DOPA and nialamide, shown by the formaldehyde-fluorescence method. X450.

Left side, from above:

Rat, nucleus caudatus-putamen

Rat, cerebral cortex

Rat, cerebellum (molecular layer)

Right side, from above:

Cat, nucleus caudatus-putamen

Cat, cerebral cortex

Cat, cerebellum (molecular layer)

If it is assumed that the capillary walls in the three brain regions are alike with respect to their capacity to accumulate DA after incubation with L-DOPA and nialamide, then this species difference is significant at a level $p = 0.001$ (Fisher exact probability test, two-tailed)

As an estimation of capillary wall fluorescence, by eye, is made by comparing the intensity of capillary wall fluorescence with that of the surrounding parenchyma, the intensity of the fluorescence in the neuropil of the 3 regions was measured, and the results are shown in table 22.

	Fluorescence intensity. Arbitrary units		
	Nuc. caudatus-putamen	Cerebral cortex	Cerebellum, molecular layer
Rat 1	21	56	31
2	45	43	68
Cat 1	62	41	27
2	106	45	56

Table 22. The fluorescence intensity of the neuropil of three brain regions in sections from tissue slices incubated in a medium containing L-DOPA and nialamide before preparation by the formaldehyde-fluorescence method. Each value represents the mean of 18 readings of fluorescence intensity from three sections from one tissue slice. (The intensity of typical capillary wall fluorescence in each section was compared, by eye, with the measured intensity of fluorescence of the neuropil.)

It can be seen that there were variations in the intensity of fluorescence in the neuropil between different tissue slices. However, there are examples of a similar level of fluorescence between tissue slices from the two species, and although the neuropil of some cat tissue samples showed greater fluorescence intensity than rat tissue samples, differences in the reverse direction were also seen. It follows that the observed species difference in capillary wall fluorescence was due to an actual difference in the intensity of the capillary walls, and not to an apparent difference depending on the levels of fluorescence in the neuropil.

These findings were confirmed in two subsequent experiments.

Discussion

The results shown by the rat tissue samples show that L-DOPA passed into the capillary walls where there was a marked accumulation of formaldehyde-induced fluorescence. This was not seen in the capillary walls of the cat tissue samples, and there are many possible reasons for this species difference,

such as a species difference in factors affecting the entry or exit of L-DOPA to or from the cells of the capillary walls, or a species difference in the metabolism of L-DOPA in the endothelial cells and pericytes.

It is likely that, in the rat and mouse, incubation of tissue in the presence of L-DOPA provides a model for the accumulation of DA in the capillary walls after intraperitoneally administered L-DOPA, as in both types of experiment, a similar histochemical appearance of the capillary walls has been described. Therefore, the results of this study suggest that there would be a marked species difference between the rat and cat in the accumulation of DA in the capillary walls after intraperitoneal L-DOPA administration. Unfortunately, this is difficult to verify because a dose of L-DOPA/kg body wt. leads to a larger increase in parenchymal fluorescence in the cat than in the rat, in the three regions studied. (Section C;B) However I did not observe a contrast between capillary wall fluorescence and parenchymal fluorescence in the cat nucleus caudatus-putamen, cerebral cortex or cerebellum after L-DOPA 250 mg/kg intraperitoneally, 1 hr. before killing, with or without pretreatment with nialamide 100 mg/kg intraperitoneally, 2 hrs. before killing, although these doses produced brightly fluorescent capillary walls in rat tissue, relative to the parenchymal fluorescence. In addition, two different doses of L-DOPA/kg body wt. which produced a similar increase in parenchymal fluorescence in the nucleus caudatus-putamen of both species, gave rise to intensely fluorescent capillary walls (relative to the surrounding parenchymal fluorescence) in the rat tissue, but the capillaries in the cat tissue did not stand out in this way. (Section C;B, Table 21)

Evidence of a species difference in the accumulation of DA in the capillary walls of animal models makes it impossible to predict the nature of a blood-brain barrier to therapeutically administered L-DOPA in man. Preliminary investigations of three thalamic biopsy samples, obtained during stereotaxic surgery for Parkinsonism, (71) which were incubated in the

presence of L-DOPA and nialamide, have failed to demonstrate the accumulation of formaldehyde-induced fluorescence in the capillary walls. (See Section C;E)

Summary

Tissue slices from three brain regions of the rat and cat were incubated in a medium containing L-DOPA and nialamide, and studied by the formaldehyde-fluorescence method.

Unlike the rat brain capillary walls, which showed a marked increase in fluorescence due to uptake of L-DOPA and subsequent DA formation and accumulation, the capillary walls of the cat brain samples did not show evidence of DA accumulation.

This species difference may have contributed to the species difference in the effect of administered L-DOPA which has been previously described. (Section C;B)

The nature of a blood-brain barrier to L-DOPA in man is discussed with regard to this finding.

Further investigation, in animal models, of factors affecting species differences in the fluorescence intensity of the brain parenchyma after L-DOPA administration may give more information on the fate of L-DOPA used for the treatment of Parkinsonism.

D. The effects of pretreatment with the dopa-decarboxylase inhibitor Ro4-4602 on the intensity of formaldehyde-induced diffuse fluorescence, resulting from L-DOPA administration, in three regions of the rat brain.

Introduction

In the rat, regional differences in the effects of L-DOPA administration on the diffuse formaldehyde-induced fluorescence in the neuropil have been described. (Section C;B) A sufficient dose of L-DOPA produced an increase in the intensity of fluorescence in the neuropil of the nucleus caudatus-putamen which was greater than the increase in the neuropil of the cerebral cortex or molecular layer of the cerebellum. The regional difference between the neostriatum and cortex, and between the neostriatum and cerebellum, (produced by a range of doses of L-DOPA from 150 to 400 mg/kg) was each significant at a level P = 0.038. (Sign test, two-tailed)

Further investigation, in animal models, of factors affecting these regional differences in the fluorescence intensity of the brain parenchyma after L-DOPA administration, and of factors affecting the intensity of L-DOPA-induced fluorescence in each brain region may give more information on the fate of L-DOPA used for the treatment of Parkinsonism.

The present study investigated the effects of pretreatment with Ro4-4602 on the regional differences in the increase of diffuse parenchymal formaldehyde-induced fluorescence after L-DOPA administration.

Materials and Methods

Twelve male P.V.G. rats were used, 120-140 g in weight. They were decapitated under chloroform anaesthesia and dissected samples were prepared for fluorescence microscopy and examined as previously described. (Section A; Introduction,A,G) The sections were stored for 2 days before being mounted in Entellan. Solutions of L-DOPA for injection were prepared in 0.08N HCl, and solutions of Ro4-4602 (N^1-(DL-seryl)-N^2-(2, 3, 4-trihydroxybenzyl)-hydrazine) were prepared in distilled water. Both solutions were at a concentration of 10 mg/ml.

Two samples were dissected from each rat brain, one containing regions of the nucleus caudatus-putamen and cerebral cortex, the other containing a region of cerebellum. Details of the samples and regions examined have been described. (Section A; A., Section C; B)

The intensity of diffuse parenchymal fluorescence was measured from circular areas each with a diameter of 10 μm. The areas for fluorimetry were selected from the neuropil and were arbitrarily chosen in each region. Three adjacent sections were taken to represent each tissue sample, and measurements of the intensity of fluorescence were taken from 6 areas from the region or regions to be examined in each section. The mean of the 18 readings obtained from each brain region of each rat was taken as the value for the fluorescence intensity.

Five rats received Ro4-4602 50 mg/kg i.p. 1½ hrs. before killing and L-DOPA 100 mg/kg 1 hr. before killing. Another 5 rats received Ro4-4602 50 mg/kg i.p. 1½ hrs. before killing and L-DOPA 250 mg/kg 1 hr. before killing. The remaining 2 rats were not treated with drugs and served as controls.

It has been reported (32) that this dose regimen of Ro4-4602 inhibited extracerebral dopa-decarboxylase thus increasing blood levels of L-DOPA after a given dose of L-DOPA. In addition, the dopa-decarboxylase in capillary walls was reported to be inhibited by Ro4-4602 50 mg/kg 1½ hrs. before killing, although dopa-decarboxylase in the neostriatal parenchyma probably retained significant activity during the hour before killing.

Results

Treatment	Rat	Fluorescence intensity of neuropil (Arbitrary units) Nuc.caudatus -putamen	D	Cerebral cortex	D	Cerebellum, molecular layer	D
Ro4-4602	1	102	41	80	46	102	66
50mg/kg	2	102	41	84	50	97	61
and	3	100	39	88	54	112	76
L-DOPA	4	106	45	86	52	95	59
100mg/kg	5	128	67	91	57	106	70
Mean values		107	47	86	52	102	66
Ro4-4602	6	169	108	141	107	186	150
50mg/kg	7	174	113	154	120	186	150
and	8	170	109	142	108	194	158
L-DOPA	9	132	71	134	100	174	138
250mg/kg	10	146	85	124	90	136	100
Mean values		158	97	139	105	175	139
None	11	56		32		37	
None	12	65		35		35	
Mean values		61		34		36	

Table 23. The fluorescence intensity of the neuropil of 3 regions of the rat brain, in tissue sections prepared by the formaldehyde-fluorescence method. Each value is the mean of 18 readings of fluorescence intensity. D = Difference between each value from rats 1-10 and the mean of the values for the corresponding region from the untreated rats. Each difference (D) therefore represents the increase of diffuse formaldehyde-induced fluorescence intensity resulting from the administration of L-DOPA and Ro4-4602.

Ro4-4602 was given i.p. 1½ hrs. before killing.

L-DOPA was given i.p. 1 hr. before killing.

For each rat treated with Ro4-4602 and L-DOPA, a calculation was made of the difference (D) between the value of the measured fluorescence intensity and the mean value for the corresponding region from the untreated rats. This difference (D) represents the increase in fluorescence intensity due to Ro4-4602 and L-DOPA administration, and was greater in the cerebellum than in the other regions studied. In both groups of rats (i.e. rats 1-5 and 5-10) the increase in cerebellar fluorescence intensity was significantly greater, at a level $P = 0.031$ (Sign test, one-tailed), than the increase in either the nucleus caudatus-putamen or the cerebral cortex. As this contrast had been previously found in an experiment involving rats which received Ro4-4602 50 mg/kg i.p. 1½ hrs. before killing and L-DOPA 100 mg/kg or 250 mg/kg i.p. 1 hr. before killing, the "one-tailed" Sign test results can be considered significant at this level. (84)

These conclusions are based on the assumption that the values for the 3 regions in the untreated rats gave a sufficiently accurate prediction of the contribution, to each value of fluorescence intensity, in rats 1-10, which did not result from L-DOPA administration.

The results of Section B;A, (in which the neostriatal samples showed a similar ratio of formaldehyde-induced fluorescence to autofluorescence as did rats 11, 12 in this study), showed that the range of intersample differences in neostriatal fluorescence between 7 rats was 23% of the mean value for the samples. Thus, in rats 6-10, it is unlikely that errors due to miscalculation of the contribution of increased L-DOPA-induced fluorescence to the total measured fluorescence, are responsible for the relatively large values for increased fluorescence intensity (D) in the cerebellum, compared with the other brain regions.

As the regional differences in the increase in fluorescence were more marked in rats 6-10 (treated with the larger dose of L-DOPA) than in rats 1-5, it is almost certain that the regional differences were genuine, and not due to concentration-dependent quenching in the cortex and neostriatum. (See General Introduction)

Rats 1-10 received a constant dose of Ro4-4602, while rats 1-5 received L-DOPA 100 mg/kg and rats 5-10 were given L-DOPA 250 mg/kg. The larger dose of L-DOPA probably resulted in the penetration of larger amounts of L-DOPA into the brain parenchyma, and the effects of the change in the dose of L-DOPA on the regional distribution of L-DOPA-induced diffuse fluorescence can be examined by comparing the increases in fluorescence in the 3 regions for each dose regimen. (Table 24)

Ratios of mean increases in fluorescence intensity

	Cerebral cortex	Cerebellum	Cerebellum
Ratio of:-	Nuc. caudatus-putamen	Nuc. caudatus-putamen	Cerebral cortex
Ro4-4602 and L-DOPA 100mg/kg	1.11	1.40	1.27
Ro4-4602 and L-DOPA 250mg/kg	1.08	1.44	1.32

Table 24. Ratios of mean values (D, see table 23) of increases in diffuse formaldehyde-induced fluorescence resulting from L-DOPA administration in rats pretreated with a constant dose of Ro4-4602.

It can be seen that the results in table 24 do not indicate any contrast between the effects of the two dose regimens on the regional distribution of L-DOPA-induced diffuse fluorescence in the regions studied. This finding was confirmed in a subsequent experiment which also found no evidence of a difference in the regional distribution of L-DOPA-induced fluorescence between the nucleus caudatus-putamen and cerebral cortex after a constant dose of L-DOPA (200 mg/kg i.p. 1 hr. before killing), in rats pretreated with Ro4-4602 (i.p. 1½ hrs. before killing) either 50 mg/kg or 150 mg/kg.

Discussion

In the rat, doses of L-DOPA sufficient to produce an increase in diffuse parenchymal fluorescence have been found to produce a larger increase in the nucleus caudatus-putamen, compared with the cerebral cortex or molecular

layer of the cerebellum. (Section C;B)

The results of the present study indicate that after pretreatment with Ro4-4602, L-DOPA administration leads to the largest increase in diffuse fluorescence in the molecular layer of the cerebellum, compared with the other two regions. In addition, the results suggest that pretreatment with Ro4-4602 annulled the contrast between the intensity of L-DOPA-induced fluorescence in the nucleus caudatus-putamen and cerebral cortex.

The reported effects of Ro4-4602 50 mg/kg, 1½ hrs. before killing (32) are to inhibit the dopa-decarboxylase in the capillary walls of most brain regions of the rat, although the enzyme in the neostriatal parenchyma probably remains significantly active during the hour before killing. In addition Ro4-4602 lowers blood levels of L-DOPA for a given dose of L-DOPA. Ro4-4602 may have other effects on the fate of administered L-DOPA, which are as yet unknown.

Summary

The results indicate that pretreatment of rats with the dopa-decarboxylase inhibitor Ro4-4602 affected regional differences in the effects of L-DOPA on the intensity of formaldehyde-induced fluorescence in the parenchyma, compared with the effects of L-DOPA alone.

Inhibition of dopa-decarboxylase in the capillary walls of most regions of the rat brain may have contributed to these effects of Ro4-4602.

Further investigation, in animal models, of factors affecting formaldehyde-induced fluorescence intensity in different regions and in the same region of the brain parenchyma after L-DOPA administration, may give more information on the fate of L-DOPA used for the treatment of Parkinsonism.

E. An investigation of the effects of therapeutically administered L-DOPA.

Introduction

The fluorescence intensity of brain parenchyma after L-DOPA administration has been investigated in regions of the rat and cat brain. (Section C)

The study which involved the incubation of tissue slices from 3 brain regions of the rat and cat in a medium containing L-DOPA and nialamide, reported a marked increase in formaldehyde-induced fluorescence in the majority of rat brain capillary walls due to uptake of L-DOPA and subsequent DA formation and accumulation, in contrast to the capillary walls of the cat brain samples which did not show evidence of DA accumulation.

As some or all the DA formed in rat capillary walls is metabolized in situ (86) any such DA formation and metabolism in man could reduce the therapeutic activity of L-DOPA in Parkinsonism by limiting the passage of L-DOPA into the brain. However, evidence of a species difference in the accumulation of DA in the capillary walls of animal models (Section C;C) makes it impossible to predict the nature of a blood-brain barrier to therapeutically administered L-DOPA in man.

This study reports an investigation of the nature of a blood-brain barrier to administered L-DOPA in man, involving the examination of biopsy samples taken during stereotaxic surgery for Parkinson's disease.

Materials and Methods

Experiment A

A biopsy was obtained from the ventro-lateral nucleus of the thalamus from a patient with Parkinson's disease, weighing approximately 12 stones, who had received 2 grams of L-DOPA, orally, 3 hrs. previously. (It has been reported (52,p.101) that 80% of orally administered L-DOPA is rapidly absorbed in man usually giving peak plasma levels in 1-3 hrs.) The technique of stereotaxic biopsy has been described by Kalyanaraman and Gillingham. (71) The size of biopsy obtained was approximately 1 mm in

diameter X 3 mm in length. It was stored on moist gauze at room temperature before being frozen in liquid nitrogen 2 hrs. later. A male P.V.G. rat, wt. 140 g, was killed by decapitation under chloroform anaesthesia, 30 mins before freezing of a sample which contained regions of nucleus caudatus-putamen and cerebral cortex as previously described. (Section A;A) This sample was frozen at the same time as the biopsy sample and they were both processed together.

The purpose of this experiment was to find out if the capillary walls in the biopsy showed evidence of accumulation of formaldehyde-induced fluorescence.

Experiment B

Three biopsy samples were obtained from the globus pallidus from a patient with Parkinson's disease. The size of each sample was approximately 1 mm in diameter X 2-3 mm in length. Within 5 mins of the biopsies being taken, during which they were stored on moist gauze at room temperature, they were placed in 5 ml of an incubation medium contained in a 50 ml stoppered conical flask, which was then continually shaken for 40 mins at approximately 37°C. The medium was a Krebs-Ringer bicarbonate buffer, as previously described (Section C;C), saturated with 95% O_2 and 5% CO_2, containing L-DOPA 3 mg/100 ml and the monoamine oxidase inhibitor pargyline 15 mgs/100 ml. The latter was designed to inhibit the metabolism of DA to non-fluorogenic products. (15) The samples were frozen in liquid nitrogen within 3 mins of the end of the incubation period.

A male P.V.G. rat, (wt. 130 g) was killed by decapitation under chloroform anaesthesia and 3 samples of a similar size to that of the biopsy samples, were dissected from the globus pallidus. Another sample of the rat brain, containing a region of the cerebral cortex as previously described (Section A;A) was also examined. The samples of globus pallidus from the

rat brain were taken from that region extending approximately 1 mm posteriorly from the median part of the anterior commissure. Within 5 mins of killing the samples of globus pallidus were placed in a further 5 ml of the incubation medium, contained in a stoppered 50 ml conical flask, which was then continually shaken for 40 mins, at approximately 37°C. (The sample containing a region of cerebral cortex was not incubated). Within 3 mins of the end of the incubation period, the samples were frozen in liquid nitrogen. Although the biopsy samples were frozen before the rat brain samples, previous experiments had shown that the time of storage in liquid nitrogen, even up to several weeks, did not have a detectable effect on increased capillary wall fluorescence. When all the samples had been frozen in liquid nitrogen they were all transferred to the freeze dryer and processed together.

In this experiment the incubation medium contained L-DOPA 3 mg/100 ml. while the incubation medium used in the study of rat and cat brain slices (Section C;C) contained L-DOPA 20 mg/100 ml. This was because preliminary experiments with 'biopsy-sized' tissue samples as small as those examined in this study, showed that an incubation medium containing L-DOPA 20 mg/100 ml produced a most intense diffuse parenchymal fluorescence throughout the sample, which made it impossible to judge, by eye, the degree of increase in capillary wall fluorescence relative to the intensity of the surrounding parenchyma. (See Section C;C) (In the study in which rat and cat brain slices were incubated, all the sections examined contained a region, corresponding to the region adjacent to the surface of each tissue sample which showed intense diffuse parenchymal fluorescence compared with the rest of the sample, and was sufficient to interfere with the observation of capillary wall fluorescence. (Section C;C))

In the present study, the intensity of typical capillary wall fluorescence in each sample was compared, by eye, with the measured intensity of the surrounding diffuse formaldehyde-induced fluorescence and parenchymal autofluorescence.

The samples were prepared for fluorescence microscopy and examined as previously described. (Section A;Introduction,A,G) In both experiments, A and B, the sections were stored for 2 days before being mounted in Entellan.

The intensity of fluorescence in the neuropil was measured from circular areas each with a diameter of 10 µm. Measurements were taken from 6 arbitrarily chosen areas from each section, from regions with capillaries judged as having capillary wall fluorescence typical of the section. Three adjacent sections were selected for each tissue sample and the mean of the 18 readings from each sample was taken as an estimate of fluorescence intensity.

Results

Experiment A

The value for the fluorescence intensity of the neuropil of the rat nucleus caudatus-putamen was 84 arbitrary units, while the neuropil of the cerebral cortex gave a value of 40 units. The biopsy gave a value of 34 units for the fluorescence intensity of the neuropil, and the capillary walls showed no evidence of an accumulation of fluorophore.

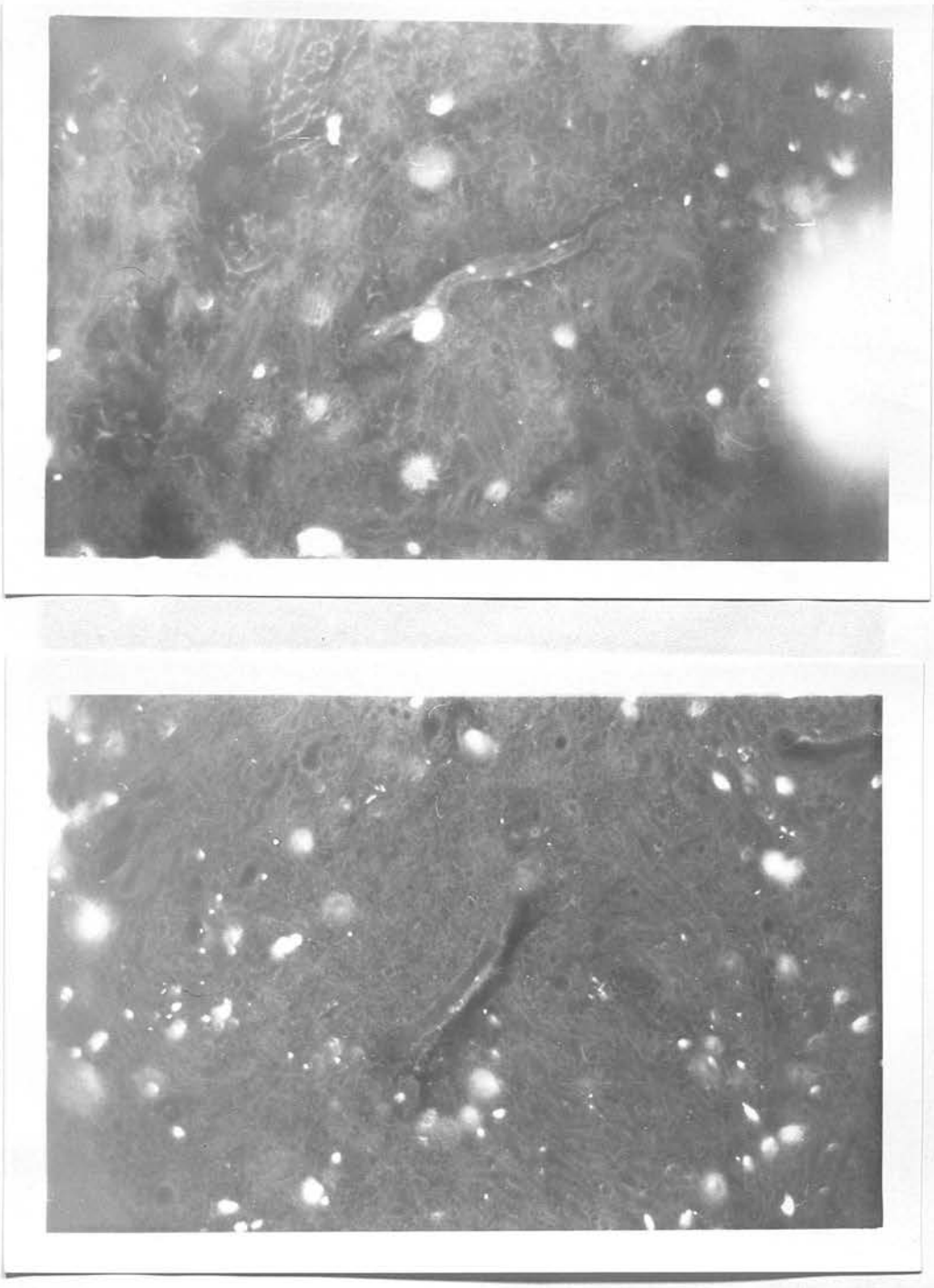


Figure 65. Fluorescence of sample of thalamus, obtained during stereotaxic surgery from a patient with Parkinson's disease, in tissue sections prepared by the formaldehyde-fluorescence method. X600.

Two typical capillaries are shown, in figure 65. There was no evidence of an increase in the fluorescence intensity of capillary walls, relative to the intensity of the surrounding parenchyma. Numerous regions of intense fluorescence are seen. These appeared yellow, and a subsequent experiment when a thalamic biopsy sample was prepared without exposure to formaldehyde gas, indicated that these were regions of autofluorescence. These autofluorescent regions had a maximum dimension of up to 100 μm, and could be seen to be composed of an aggregation of individual spherical granules approximately 2 μm in diameter. (See figure 66)

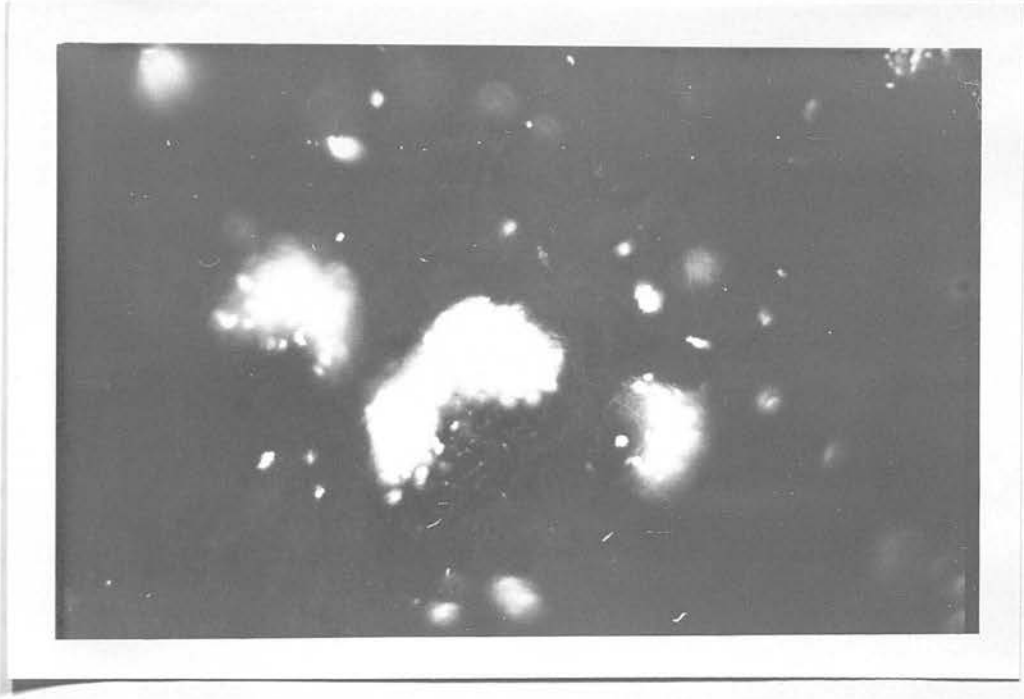


Figure 66. Autofluorescence of a sample of thalamus, obtained during stereotaxic surgery from a patient with Parkinson's disease, in tissue sections prepared by the formaldehyde-fluorescence method. X520. The autofluorescent regions were composed of aggregations of granules, approximately 2 μm in diameter, some of which are visualized.

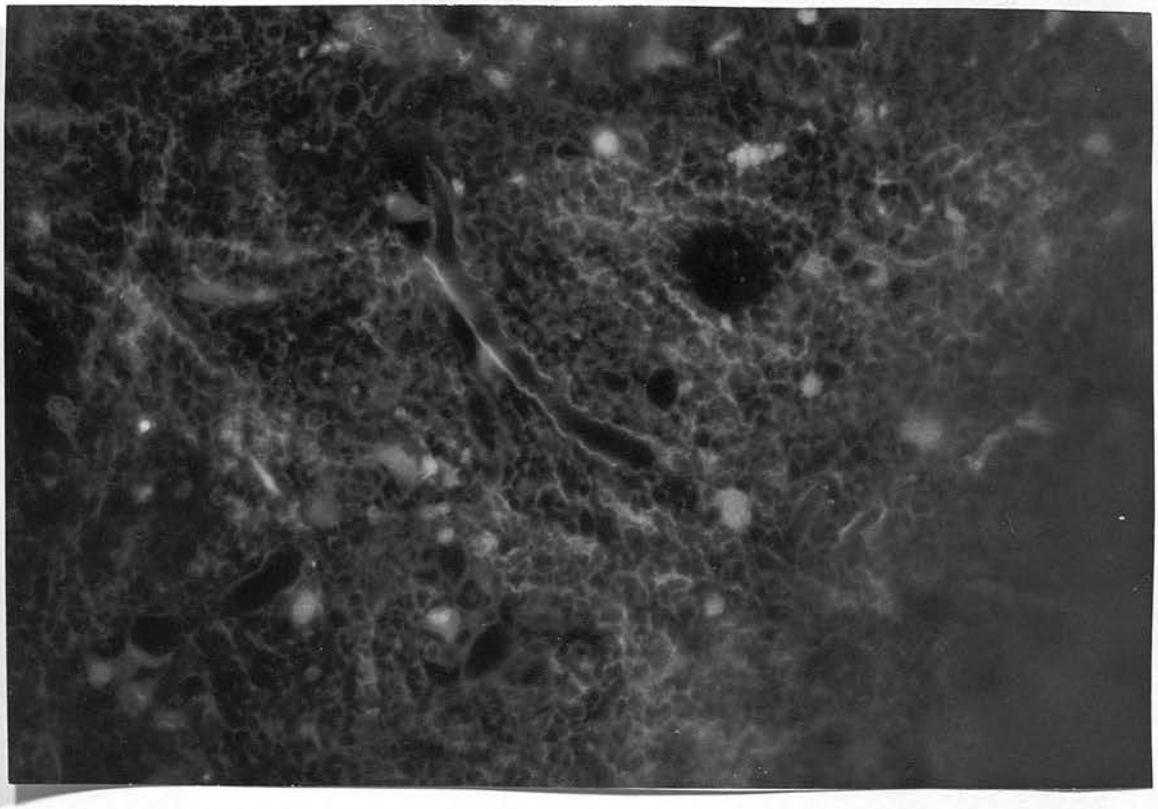
The location of many of these autofluorescent granules was often uncertain; however sometimes an aggregation was seen to be contained in a cell body.

The measurements from the rat brain sample showed that the reaction conditions in this experiment were suitable for the development of formaldehyde-induced fluorescence. However there was no evidence suggesting an increase in the diffuse parenchymal fluorescence or capillary wall fluorescence in the biopsy. Although an interval of 2 hrs. between killing and freezing of the samples had been previously associated with the demonstration of increased capillary wall fluorescence in the rat, the effect of this unavoidable interval during this experiment could have had a significant effect on the histochemical results.

An increase in capillary wall fluorescence in the biopsy would have suggested DA accumulation, however no conclusions can be drawn from this negative finding.

Experiment B

The 3 rat brain samples showed a marked increase in capillary wall fluorescence, relative to surrounding parenchymal fluorescence, in the majority of capillaries observed. This was in contrast to the 3 biopsy samples in which the capillary walls did not appear to fluoresce more intensely than the surrounding neuropil.



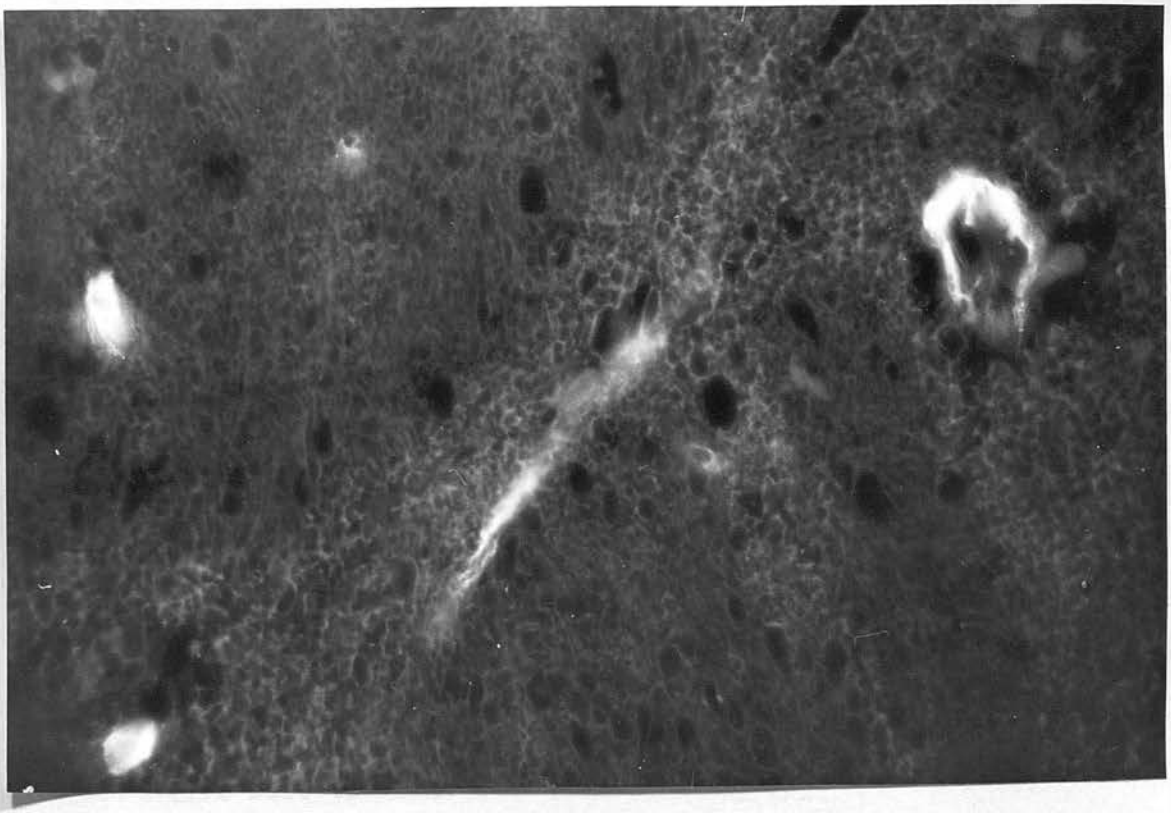


Figure 67. Typical appearance of capillary walls, relative to the

intensity of fluorescence in the surrounding parenchyma, after incubation of samples in a medium containing L-DOPA and pargyline, shown by the formaldehyde-fluorescence method. X750.

Above: Human biopsy samples (globus pallidus)

Below: Rat brain samples (globus pallidus)

As an estimation of capillary wall fluorescence, by eye, is made by comparing the intensity of capillary wall fluorescence with that of the surrounding parenchyma, the intensity of the parenchymal fluorescence in the samples was measured. (Table 25)

	Fluorescence intensity of neuropil (arbitrary units)		
Biopsy samples (globus pallidus)	37	17	17
Rat brain samples (globus pallidus)	41	40	17

Table 25. The fluorescence intensity of the neuropil of the globus pallidus from samples incubated in a medium containing L-DOPA and pargyline before preparation by the formaldehyde-fluorescence method. Each value represents the mean of 18 readings of fluorescence intensity. (The intensity of typical capillary wall fluorescence in each section was compared, by eye, with the measured intensity of fluorescence in the neuropil).

It can be seen that there were variations in the intensity of fluorescence in the neuropil between different samples. However there are examples of a similar level of fluorescence between the biopsy samples and the rat brain samples, and although the neuropil of some rat brain samples showed greater fluorescence intensity than the biopsy samples, a difference in the reverse direction was also seen. It follows that the observed differences in capillary wall fluorescence between the biopsy samples and rat brain samples were at least partly due to actual differences and not just to apparent differences depending on the levels of diffuse fluorescence in the neuropil.

The value for the fluorescence intensity of the neuropil of the sample of rat cerebral cortex, (which was not incubated) was 8 of the same arbitrary units of fluorescence intensity. If it is assumed that the intensity of autofluorescence in all the samples of globus pallidus was of a similar intensity, then all the samples examined showed a marked increase in formaldehyde-induced parenchymal fluorescence.

Discussion

Although no conclusions can be drawn from the negative finding of experiment A, regions of intense autofluorescence were noted and described.

Experiment B does not provide sufficient data for a general conclusion to be drawn, but the results suggest that the capillary walls in samples of globus pallidus from a patient with Parkinsonism did not accumulate DA, derived from L-DOPA in the incubation medium, in contrast with the majority of the capillary walls in the samples from the globus pallidus of the rat, which showed an accumulation of fluorophore.

It is likely that, in the rat, incubation of tissue in the presence of L-DOPA and a monoamine oxidase inhibitor provides a model for the accumulation of DA in the capillary walls after intraperitoneally administered L-DOPA, as in both types of experiment a similar histochemical appearance of the capillary walls has been described. (See Section C;C) Therefore the results of this experiment suggest that capillary walls of the regions of rat brain studied should not be used as a model for a blood-brain barrier to administered L-DOPA in Parkinsonism. This assumes that the capillary walls of the globus pallidus in man are representative of the capillary walls in most brain regions with respect to their capacity to metabolize administered L-DOPA.

Summary

This study reports an investigation of the properties of a blood-brain barrier to administered L-DOPA in man. Biopsy samples, obtained during stereotaxic surgery for Parkinsonism, were incubated in a medium containing L-DOPA and a monoamine oxidase inhibitor.

Although there were insufficient data for a general conclusion to be drawn, the results suggest that the capillary walls in samples of globus pallidus from a patient with Parkinsonism did not show evidence of accumulation of DA, (derived from L-DOPA in the incubation medium), in contrast to the majority of the capillary walls in samples of the globus pallidus from a rat brain.

The nature of a blood-brain barrier to therapeutically administered L-DOPA is discussed with regard to this finding.

F. Drugs which may potentiate the therapeutic effect of L-DOPA in Parkinsonism.

Introduction

L-aminoacid decarboxylase inhibitors (DCIs) and monoamine oxidase inhibitors (MAOIs) have been used in combination with L-DOPA in attempts to potentiate its therapeutic effect. (28) In addition, dimethyl sulphoxide (DMSO) has been reported to assist the transport of L-DOPA across the rat capillary walls which led to the suggestion that a combination of DMSO and L-DOPA may be useful in the treatment of Parkinsonism. (102)

There is evidence (28) that Parkinsonism is usually associated with a decrease in the level of neostriatal DA. The therapeutic effect of L-DOPA may be largely due to its passage into the neostriatum where it could be decarboxylated to DA by the enzyme in the terminal parts of the remaining nigro-striatal neurones. (See General Introduction)

Monoamine oxidase inhibitors (alone) have been reported to have a therapeutic effect in Parkinsonism, although other reports have denied this. (28) In addition, MAOIs have been reported to potentiate the therapeutic actions of L-DOPA. (17) However, troublesome side effects of this combination have been described. (28) In previous experiments (Section C) the MAOI nialamide, when combined with L-DOPA, increased the diffuse formaldehyde-induced fluorescence in the parenchyma of regions of the rat neostriatum, cerebral cortex and cerebellum, compared with the effects of the same dose of L-DOPA alone. Pretreatment with nialamide produced a marked increase in the rat neostriatal DA concentration, compared with the effect of L-DOPA alone, and enhanced the accumulation of fluorophore in rat capillary walls. (See Section C; C)

Dopa-decarboxylase inhibitors have been reported to potentiate the therapeutic effect of L-DOPA. (14) This action may be due to an inhibition of extracerebral metabolism of L-DOPA to DA by a dose of DCI which is not sufficient to significantly inhibit the enzyme in the brain parenchymas. (32) Thus more L-DOPA is available to penetrate the CNS. In the rat, DCI inhibits the 'trapping' of L-DOPA by dopa-decarboxylase in the capillary walls of most

brain regions, (32) thus reducing 'wastage' of L-DOPA due to DA accumulation and metabolism in the capillary walls. If a similar blood-brain barrier mechanism occurs in man, this effect of a DCI could also potentiate the therapeutic action. Extracerebral decarboxylase inhibition may also reduce side effects of L-DOPA administration, such as cardiac arrhythmias, due to increased circulating catecholamines formed by extracerebral metabolism of L-DOPA.

In a previous experiment (Section C;A) pretreatment with the DCI Ro4-4602 potentiated the effect of a dose of L-DOPA on the increased diffuse formaldehyde-induced neostriatal fluorescence (probably largely derived from a mixture of DA and L-DOPA) and increased the concentration of rat neostriatal DA.

de la Torre (102) reported, in the rat, the effects of mixing a dose of L-DOPA with dimethyl sulphoxide (DMSO) on the level of increased diffuse formaldehyde-induced fluorescence in regions of the brain parenchyma. Rats pretreated with nialamide 250 mg/kg i.p. 3 hrs. before killing were given L-DOPA 75 mg/kg i.p. 2 hrs. before killing. In some rats the L-DOPA was given "dissolved in undiluted DMSO" 750 mg/kg. It was reported that DMSO reduced the intensity of the increased capillary wall fluorescence which is associated with accumulation of DA, derived from L-DOPA, in the capillary walls. The above combination of drugs also caused a relatively large increase in the diffuse parenchymal formaldehyde-induced fluorescence, in several brain regions, compared with the effects of L-DOPA and nialamide without DMSO. It was concluded that DMSO assisted the transport of L-DOPA across the capillary walls in several brain regions including the neostriatum. However if DMSO was given 1 hr. before the administration of L-DOPA, the above effects were not seen.

The present study investigated the effects of DMSO on diffuse formaldehyde-induced parenchymal fluorescence, derived from administered L-DOPA, by the use of a microfluorimetric method.

Materials and Methods

Six male P.V.G. rats were used weighing approximately 150 g and were decapitated under chloroform anaesthesia. Two rats received nialamide 250 mg/kg i.p. 3 hrs. before killing and L-DOPA 75 mg/kg i.p. 2 hrs. before killing. Two rats were untreated by drugs.

Solutions for L-DOPA and nialamide for injection were prepared in 0.08N HCl at a concentration of 10 mg/ml. 1 g of dimethyl sulphoxide (Koch-Light) was diluted to 30 ml with distilled water before measuring the dose to be injected, which was contained in approximately 3.4 ml of the diluted solution. The solution of DMSO and L-DOPA were mixed a few minutes prior to injection.

A sample containing regions of nucleus caudatus-putamen and cerebral cortex was dissected from each rat brain. The samples were prepared for fluorescence microscopy and examined as previously described. (Section A; Introduction,A,G) The sections were stored for 2 days before being mounted in Entellan. The details of the sample and the regions examined have been described. (Section A;A)

In addition to observations of capillary wall fluorescence, measurements of the intensity of diffuse parenchymal fluorescence were taken from circular areas of the neuropil, each with a diameter of 10 μm. Six areas for fluorimetry were arbitrarily chosen from each of the 2 selected regions of each section. Three adjacent sections were selected for fluorescence microscopy from each tissue sample, and the mean of the 18 readings for each of the 2 brain regions for each rat was taken as the value for the fluorescence intensity.

Treatment	Fluorescence intensity (Arbitrary units) Nuc. caudatus-putamen	Cerebral cortex
Nialamide and L-DOPA	93 83	38 34
Nialamide, L-DOPA and DMSO	88 87	33 35
None	47 44	24 25

Table 26. The fluorescence intensity of the neuropil in 2 regions of the rat brain, in tissue sections prepared by the formaldehyde-fluorescence method. Each value is the mean of 18 readings of fluorescence intensity.

It can be seen that there was no evidence of an effect of DMSO on the intensity of L-DOPA-induced parenchymal fluorescence, such as had been previously reported. (102) The majority of the capillary walls in all the rats treated with L-DOPA appeared to show a similar degree of intense formaldehyde-induced fluorescence. (See Section C;C)

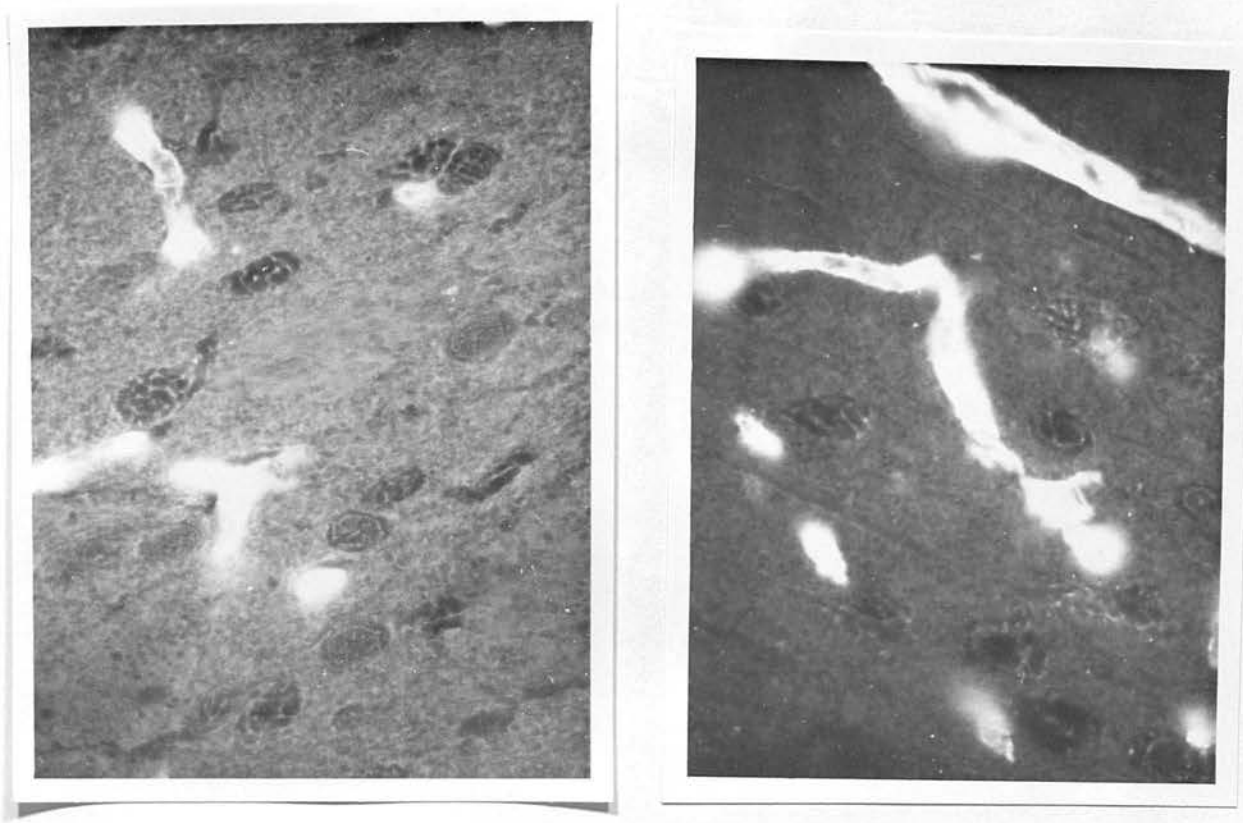


Figure 68. Fluorescence in the nucleus caudatus-putamen (left) and cerebral cortex (right) in a rat treated with nialamide 250 mg/kg, L-DOPA 75 mg/kg and DMSO 750 mg/kg. X590. The sections were prepared by the formaldehyde-fluorescence method. The parenchyma showed an increased diffuse fluorescence compared with untreated rats, and the capillary walls showed an intense formaldehyde-induced fluorescence.

Thus, another of de la Torre's reported findings (102), that DMSO reduced the intensity of capillary wall fluorescence produced by a combination of L-DOPA and nialamide, was not confirmed.

Discussion

The results of the present study do not confirm previously reported effects of DMSO on formaldehyde-induced fluorescence produced by L-DOPA and nialamide. One difference between de la Torre's study and the present study is that in the latter, L-DOPA was not dissolved in pure DMSO. This was not

attempted because of the risk of error in the measurement of the required dose of DMSO from undiluted DMSO. It is difficult to understand how the dose of L-DOPA was dissolved in undiluted DMSO in de la Torre's study.

In subsequent experiments, DMSO 750 mg/kg 1 hr. (i.e. mixed with the dose of L-DOPA) or 2 hrs. before killing had no obvious effect on L-DOPA-induced fluorescence after L-DOPA 200 mg/kg 1 hr. before killing, in the rat nucleus caudatus-putamen or cerebral cortex.

Summary

Drugs which may potentiate the therapeutic activity of L-DOPA are discussed.

Amongst these, dimethyl sulphoxide (DMSO) has been suggested as a possible potentiator of the therapeutic activity of L-DOPA, on the basis of its previously reported effects on the intensity of parenchymal formaldehyde-induced fluorescence of the rat brain following L-DOPA administration. However these effects were not confirmed in the present study.

Abbreviations

DA	Dopamine
L-DOPA	L-3, 4-dihydroxyphenylalanine
i.p.	Intraperitoneal injection
Parenchyma	Tissue, excluding capillary walls
Neuropil	Part of parenchyma, consisting of meshwork of cell processes
NA	Noradrenaline/Numerical aperture
A	Adrenaline
5HT	5-hydroxytryptamine
HVA	Homovanillic acid
5HTP	5-hydroxytryptophan
ACh	Acetyl choline
Interquartile range	A range of values, excluding the highest 25% and the lowest 25%
EDTA	Ethylenediamine tetra-acetic acid

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Declaration

I declare that this thesis has been composed by myself and that the work reported is my own.

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Life Sciences	13, 23-29. 1973.	
Neuropharmacology	(In press, for October 1973)	*
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Histochemie	(In press, 1973)	***

* 1973, 12, 949-953

** 1973, 29, 1122-1123

*** 1973, 37, 75-79

Appendix

Changes in the intensity of formaldehyde-induced fluorescence during storage of tissue samples.

Introduction

In a previous experiment (see p.159) differences were reported in the intensity of formaldehyde-induced fluorescence of the rat striatal neuropil associated with different sets of storage conditions of tissue sections. After sectioning, the sections were stored for five days under two sets of conditions. In the first set, some sections were mounted in Entellan within 2 h of sectioning before being stored for five days, in the dark, prior to examination. In the second set, the remaining tissue sections were stored as ribbons for three days, before being mounted in Entellan. The mounted sections were then stored in darkness for a further two days before examination. Sections associated with both sets of storage conditions were examined during the same period of microscopy.

Measurements of the diffuse formaldehyde-induced fluorescence of the straital neuropil showed that sections stored under the second set of reaction conditions had a higher intensity of fluorescence than the sections stored under the first set.

One or both of the following factors may have been responsible for this difference:-/

/difference:-

(a) An increase in specific striatal fluorescence may occur during storage of tissue as ribbons.

(b) A decrease in specific striatal fluorescence may take place during storage of tissue as mounted sections.

The present study was designed to investigate these possibilities further.

From each tissue sample some sections were examined on the same day as sectioning while others were examined after storage for five days under two sets of reaction conditions. In one set, the sections were stored as ribbons and in the other set they were stored as sections mounted in Entellan. In addition, further sections were cut from the blocks at the end of the storage interval, so that the effect of storage of tissue embedded in a wax block could also be investigated.

In this study the contribution of the autofluorescence to the total fluorescence of the striatal neuropil has been inferred from measurements of the fluorescence in the neuropil of a region of the cerebral cortex, by assuming that the specific component of the cortical fluorescence is sufficiently small to be ignored and that the autofluorescence is similar in both regions (see pp.126,159).

Materials and method

Eight male albino rats approximately 140 g were used. One sample from each rat, each containing the regions/

/regions of the nucleus caudatus-putamen and cerebral cortex as previously specified (see p.125), was prepared and examined. Any differences in the striatal fluorescence intensity between sections cut within these limits from the same tissue sample were assumed to be sufficiently small to be ignored. (See pp. 96-122, 173). Sections were cut on the day after the embedding of the formaldehyde-treated tissue in paraffin wax and the tissue was then stored, at a room temperature of 23°C, under 4 sets of conditions.

Set 1: Sections were mounted in Entellan within 1 h of sectioning and were examined within 4 h of sectioning.

Set 2: Sections were stored as ribbons in the dark on slide trays in non-airtight plastic boxes for five days before they were mounted in Entellan. They were then examined within 3 h of mounting.

Set 3: Sections were mounted in Entellan within 1 h of sectioning and were stored in the dark for five days before examination.

Set 4: After removal of the initial sections, the cut surfaces of the paraffin wax blocks, each containing the remaining part of the tissue sample, were sealed with paraffin wax. The blocks were then stored in the dark for five days, after which further sections were cut. These sections were mounted in Entellan within 1 h of sectioning and examined within 4 h of sectioning.

Sections stored under the conditions of sets 2-4 were examined during the same period of microscopy. Measurements of diffuse fluorescence of the neuropil of the striatum and cerebral cortex were obtained as previously described. (See p.126).

Results

TABLE 1.

Fluorescence intensity (arbitrary units)

	Total fluorescence of caudate nucleus (CN)	Cerebral cortex (CC)	Specific fluorescence of CN. CN-CC
Rat			
1	74	38	36
2	72	50	22
3	67	37	30
4	71	38	33
5	71	46	25
6	76	40	36
7	73	46	27
8	63	40	23
Mean value	71	42	29

The intensity of fluorescence of the neuropil of the caudate nucleus, measured on the same day as sectioning, which was the day after paraffin wax embedding of the freeze-dried formaldehyde-treated tissue. The fluorescence of the cerebral cortex, which gives an estimate of the striatal autofluorescence, was also measured. Each value represents the mean of 18 readings of fluorescence intensity.

TABLE 2.

The fluorescence intensities of formaldehyde-treated tissue sections kept under different storage conditions before measurement.

	Fluorescence intensity (arbitrary units)								
	Total fluorescence of caudate nucleus (CN)			Cerebral cortex (CC)			Specific fluorescence of CN. (CN-CC)		
Storage conditions	Set 2	Set 3	Set 4	Set 2	Set 3	Set 4	Set 2	Set 3	Set 4
Rat 1	86	58	61	35	41	34	51	17	27
2	91	58	56	42	44	37	49	14	19
3	82	52	56	34	36	31	48	16	25
4	103	59	64	40	40	30	63	19	34
5	89	53	63	39	36	43	50	17	20
6	104	59	60	44	38	37	60	21	23
7	84	54	69	42	44	45	42	10	24
8	93	55	65	45	42	37	48	13	28
Mean	92	56	62	40	40	37	52	16	25

The intensity of fluorescence of the neuropil of the caudate nucleus, six days after embedding of the freeze-dried tissue and five days after sectioning. The effects of three sets of conditions for tissue storage are shown. The fluorescence of the cerebral cortex, which gives an estimate of the autofluorescence of the striatal neuropil was also measured. Each value represents the mean of 18 readings of fluorescence intensity. CN-CC represents the formaldehyde-induced component of the total striatal fluorescence.

Discussion

In Table 2 it can be seen that the part of each sample which/

/which had been stored as ribbons for five days (Set 2 conditions) showed a markedly greater intensity of formaldehyde-induced striatal fluorescence (CN-CC) compared with the parts which had been stored as mounted sections or as blocks embedded in paraffin wax. It is possible that exposure of the tissue sections to the humidity or oxygen of the atmosphere during storage as ribbons was responsible for this finding.

In addition, the values for the specific striatal fluorescence (CN-CC) associated with storage as sealed blocks (Set 4 conditions) are significantly higher than those associated with storage as mounted sections (Set 3 conditions), t test, $p = < 0.01$, although the difference between their mean values is relatively small compared with the difference between the mean value for Set 2 conditions and the mean value for the conditions of Set 3 or Set 4.

Differences between the values for the autofluorescence of the neuropil of the cerebral cortex were relatively small in Sets 2-4 compared with the differences between the values for striatal fluorescence, therefore the contrast in striatal fluorescence (CN) between Set 2 and Set 3 or 4 was almost certainly due to differences in the formaldehyde-induced component of the total striatal fluorescence, if it is assumed that both regions have a similar level of autofluorescence after a period of storage as ribbons or as mounted sections. The justification for such an/

/an assumption has been discussed. (See p.164). It should be noted that the arbitrary unit of fluorescence intensity in Table 1, while similar to that of Table 2, is not necessarily identical, as there may have been differences in the intensity of the exciting light between the two periods of microscopy. One possible reason for such a difference is that the emission intensity of the light source can alter between different periods of microscopy. In addition, the position of a mirror which determined the path of the exciting light often had to be readjusted before each period of microscopy and any slight change in its position would also have affected the intensity of the exciting light reaching the specimen . (This readjustment was necessary if other users of the microscope had changed the path of the exciting light during the interval between two periods of microscopy). Although the intensity of fluorescence of a fluorescent glass standard can be compared between two periods of microscopy, the interpretation of any difference is difficult because a change in the intensity of a fluorescent standard may not correspond to a similar degree of change in measurements of fluroescence in tissue samples. (See p.168).

The values for the cerebral cortex in Table 1 are similar to those in Table 2 and although it is possible that the effects of any change in the intensity of the exciting light between the two periods of microscopy could have/

/have been cancelled out by the effects of a change in autofluorescence during the storage interval, it is likely that there was no significant change in the intensity of the exciting light between the two periods of microscopy and that the cortical autofluorescence showed, at most, a relatively small change during the storage period compared with the changes in the specific striatal fluorescence.

If it is assumed that any difference between the arbitrary units of fluorescence in Tables 1 and 2 are sufficiently small to be ignored, the values of the Tables 1 and 2 can be compared. They suggest that there was a marked increase in specific striatal fluorescence during storage of the sections as ribbons. Even if the values of Table 1 are not directly compared with those of Table 2, it can be seen that the contrast between cortical and striatal fluorescence was much greater after storage of the section as ribbons compared with the sections which were examined on the same day as sectioning.

If the results of Tables 1 and 2 are compared they also suggest that storage of tissue embedded in a paraffin wax block was not associated with any significant change in striatal fluorescence and that there was a decrease in the specific striatal fluorescence of the sections which had been mounted in Entellan during the storage interval.

Summary

This study investigated changes in formaldehyde-induced striatal fluorescence during different storage conditions of the tissue samples after they had been embedded in paraffin wax. Storage as ribbons, cut at 8 μm, was associated with greater striatal fluorescence compared with storage of tissue embedded in a block of paraffin wax or storage as 8 μm sections mounted in Entellan. Storage as sections mounted in Entellan was associated with the lowest intensity of striatal fluorescence. The results suggest that there had been an increase in specific fluorescence of the striatum during storage of the ribbons, while storage as sections mounted in Entellan might result in a decrease in specific fluorescence of the striatum.

The effect of the interval between killing and freezing of brain samples on the intensity of formaldehyde-induced fluorescence of the rat striatum.

Introduction

In all the experiments reported in the thesis, the time between killing of the animals and freezing of the tissue depended on the number of animals in each experiment and although this interval varied up to a maximum of approximately 2 h, it was usually less than 30 min. The interval between killing and freezing of the tissue was almost identical (i.e. within a range of 5 min) for all the samples in each experiment. (The tissue samples were dissected within 2 min of removal of the brain from the skull). However, the times (i) between killing and removal of the brain from the skull and (ii) between removal of the brain from the skull and freezing of the sample were not constant for each interval between killing and freezing and Joyce (70) has suggested that these variables can significantly affect the nature of post-mortem changes.

The present study investigated the effects of these variables on the formaldehyde-induced fluorescence of the rat striatum.

Materials and method

Sixteen male albino rats (approximately 140 g) were used. One sample from each rat, each containing the regions of the nucleus caudatus-putamen and cerebral cortex which have been previously specified (see p. 125), was prepared and examined. (See pp. 96-122, 173).

After sectioning, the ribbons were stored in the dark in non-airtight plastic boxes for three days before being mounted in Entellan. A three-day interval was chosen as this was approximately the average period of storage in previous experiments. The sections were examined within 3 h of mounting.

The brain samples had been prepared in four groups:-

Group 1. Each sample was dissected and frozen in liquid nitrogen within 4 min of killing.

Group 2. Each sample was dissected within 4 min of killing and kept for 56 min at a room temperature of 23°C before being frozen. During this interval the samples were kept on a metal tray in a closed vessel, containing moist filter paper to provide additional humidity. This reduced drying of the tissue surface.

Group 3. Each sample was prepared as described in group 2, except that the dissected samples were kept for 1 h 56 min before freezing.

Group 4. Each skull was opened 1 h 56 min after killing, so that the sample could be dissected before being frozen 2 h after killing.

Measurements of diffuse fluorescence of the striatal neuropil were taken as previously described. (See p.126). Measurements of cortical autofluorescence were also taken, so that the contribution of the autofluorescence to the total fluorescence of the striatal neuropil could be/

~~by~~ inferred by assuming the similarity of the autofluorescence in both regions. (See pp.126, 159).

Results

	Fluorescence of caudate N.	Fluorescence of cerebral cortex	Time between killing and freezing	Details of preparation
	(Arbitrary units)			
Group 1 (4 rats)	94,89 88,103	40,42 34,40	Approx 4 min	Tissue frozen within 4 min
	Mean = 93	Mean = 39		
Group 2 (4 rats)	95,103 91,99	38,39 35,45	1 h	Brain removed and dissected within 4 min of killing
	Mean = 97	Mean 39		
Group 3 (4 rats)	96,103 93,76	45,47 43,38	2 h	As in Group 2
	Mean = 92	Mean = 43		
Group 4 (4 rats)	84,82 79,75	41,40 40.39	2 h	Brain remained in skull until 4 min prior to freezing of dissected tissue
	Mean = 80	Mean = 40		

Fluorescence intensity of the neuropil in tissue prepared by the formaldehyde-fluorescence method. Each value represents the mean of 18 readings of fluorescence intensity. The contribution of autofluorescence to the total striatal fluorescence can be inferred from the values for the cerebral cortex by assuming that the/

/the formaldehyde-induced component of the cortical fluorescence is sufficiently small to be ignored and that the autofluorescence is similar in both regions.

Discussion

The mean total fluorescence intensity of the striatal neuropil was low in group 4 compared with the other groups. However, the differences between the groups are not significant at the 5 per cent level, (Kruskal-Wallis one-way analysis of variance, $p = \langle 0.1$) (84). Nevertheless, this suggests that it would be a sensible precaution to control for the length of time between killing and removal of the brain from the skull. The relatively low intensity of striatal fluorescence in group 4 compared with group 3 may have been the result of a higher post-mortem mean temperature in the brains which remained in the skull for the longer post-mortem period. The higher temperature may have resulted in a relatively rapid metabolism of striatal dopamine to non-fluorogenic products.

The results of groups 1-3 do not demonstrate any significant change associated with the different time intervals between dissection and freezing during which the samples were maintained at a room temperature of $23^{o}C$. Although it is possible that as the post-mortem time increased there was an increasing degree of diffusion of dopamine in the striatal neuropil, this could have been masked by diffusion of dopamine during the exposure of the freeze-dried tissue to formaldehyde gas.

Summary

The fluorescence intensity of the rat striatal neuropil /

/neuropil was examined after preparation by the formaldehyde-fluorescence method. Some tissue samples were dissected within 4 min of killing and were then frozen after post-mortem times of up to 2 h. The length of this interval had no significant effect on the intensity of striatal fluorescence. However, other samples obtained from brains which had remained in the skull for 1 h 56 min after killing before being frozen 2 h after killing showed less intense striatal fluorescence compared with samples which had been dissected within 4 min of killing and maintained at 23°C for 1 h 56 min before freezing. Although the difference was not statistically significant at the 5 per cent level, it suggests that the interval between killing and removal of the brain from the skull should be controlled.