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Time course of the effects of 6-hydroxydopamine on catecholamine-containing neurones in rat hypothalamus and striatum

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Summary

1. The effects of intraventricular injection of 6-hydroxydopamine (6-OHDA) on tyrosine hydroxylase activity, uptake of ^{3}H-noradrenaline and endogenous catecholamine concentration in rat hypothalamus and striatum were investigated at various times after the injection of 6-OHDA.

2. In the hypothalamus after the injection of 250 μg of 6-OHDA there was a rapid decrease in tyrosine hydroxylase activity, ^{3}H-noradrenaline uptake and noradrenaline content, which was essentially complete within 2 hours.

3. In the striatum after this dose of 6-OHDA there was a much slower reduction in tyrosine hydroxylase activity and ^{3}H-noradrenaline uptake during the first 48 h after drug injection. For the first 24 h the dopamine concentration in this brain area was increased significantly above control values, but had fallen below control values by 48 hours.

4. After the injection of a smaller dose of 6-OHDA (25 μg) the only detectable change in the striatum was a rapid increase in the dopamine concentration. In the hypothalamus this dose induced a rapid depletion of noradrenaline, not accompanied initially by any significant reduction in tyrosine hydroxylase activity.

5. These results are consistent with the hypothesis that 6-OHDA causes a rapid degeneration of catecholamine-containing nerve terminals in the central nervous system (CNS). These degenerative changes, indicated by the loss of tyrosine hydroxylase and noradrenaline uptake sites, did not appear to be preceded by an initial displacement of endogenous catecholamines by 6-OHDA, except possibly at early times after the administration of small doses of the drug.

Introduction

Systemically administered 6-hydroxydopamine (6-OHDA) produces a selective degeneration of adrenergic nerve terminals in peripheral sympathetically innervated organs (Tranzer & Thoenen, 1968; Malmfors & Sachs, 1968). Catecholamine-containing neurones in the rat brain appear to be similarly affected after the injection of 6-OHDA into the cerebrospinal fluid (Bloom, Algeri, Groppetti & Costa, 1969; Uretsky & Iversen, 1969, 1970; Bartholini, Richards & Pletscher, 1970). After treatment with large doses of 6-OHDA, tyrosine hydroxylase activity, endogenous catecholamines and uptake sites for ^{3}H-noradrenaline all undergo a long-

lasting reduction in rat brain, suggesting that degenerative changes occur in CNS catecholamine neurones (Uretsky & Iversen, 1970).

Bartholini *et al.* (1970), however, have suggested that the decrease in CNS catecholamines caused by 6-OHDA may be due in part to a displacement of the catecholamines by 6-OHDA, or to an inhibition of catecholamine biosynthesis. Thoenen & Tranzer (1968), Jonsson & Sachs (1970) and Bennett, Burnstock, Cobb & Malmfors (1970) have shown that after the administration of small doses, 6-OHDA is taken up and stored by peripheral adrenergic neurones and can probably be released as a 'false transmitter.'

In the present study we have sought to establish whether 6-OHDA can displace catecholamines in the rat brain, and to distinguish this effect from the chronic degenerative changes induced by the drug. For this purpose we have studied changes in brain catecholamines, tyrosine hydroxylase and noradrenaline uptake at early times after 6-OHDA administration, and after the administration of doses of 6-OHDA smaller than those previously found to cause maximum degenerative changes (Uretsky & Iversen, 1970). If 6-OHDA acts initially by displacing noradrenaline or dopamine before degeneration occurs, one might expect amine levels to be reduced before the other biochemical systems were affected. These studies were carried out on an area of the brain rich in noradrenaline terminals (hypothalamus), and an area rich in dopamine terminals (striatum) to compare the responses of these two types of catecholamine-containing neurone to 6-OHDA treatment.

Methods

Male or female albino Wistar rats (150–200 g) were used. Twenty μl of a solution of 6-OHDA hydrobromide (containing 25 or 250 μg of free base) was injected into the lateral ventricle of the brain as described by Uretsky & Iversen (1970). Control animals received 20 μl of the vehicle solution. Animals were stunned and killed by decapitation at various times after drug injection. Brains were rapidly removed and placed on an ice-cooled metal surface. The hypothalamus and both corpora striata were dissected by the procedure of Glowinski & Iversen (1966) and used for the estimation of tyrosine hydroxylase activity, or endogenous catecholamines, or the uptake of ^{3}H-noradrenaline.

Tyrosine hydroxylase assay

The hypothalamus or striatum was weighed and homogenized in 10 volumes of ice-cold 10% sucrose solution. Tyrosine hydroxylase activity was assayed in samples of the homogenates by measuring the rate of formation of ^{3}H-DOPA from ^{3}H-L-tyrosine, using two different procedures. In one, the activity was assayed by a modification of the method of McGeer, Gibson & McGeer (1967) as described by Uretsky & Iversen (1970). The other assay method was similar, but included treatment of the homogenates with Triton X-100 to release particle-bound enzyme activity, and the addition of mercaptoethanol and a reduced pteridine cofactor (Iversen & Uretsky, 1970).

Endogenous catecholamine assays

Hypothalamic or striatal tissue from two or three rats was pooled for the fluorimetric estimation of noradrenaline or dopamine. The catecholamines were extracted

from the tissue and adsorbed on Amberlite CG-120 resin columns (Uretsky & Iversen, 1970). Noradrenaline was eluted with 10 ml of 1 N hydrochloric acid (3 ml discarded, 5 ml collected, 2 ml discarded) and dopamine with a further 8 ml of 2 N hydrochloric acid.

For some samples a double column procedure was used. After centrifuging neutralized tissue extracts to remove the potassium perchlorate precipitate, the supernatant was mixed with 300 mg alumina and 0·3 volume of 0·2 M EDTA, and the pH adjusted to 8·5–9·0 with sodium hydroxide solution. The alumina suspension was poured onto columns, and after washing with 15 ml of distilled water, the catecholamines were eluted with 6 ml of 0·2 N hydrochloric acid. After the addition to the eluates of 0·25 ml of a mixture containing 4% EDTA and 0·4% ascorbic acid they were adjusted to pH 3·0–4·0 with sodium hydroxide and poured onto columns of Amberlite CG-120 resin. The catecholamines were finally eluted from these columns as described above.

The catecholamines were assayed fluorimetrically as described by Uretsky & Iversen (1970). Except where stated, the results were not corrected for loss of catecholamines during their isolation, and are expressed as percentages of the values obtained in vehicle treated controls.

Uptake of ^{3}H-noradrenaline

Uptake of ^{3}H-noradrenaline was measured by two different methods. In the first method, slices from hypothalamus or striatum were incubated for 5 min in Krebs-Ringer phosphate solution containing ^{3}H-L-noradrenaline (0·05 μM) as described by Uretsky & Iversen (1970). In the second method, ^{3}H-noradrenaline uptake into synaptosomes was measured in homogenates of the two brain regions by the method of Snyder & Coyle (1969). The latter method had the advantage that aliquots of the same homogenates could be used for both uptake and tyrosine hydroxylase measurements; this method also measures only the uptake of ^{3}H-noradenaline by adrenergic nerve terminals.

Radioactive materials

L-7-^{3}H-Noradrenaline (specific activity, 2·18 Ci/mmol); L-2,3-^{3}H-tyrosine (specific activity, 5·3 Ci/mmol) and ^{3}H-dopamine (generally labelled) (specific activity, 280 mCi/mmol) were obtained from the Radiochemical Centre, Amersham.

Results

Two series of experiments were performed. In one, animals were killed at various intervals (0·5 h–16 days) after the injection of a single dose of 250 μg of 6-OHDA and their brains analysed (Fig. 1). In the other, measurements were made at 0·5 h and 24 h after the injection of a single dose of 25 μg of 6-OHDA (Fig. 2).

Hypothalamus

Noradrenaline concentration

After 250 μg of 6-OHDA there was a progressive fall in hypothalamic noradrenaline which was significant 0·5 h after 6-OHDA injection and complete within 24 h

(Fig. 1). A significant reduction in hypothalamic noradrenaline was also observed 0·5 h after the injection of 25 μg of 6-OHDA (Fig. 2).

Tyrosine hydroxylase

Both assay pocedures produced similar results. Enzyme activity was reduced by approximately 50% within 2 h after injection of 250 μg of 6-OHDA, and thereafter

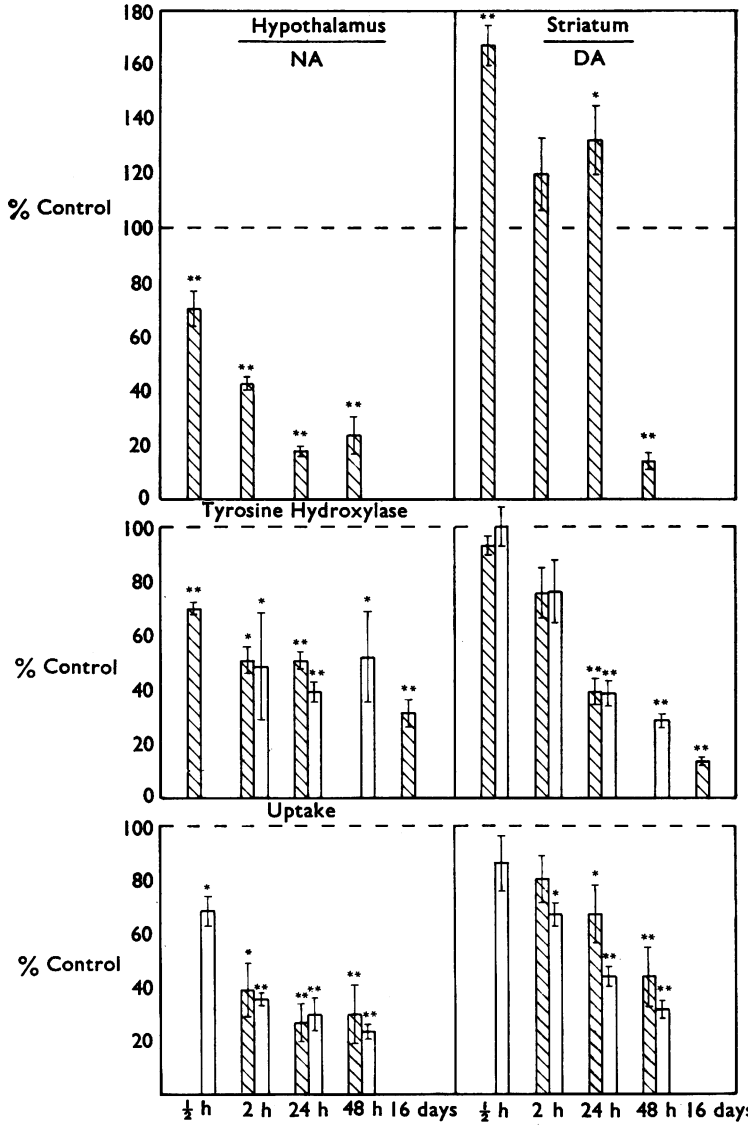

FIG. 1. Time course of changes in noradrenaline (NA), dopamine (DA), tyrosine hydroxylase and ^{3}H-noradrenaline uptake in rat hypothalamus and striatum after the intraventricular injection of 250 μg 6-hydroxydopamine. All values are expressed as percentages of those obtained in vehicle treated rats and are means ± S.E. for three to six experiments. The endogenous NA and DA values represent the pooled results obtained by two different methods of extraction (compare Fig. 3). Tyrosine hydroxylase results by method of McGeer *et al.* (1967), hatched columns; by method of Iversen & Uretsky (1970), open columns. Results for noradrenaline uptake in tissue slices, hatched columns; uptake in tissue homogenates, open columns. $*=P<0{\cdot}05$, $**P=<0{\cdot}01$, when compared with control values.

there were no futher significant changes (Fig. 1). There was no recovery of enzyme activity 16 days after 6-OHDA injection.

After the lower dose of 6-OHDA (25 μg) there was no significant reduction in enzyme activity at 0·5 h, although at this time a significant depletion of noradrenaline had occurred (Fig. 2). However, by 24 h after this dose of 6-OHDA, tyrosine hydroxylase was significantly reduced.

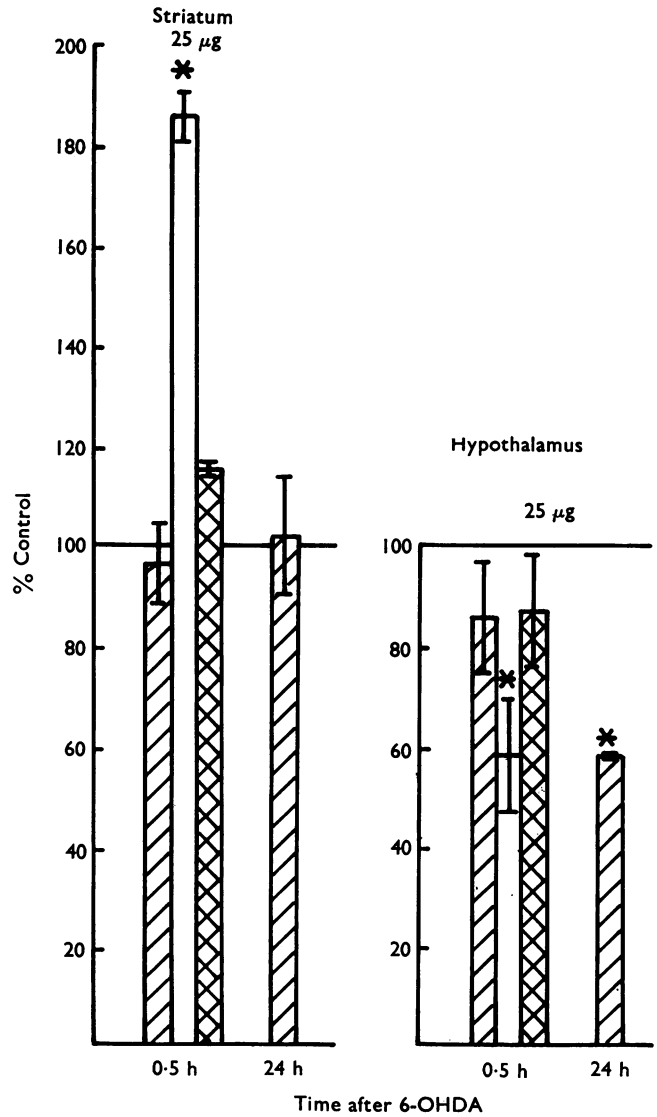

FIG. 2. Changes in hypothalamic noradrenaline (NA), striatal dopamine (DA), tyrosine hydroxylase (McGreer *et al.*, 1967, assay), and ^{3}H-noradrenaline uptake (tissue slices) in rat hypothalamus and striatum after the intraventricular injection of 25 μg of 6-hydroxydopamine. Values are expressed as percentages of those in vehicle treated controls (Table 1) and are means ± S.E. for three to six experiments at each point; *$=P<0·05$ when compared with control values. [/], Tyrosine hydroxylase; □, NA or DA; [×], uptake of ^{3}H-noradrenaline.

^{3}H-Noradrenaline uptake

Again, measurements by the two procedures used were in close agreement. The ability to concentrate ^{3}H-noradrenaline in slices or homogenates of hypothalamus was significantly reduced at 0·5 h and by approximately 60% at 2 h after the injection of 250 μg of 6-OHDA (Fig. 1).

With the lower dose of 6-OHDA, however, there was no significant reduction in ^{3}H-noradrenaline uptake 0·5 h after the drug injection (Fig. 2).

Striatum

Dopamine concentration

During the first 24 h after 6-OHDA (250 μg) there was an unexpected increase in the dopamine concentration of striatal tissue. This increase was most pronounced 0·5 h after the injection of 6-OHDA, and was followed by a substantial reduction in dopamine concentration between 24 and 48 h after the drug injection (Fig. 1).

A similar large increase in striatal dopamine was observed 0·5 h after the injection of the lower dose of 6-OHDA (25 μg) (Fig. 2).

Tyrosine hydroxylase

The reduction in tyrosine hydroxylase activity in the striatum after 6-OHDA (250 μg) was much slower than that observed in the hypothalamus. No significant reduction occurred within the first 2 h after drug injection, and the reduction which occurred thereafter was progressive, reaching a maximum 85% reduction at 16 days (Fig. 1).

After the lower dose of 6-OHDA (25 μg) there was no significant reduction in enzyme activity at either 0·5 h or 24 h (Fig. 2).

^{3}H-Noradrenaline uptake

There was a progressive reduction in the ability of striatal tissue to accumulate exogenous noradrenaline during the first 48 h after 250 μg of 6-OHDA, with no significant reduction becoming apparent until 2 h after injection.

There was no significant reduction in uptake 0·5 h after the injection of the lower dose of 6-OHDA (25 μg).

TABLE 1. *Control values for catecholamines, tyrosine hydroxylase and noradrenaline uptake in rat hypothalamus and striatum*

	Units	*n*	Striatum	*n*	Hypothalamus
Noradrenaline*	μg/g	—	—	15	1·7±0·06
Dopamine*	μg/g	15	5·3±0·64	—	—
Tyrosine hydroxylase	(nmol/g)/h				
McGeer *et al.* (1967) assay)		15	27·0±1·35†	16	6·5±0·35†
Iversen & Uretsky (1970) assay		4	21·4±1·26	4	7·8±0·32
^{3}H-Noradrenaline uptake: concentration ratio					
Slices		8	15·8±1·40	8	5·9±0·20
Homogenates		8	19·7±2·54	8	6·0±0·43

Values are overall means of *n* determinations ±S.E.
* Noradrenaline and dopamine values corrected for average recovery of 65% in the isolation procedures.
† Extrapolated V_{max} values.

Checks on the specificity of the isolation and assay procedures for striatal dopamine

Because of the large and unexpected increases in endogenous dopamine concentrations in the striatum after 6-OHDA, the isolation and assay procedures were investigated in an attempt to eliminate the possibility that the apparent rises in dopamine might be due to contamination with 6-OHDA. Laverty, Sharman & Vogt (1965) reported that 6-OHDA was eluted in the dopamine fraction (2 N hydrochloric acid), following elution of noradrenaline from Dowex-50 resin columns. When 6-OHDA

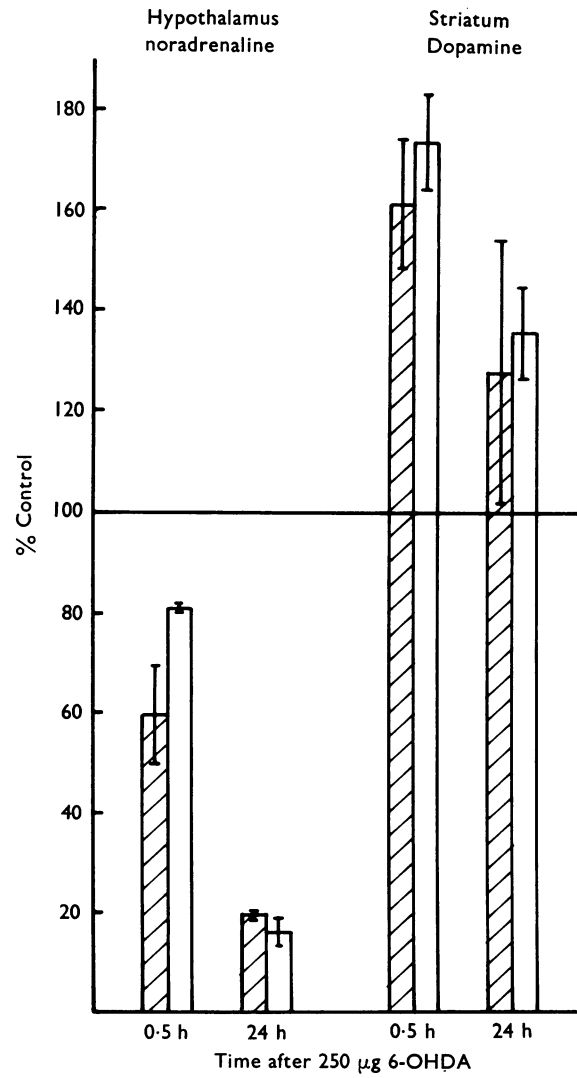

FIG. 3. A comparison of endogenous catecholamine concentrations measured after extraction by Amberlite alone (hatched columns) and Amberlite+alumina columns (open columns); for details see **Methods**. Samples from animals killed 0·5 h or 24 h after intraventricular injection of 6-OHDA (250 μg) were assayed after isolation of catecholamines by each procedure. Hypothalamic noradrenaline and striatal dopamine concentrations are expressed as percentages of control values obtained from untreated animals with each procedure. Each value is the mean ± S.E.M. for three samples of pooled tissue.

was added to samples before adsorption on Amberlite-CG-120 resin columns of the type used in the present study, however, most of the 6-OHDA (>90%) (measured by native fluorescence at 295/340 nm) was eluted in the noradrenaline fraction (1 N hydrochloric acid). Laverty *et al.* (1965) also reported that 6-OHDA yielded products with a much lower fluorescence intensity than those derived from noradrenaline or dopamine after the oxidation procedure used in the present assay of dopamine. It seems unlikely, therefore, that 6-OHDA could seriously interfere with the assay of striatal dopamine. Furthermore, any 6-hydroxynoradrenaline which might be formed from 6-OHDA would not be expected to elute from the resin columns in the dopamine fraction.

Bloom *et al.* (1969) used a combination of Dowex-50 and alumina columns for the extraction of catecholamines from rat brains after 6-OHDA treatment. They found that 6-OHDA did not appear in the eluates from alumina columns. In control experiments in which 6-OHDA was added in large amounts (250 μg) to brain samples which were then processed by a similar two-column procedure (see **Methods**) we have confirmed this finding. Figure 3 illustrates the results obtained in separate experiments using the single- or double-column procedures for the isolation of catecholamines from brain tissue after 6-OHDA injection. The results were similar, suggesting that the apparent increases in striatal dopamine after 6-OHDA cannot be accounted for by contamination with 6-OHDA.

Furthermore, eluates obtained by the double-column procedure from hypothalamic tissue samples were also assayed for dopamine ; the amounts present in both control and 6-OHDA treated animals were similar, and too low to be accurately measured. The absence of any increase in the apparent dopamine content of the hypothalamic samples after 6-OHDA also argues against the possibility that the rises observed in the striatum were artifacts. Finally, such contamination might be expected to be greater after a dose of 250 μg of 6-OHDA than after 25 μg. The increase in striatal dopamine, however, was similar after the administration of either dose (Figs. 1 and 2).

Discussion

In this study we have sought to establish the time course of the onset of the selective degenerative changes induced in catecholamine neurones in the rat brain by 6-OHDA. As indices of these degenerative changes the disappearance of biochemical activities (tyrosine hydroxylase and noradrenaline uptake) specifically associated with adrenergic neurones have been measured. Two different methods were used for the measurement of each of these parameters ; in each case these yielded similar results.

The time course of the disappearance of enzyme and uptake sites was compared with that for the changes in catecholamine content following intraventricular administration of 6-OHDA. In this way we hoped to establish whether displacement of catecholamine by 6-OHDA could occur without degenerative changes, either at early times after drug injection or after the administration of small doses of 6-OHDA.

It might be argued that an apparent loss of tyrosine hydroxylase or noradrenaline uptake at early times after drug administration could be due to an acute inhibition caused by 6-OHDA. We consider this unlikely, however, because any free drug remaining in treated tissues would be considerably diluted in the *in vitro* enzyme or uptake assay systems. Furthermore, we found that the combination of experimental

and control tissue homogenates did not lead to any inhibition of control tyrosine hydroxylase activity, suggesting that no reversible inhibitor of the enzyme was present in the experimental tissue.

The amine depletion in the hypothalamus after 6-OHDA treatment has a more rapid onset than that in the forebrain following surgical lesions in the lateral hypothalamus (Moore & Heller, 1967). This may be due to the direct and selective action of 6-OHDA on nerve terminals which contain the storage sites.

In the hypothalamus after the injection of 250 μg of 6-OHDA there was no indication that noradrenaline depletion followed a different time course from that for the loss of tyrosine hydroxylase or noradrenaline uptake sites. The loss of catecholamine and the degenerative changes appeared to occur simultaneously, and were essentially complete within 2 h of the drug administration. Marked changes in amine, enzyme and uptake had already occurred at 0·5 h. Only after the administration of a small dose of 6-OHDA (25 μg) was there any evidence that noradrenaline depletion might precede the onset of degenerative changes (Fig. 2).

In the striatum there was also no evidence that dopamine depletion preceded the onset of degenerative changes; indeed in this brain region the converse appeared to occur. A depletion of dopamine was not detected until 48 h after administration of 250 μg of 6-OHDA, whereas significant reductions in tyrosine hydroxylase and noradrenaline uptake were evident at 24 hours. During the first 24 h after 6-OHDA there was a striking increase in the dopamine content of the striatum; this was observed even after precautions had been taken to eliminate possible interference by 6-OHDA in the assay procedure. The rise in striatal dopamine did not appear to be directly related to the concurrent degenerative changes induced by the drug, since a similar rise was observed after a small dose of 6-OHDA which did not lead to any degenerative changes. One possible explanation of these findings is that 6-OHDA might act as a potent and selective inhibitor of monoamine oxidase activity in the striatum, thus preventing the normal degradation of the catecholamine.

Comparison of the results from the hypothalamus and striatum indicates that the time course of the degenerative changes in these two brain areas is different. The onset of degenerative changes in the hypothalamus occurred with 0·5 h of 6-OHDA administration, whereas even 2 h after the drug only small changes had occurred in the striatum. Similarly the changes in the hypothalamus were essentially complete with 2 h, whereas those in the striatum progressed over a period of at least 48 h. The delayed onset of 6-OHDA induced changes in the dopamine neurones of the striatum is in agreement with the results of Taylor & Laverty (personal communication).

There are various possible explanations of the different time course of the 6-OHDA induced changes in noradrenaline and dopamine terminals. It is known from previous studies that noradrenaline terminals are more sensitively affected by 6-OHDA treatment than are dopamine terminals (Uretsky & Iversen, 1970). This was confirmed in the present study by the finding that a low dose of 6-OHDA induced degenerative changes in the hypothalamus but not in the striatum. This may be related to the observation that 6-OHDA has a higher affinity for catecholamine uptake sites in the noradrenaline nerve terminals of the hypothalamus than for the dopamine neurones of the striatum (Iversen, 1970). The drug may, therefore, be more effectively concentrated by noradrenaline terminals. Thoenen &

Tranzer (1968) suggest that the destruction of peripheral sympathetic nerve endings might depend upon the uptake of a critical amount of 6-OHDA by such terminals. It is possible that the onset and rate of degenerative changes induced by 6-OHDA in the CNS may also depend upon the amount of drug initially accumulated by central adrenergic neurones. Thus in the hypothalamus, a significant reduction in tyrosine hydroxylase activity occurred within 0·5 h after the injection of 250 μg of 6-OHDA, but the onset of such a change was delayed beyond 0·5 h after the injection of 25 μg of 6-OHDA.

The present findings are in agreement with the previously stated hypothesis that the long-lasting reduction in the catecholamine content of rat brain following 6-OHDA treatment is associated with degenerative changes induced by the drug (Uretsky & Iversen, 1970). A displacement of endogenous brain catecholamines by 6-OHDA does not seem to be important in the long-term effects of the drug on CNS catecholamines.

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