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Pathways of Dopamine Metabolism in the Rabbit Caudate Nucleus in vitro

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Summary. Slices of rabbit caudate nucleus were preincubated with ^{3}H-dopamine and then superfused. ^{3}H-dopamine and its metabolites were separated by column chromatography.

The basal outflow of tritium consisted of 68% DOPAC, 21% OMDA metabolites (most of which was HVA), 6% dopamine and 5% MTA. Except for an increase in ^{3}H-dopamine, the basal outflow was little changed by nomifensine or cocaine. Amezinium reduced the outflow of ^{3}H-DOPAC and ^{3}H-OMDA metabolites and increased that of ^{3}H-dopamine and ^{3}H-MTA; its effect was antagonized by nomifensine. Haloperidol 10^{-7} M selectively enhanced the outflow of ^{3}H-DOPAC and ^{3}H-OMDA metabolites. At haloperidol 10^{-5} M, a large increase in these two fractions was accompanied by a small increase in ^{3}H-dopamine and ^{3}H-MTA. Nomifensine diminished only slightly the outflow of ^{3}H-DOPAC caused by haloperidol.

The overflow of tritium elicited by electrical stimulation at 3 Hz consisted of 74% DOPAC, 15% OMDA metabolites, 10% dopamine and 0.4% MTA. Lowering the frequency reduced the overflow of total tritium and caused a decrease in the percentage of ^{3}H-DOPAC and an increase in the percentage of ^{3}H-dopamine. Nomifensine or cocaine greatly diminished the evoked overflow of ^{3}H-DOPAC and ^{3}H-OMDA compounds and increased the evoked overflow of ^{3}H-dopamine and ^{3}H-MTA. The effects of apomorphine and bromocriptine were similar to those caused by decreasing the stimulation frequency.

The major pathways of the metabolism of previously taken up dopamine, under the conditions of our experiments, are as follows. When the neurones are at rest, dopamine metabolism is initiated by leakage of the amine into the axoplasm, where it is degraded through the aldehyde to DOPAC. Part of the DOPAC is methylated extraneuronally to HVA. Traces of MTA are formed by extraneuronal methylation of dopamine. When action potentials arrive, dopamine metabolism is initiated by exocytosis. The bulk of the extracellular dopamine is taken up back into the neurones. When the stimulation frequency is 3 Hz, most of the axoplasmic dopamine is subsequently transformed to DOPAC; little seems to be re-stored. HVA and MTA are generated essentially as during neuronal rest. When dopamine release is low (stimulation at low frequency; addition of apomorphine or bromocriptine), a larger portion seems to be re-stored, thus leading to a decrease in the percentage of DOPAC. Haloperidol, apart from its receptor blocking properties, acts on dopaminergic axons in a manner akin to the effect of reserpine.

Key words: Rabbit caudate nucleus – Dopamine release – Dopamine metabolism – Monoamine oxidase – Catechol-O-methyl transferase

Introduction

The metabolism of the cerebral neurotransmitter dopamine has been extensively studied in vivo, above all in the corpus striatum. The main metabolites that occur in the brain are DOPAC and HVA (Rosengren 1960; Andén et al. 1963; Sharman 1963). Substantial amounts of conjugates of DOPAC and HVA have also been found (Gordon et al. 1976; Swahn and Wiesel 1976). MTA levels are much lower (Carlsson and Waldeck 1964; Galli et al. 1976). The alcohol analogues of DOPAC and HVA, i.e., DOPET and MOPET, have not been identified in brain with certainty (Wilk and Zimmerberg 1973). Electrical stimulation of the nigrostriatal dopamine pathway increases striatal DOPAC and HVA levels (Korf et al. 1976; Roth et al. 1976). It has been proposed that DOPAC is formed within the dopamine neurones (Carlsson and Hillarp 1962; Roffler-Tarlov et al. 1971; Sharman 1973), although this is not certain (Westerink 1979). In contrast, since catecholamine neurones contain little, if any, COMT (Carlsson and Hillarp 1962; Kaplan et al. 1979), MTA probably is formed exclusively extraneuronally (Kehr 1976). Both intraneuronal and extraneuronal enzymes may contribute to the production of HVA (Carlsson and Hillarp 1962; Roffler-Tarlov et al. 1971; Westerink and Korf 1976).

In vitro experiments on isolated tissues have helped to elucidate the metabolism of noradrenaline in the periphery (Jonason 1969; Langer 1974) and in the central nervous system (Jonason 1969; Farah et al. 1977; Taube et al. 1977). They have some advantages over in vivo experiments. For instance, compounds in tissue and medium can be measured separately. Metabolism can be studied during neuronal rest and during stimulation at defined frequencies. Metabolites are not carried away by the blood stream. The time course of

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Abbreviations and Terminology. AD, Aldehyde dehydrogenase (E.C. 1.2.1.3.); COMT, catechol-O-methyl transferase (E.C. 2.1.1.6.); DOPAC, 3,4-dihydroxyphenylacetic acid; DOPEG, 3,4-dihydroxyphenylglycol; DOPET, 3,4-dihydroxyphenylethanol; HVA, 3-methoxy-4-hydroxyphenylacetic acid; MAO, monoamine oxidase (E.C. 1.4.3.4.); MOPET, 3-methoxy-4-hydroxyphenylethanol; MTA, 3-methoxytyramine; OMDA metabolites, O-methylated and deaminated metabolites. "Intraneuronal" refers to location in dopaminergic terminal axons, "extraneuronal" to location in other cells, neuronal as well as non-neuronal.

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metabolite outflow can easily be measured with a single piece of tissue.

Surprisingly, the metabolism of dopamine has rarely been studied in vitro. Using slices of the caudate nucleus of the rabbit, Jonason and Rutledge (1968) showed that by far the most important metabolite of ^{3}H-dopamine was ^{3}H-DOPAC. In their experiments, slices and medium were extracted together at the end of incubation with ^{3}H-dopamine. Adapting the column chromatographic method that Graefe et al. (1973) developed for the separation of noradrenaline metabolites, Westfall et al. (1976) determined the outflow of ^{3}H-compounds from rat striatal slices preincubated with ^{3}H-dopamine and the effect of electrical stimulation at 50 Hz (a frequency that exceeds the normal rate of firing which is about 3 Hz; see Murrin and Roth 1976); in spite of the fact that the chromatographic recovery of DOPAC was only 58%, recoveries were not corrected for. Finally, using another modification of the procedure of Graefe et al. (1973), Hoffmann and Cubeddu (1978) and Cubeddu et al. (1979a, b) investigated the outflow of ^{3}H-compounds from rat striatal slices labelled with ^{3}H-dopamine in more detail, including the effect of depolarization by high potassium. In the method of Cubeddu et al. (1979a, b), incomplete recoveries and cross contaminations are corrected for.

We used this method to study the metabolism of previously taken up ^{3}H-dopamine in superfused slices of the head of the rabbit caudate nucleus. Effects of electrical stimulation at 0.1 – 30 Hz, of dopamine uptake and MAO inhibitors, and of dopamine receptor agonists and antagonists which modulate the release of dopamine in this tissue (Starke et al. 1978; Reimann et al. 1979), were also tested. Some results have been reported to the German Pharmacological Society (Zumstein et al. 1979).

Methods

Preparation, Incubation, Superfusion and Stimulation. Rabbits of either sex and weighing 1.5 – 3.5 kg were killed by decapitation. The brain was quickly removed, and both caudate nuclei were chopped in a frontal plane (Sorvall TC 2). Fifteen to twenty slices (0.4 mm thick, weight 1.5 – 3 mg each) were incubated in 2 ml medium, containing 10^{-7} M ^{3}H-dopamine, at 37° C for 30 min. The medium for incubation and superfusion was as follows (mM): NaCl 118, KCl 4.8, $CaCl_2$ 1.3, $MgSO_4$ 1.2, $NaHCO_3$ 25, KH_2PO_4 1.2, ascorbic acid 0.6, disodium EDTA 0.03, glucose 11; it was saturated with 5% CO_2 in O_2. After incubation, the slices were rinsed 3 – 4 times with 3 ml medium, and two slices were transferred to each of six glass superfusion chambers, where they were held by a polypropylene mesh between two platinum plate electrodes 20 mm apart.

Slices were superfused for 130 min and at 37° C by means of a roller pump. Unless stated otherwise, the rate of superfusion was 0.5 ml/min. All tubings were of Teflon except in the pump, where silicone tubings were used. Silicone can adsorb substances from the superfusion medium and release them in subsequent experiments (Starke et al. 1978). Therefore, fluid reservoir, chambers and pump were arranged in such a way that the medium first passed through the chambers and then through the pump. The effluent from the pump was either discarded (0 – 65 min of superfusion) or collected, by means of a fraction collector, in 5-min-samples in test tubes (65 – 130 min of superfusion). The tubes contained 2.6 µg of dopamine and each metabolite, dissolved together in 78 µl 1.6 M HCl. In each experiment, the slices of three chambers received one drug (or drug combination), the slices of the other three chambers were superfused with drug-free medium throughout or received another drug (or combination). Drugs were added either from the beginning of superfusion or after 75 min. The effluent from the corresponding chambers was pooled and immediately mixed with an aqueous solution of disodium EDTA and Na_2SO_3 to give final concentrations of 2.3 and 2.8 mg/ml, respectively. An aliquot was used for determination of total tritium, the rest was chromatographed (see below).

At the end of the experiment, the slices of the three corresponding chambers (i.e., six slices) were homogenized together in 2 ml ice-cold 0.4 M $HClO_4$ containing 2 mg disodium EDTA and 2.5 mg Na_2SO_3 (glass/Teflon homogenizer, 10 strokes, 600 rpm). The homogenizer was washed three times with 2 ml ice-cold 0.4 M $HClO_4$. The homogenate was left at 4° C for 30 min and then centrifuged at 12,000 × g for 10 min. Aliquots (2 ml) of the supernatant were used for determination of total tritium and for chromatography (see below).

For electrical stimulation, rectangular pulses of 2 ms duration were delivered at 0.1 – 30 Hz from a Stimulator T (Hugo Sachs Elektronik) for 2 min, beginning after 75 min of superfusion. The six superfusion chambers were arranged in series. The voltage gradient between the electrodes was 4.5 V/cm, giving a current of 24 mA. Pulses were monitored on an oscilloscope.

Separation of ^{3}H-Compounds. Superfusate samples and tissue extracts were adjusted to pH 8.1 – 8.3 with 1 M Tris-HCl pH 8.4, and ^{3}H-compounds were separated by the column chromatographic method of Cubeddu et al. (1979b). Briefly, the samples were passed over alumina which adsorbed the catechols dopamine, DOPET and DOPAC, but not the non-catechols MTA, MOPET and HVA. The effluent from the alumina columns dripped into Dowex 50 WX 4 columns, where MTA was retained. MOPET and HVA were collected together in the effluent from these Dowex 50 WX 4 columns (= OMDA metabolites; fraction I). MTA was eluted from Dowex 50 WX 4 by strong HCl (fraction II). The catechols were stepwise eluted from the alumina columns by first acetic acid (DOPET and dopamine), and then HCl (DOPAC; fraction V). DOPET and dopamine were again separated by Dowex 50 WX 4 (fractions III and IV). We did not determine the fraction VI of Cubeddu et al. (1979b) which contains a small part of the dopamine. This omission does not affect the validity of the procedure because the ^{3}H-dopamine found in fraction IV was corrected for incomplete recovery. Likewise, other incomplete recoveries and cross contaminations were corrected for. Recoveries, measured fluorimetrically, were: HVA in fraction I 100%, MOPET in fraction I 97%, MTA in fraction II 100%, DOPET in fraction III 88%, dopamine in fraction IV 88%, DOPAC in fraction V 94% (means of 7 experiments). Thirteen percent of the DOPET were found in fraction V and 0.3% of the DOPAC in fraction III.

Analysis of Fraction I. An attempt was made to identify the ^{3}H-compounds in fraction I. In these experiments the rate of superfusion was 0.04 ml/min. The superfusate from all six superfusion chambers, from 65 – 115 min of superfusion, was pooled (12 ml) and column chromatographed.

Fraction I was acidified with 0.5 ml 12 M HCl, saturated with NaCl and extracted five times with 20 ml each of ethyl acetate. After shaking with Na_2SO_4 the organic layer was evaporated to dryness, and the residue taken up in 0.5 ml CCl_4. Fifty microliter portions of the CCl_4-solution (underspotted with unlabelled MOPET and HVA 5 µg each) were subjected to thin layer chromatography on cellulose plates (Riedel de Haën No. 37362). The plates were developed with either chloroform: methanol: ammonia (0.88% w/v) 12:7:1 or n-butanol: pyridine: water 14:4:5. MOPET and HVA were visualized with diazotized p-nitroaniline (Sugden and Eccleston 1971). The cellulose was scraped off in 1.5 cm-portions and counted for radioactivity.

Measurement of Radioactivity. Tritium was determined by liquid scintillation spectrometry. Counting efficiency was determined by internal standardization with ^{3}H-water and ranged from 21 – 41%.

Calculations. The fractional rate of outflow of total tritium or of any ^{3}H-compound was calculated as (fmol efflux per 5 min)/5 × (fmol *total tritium* in the slices at the start of the respective 5-min-period).

In a few experiments, the apparent rate constant k for the efflux of ^{3}H-DOPAC and ^{3}H-OMDA metabolites was calculated as (fmol efflux from 125 – 130 min, i.e., in the last superfusion sample)/5 × (fmol of the *same ^{3}H-compound* in the slices at the start of this 5-min-period) (see Trendelenburg et al. 1980).

The electrically evoked overflow of total tritium was calculated in a manner similar to that used in our previous experiments (Starke et al. 1978; Reimann et al. 1979). The basal outflow (as fmol per 5 min) was assumed to decline linearly from the 5-min-period before to the period 50 – 55 min after the onset of stimulation. This estimated basal outflow was subtracted from the measured outflow of tritium 0 – 50 min after onset of stimulation. The difference was expressed as percent of the total tritium in the tissue at the onset of stimulation.

The chemical composition of the evoked overflow was calculated as follows. The increase of each ^{3}H-compound, from 0 – 55 min after onset of stimulation, beyond the basal outflow in the 5 min before stimulation was determined. This yielded the stimulation-evoked overflow Δ of each ^{3}H-compound. The contribution of each ^{3}H-component was then calculated as percentage of the sum of the Δ values.

Drugs and Statistics. The following drugs were used: dopamine [ethyl-2-^{3}H(N)] (NEN, Dreieich, FRG; "nominally labelled" in the β-position with respect to the nitrogen; specific activity 11.2 Ci/mmol, Lot. No. 1068-074) was periodically checked for purity by column chromatography; significant labelling in the α-position was excluded (Starke et al. 1980). Amezinium methylsulphate (BASF, Ludwigshafen, FRG); pargyline hydrochloride (Abbott, Chicago, Ill, USA); cocaine hydrochloride (Merck, Darmstadt, FRG); nomifensine hydrogen maleate (Hoechst, Frankfurt am Main, FRG); apomorphine hydrochloride (Woelm, Eschwege, FRG); bromocriptine mesylate (Sandoz, Basel, Switzerland); haloperidol (Janssen, Beerse, Belgium). Pargyline hydrochloride and amezinium methylsulphate were dissolved in water; cocaine hydrochloride and nomifensine hydrogen maleate in superfusion medium; bromocriptine mesylate in 0.01 M tartaric acid; haloperidol in 0.01 M citric acid; apomorphine hydrochloride in freshly boiled water containing 2 mg disodium EDTA and 40 mg ascorbic acid per 100 ml. The solutions were added directly to the superfusion fluid reservoir. In control experiments, appropriate amounts of solvent were added.

Means $\pm$ S.E. are given throughout. Significance of differences between means was calculated with two-tailed *t*-test. *n*, number of experiments.

Results

Slices of the rabbit caudate nucleus were preincubated with 10^{-7} M ^{3}H-dopamine and then superfused with ^{3}H-dopamine-free medium. ^{3}H-compounds in the effluent were separated by column chromatography which yielded five fractions. ^{3}H-DOPET was not detected with certainty in any experiment and, hence, is not shown in figures and tables. Fraction I contained the OMDA metabolites ^{3}H-HVA and ^{3}H-MOPET, but might also contain various conjugates. Hence, this fraction (obtained in experiments without drugs and without electrical stimulation) was analyzed further by thin layer chromatography. When chloroform:methanol:ammonia was used as solvent system, 88.5 $\pm$ 10.9% of the radioactivity of fraction I was recovered from the plates, of which 72.5 $\pm$ 6.7% co-chromatographed with authentic HVA, and 15.6 $\pm$ 6.1% with authentic MOPET ($n = 3$). Similar results were obtained with n-butanol:pyridine:water. Hence, by far the largest part of fraction I probably was ^{3}H-HVA, with a minor contribution of ^{3}H-MOPET. No other compound was detected.

Basal Outflow

Control experiments without electrical stimulation are shown in the left-hand panels of Figs. 1 – 3. When collection began, i.e., after 65 min of superfusion, a fraction of about 0.0020 of the tissue radioactivity left the tissue per minute. Towards the end of superfusion, this rate had declined to about 0.0017 min^{-1}. The basal efflux in the interval from 70 – 75 min of superfusion consisted mainly of ^{3}H-DOPAC (68.3 $\pm$ 0.7%) and ^{3}H-OMDA metabolites (20.9 $\pm$ 0.5%), and only small amounts of ^{3}H-MTA (4.6 $\pm$ 0.3%) and ^{3}H-dopamine (6.1 $\pm$ 0.2%) were found ($n = 17$). In the last collection period (125 – 130 min), the percentage composition was similar.

Figure 1 shows the effect of amezinium, a phenylpyradizinium derivative which potently and selectively blocks the MAO inside postganglionic sympathetic neurones (Steppeler et al. 1980; Steppeler and Starke 1980). As little as 10^{-8} M amezinium slightly reduced the outflow of total tritium, ^{3}H-DOPAC and ^{3}H-OMDA metabolites, whereas ^{3}H-dopamine and ^{3}H-MTA were not changed (cf. Table 1). At amezinium 10^{-7} (Fig. 2) and 10^{-6} M, the decrease of total tritium, ^{3}H-DOPAC and ^{3}H-OMDA metabolites was more pronounced, whereas the outflow of ^{3}H-dopamine and ^{3}H-MTA was now increased. With amezinium 10^{-5} M, the increase in ^{3}H-dopamine and ^{3}H-MTA outflow exceeded the decrease of ^{3}H-

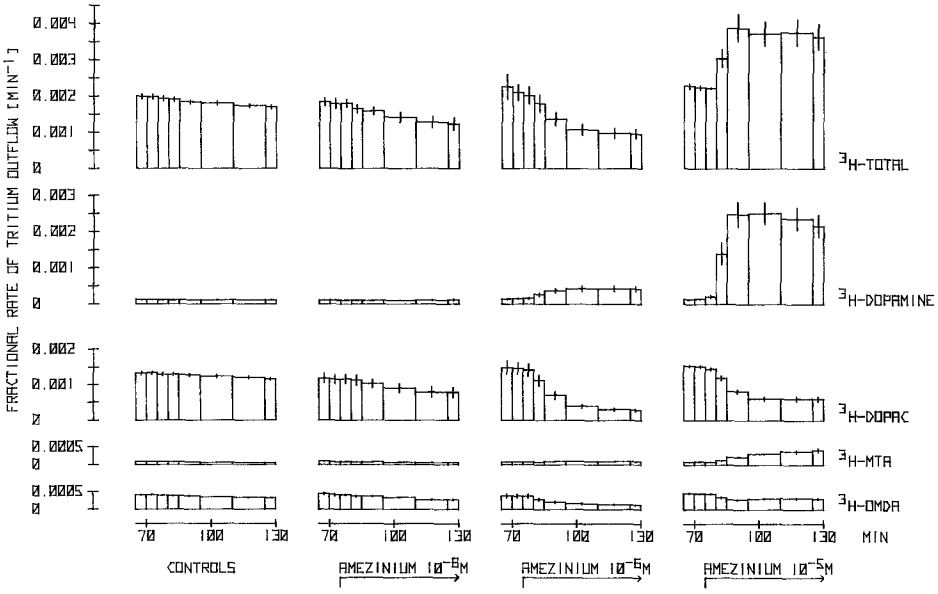

Fig. 1
Effect of amezinium on the basal outflow of ^{3}H-compounds from caudate slices preincubated with ^{3}H-dopamine. The superfusate was collected from 65 – 130 min of superfusion (*abscissa*). Amezinium was added after 75 min of superfusion. Means $\pm$ S.E. of 17 (controls) or 3 – 4 experiments

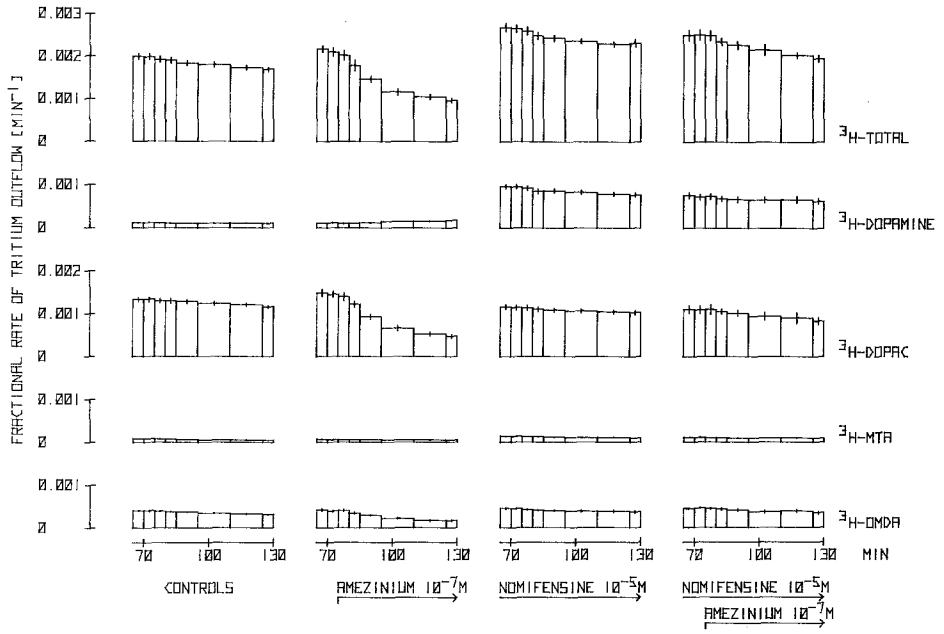

Fig. 2
Effects of amezinium and nomifensine on the basal outflow of ^{3}H-compounds from caudate slices preincubated with ^{3}H-dopamine. The superfusate was collected from 65–130 min of superfusion (*abscissa*). Nomifensine was present from the beginning of superfusion; amezinium was added after 75 min. Means ± S.E. of 17 (controls) or 3–5 experiments. Controls are identical with those of Fig. 1

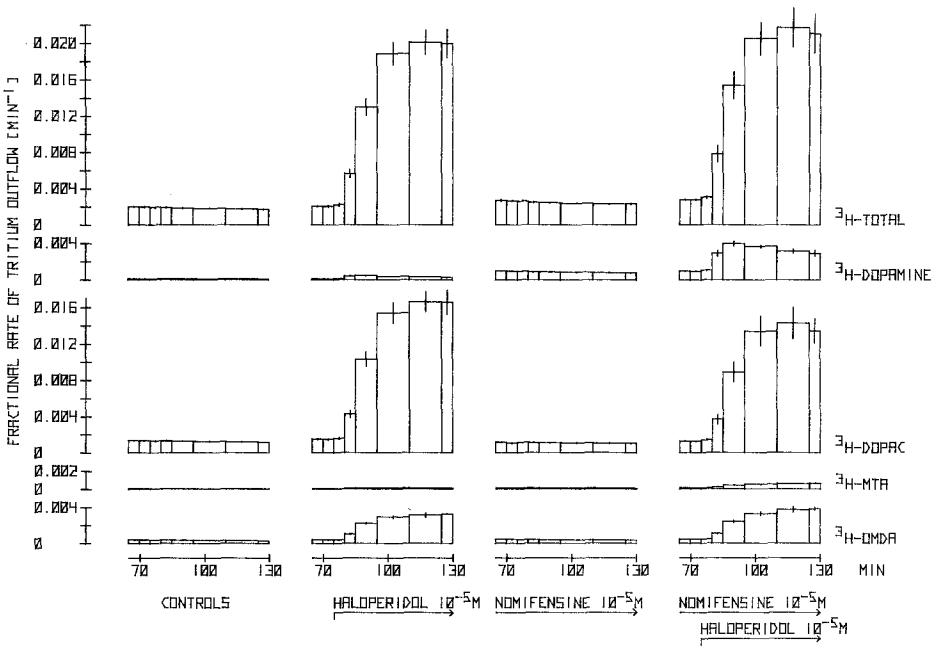

Fig. 3
Effects of haloperidol and nomifensine on the basal outflow of ^{3}H-compounds from caudate slices preincubated with ^{3}H-dopamine. The superfusate was collected from 65–130 min of superfusion (*abscissa*). Nomifensine was present from the beginning of superfusion; haloperidol was added after 75 min. Means ± S.E. of 17 (controls) or 3–5 experiments. Controls are identical with those of Fig. 1, and nomifensine experiments with those of Fig. 2

DOPAC and ^{3}H-OMDA metabolites, so that now the outflow of total tritium was increased (Fig. 1; cf. Table 1). Note that the reduction of the outflow of ^{3}H-DOPAC and ^{3}H-OMDA metabolites was much more pronounced at amezinium 10^{-6} than at 10^{-5} M.

In postganglionic sympathetic neurones, the inhibition of MAO caused by amezinium is prevented by cocaine and, hence, due to uptake of amezinium via the catecholamine transport mechanism (Steppeler et al. 1980; Steppeler and Starke 1980). An analogous mechanism seemed possible for dopamine neurones. Hence, we studied the effect of amezinium 10^{-7} M in the presence of nomifensine, a potent inhibitor of dopamine uptake (Hunt et al. 1974; Reimann et al. 1979). At the high concentration of 10^{-5} M, nomifensine, which was present throughout superfusion, accelerated the outflow of total tritium, ^{3}H-MTA, ^{3}H-OMDA metabolites and, most obviously, ^{3}H-dopamine (Fig. 2; similar results were obtained when nomifensine 10^{-5} M was added after 75 min of superfusion; Table 1). Nomifensine reduced all effects of amezinium (Fig. 2; cf. Table 1).

Apomorphine 10^{-7} and 10^{-6} M decreases the outflow of total tritium from caudate slices preincubated with ^{3}H-dopamine (cf. Starke et al. 1978). Table 1 shows that this is mainly due to a decrease in ^{3}H-DOPAC outflow, although the outflow of ^{3}H-dopamine and ^{3}H-OMDA metabolites was also diminished (not significantly). In contrast to the lower concentrations, apomorphine 10^{-5} M did not alter the outflow of ^{3}H-compounds. Haloperidol 10^{-8} M, which itself caused no change, abolished the effects of apomorphine 10^{-7} M (Table 1).

Haloperidol 10^{-5} M produced dramatic changes (Fig. 3, Table 1). The outflow of total tritium, ^{3}H-DOPAC and ^{3}H-

Table 1. Effects of various drugs on the basal outflow of ^{3}H-compounds from caudate slices preincubated with ^{3}H-dopamine (experiments without electrical stimulation). The superfusate was collected from 65 – 130 min of superfusion. Drugs were either present from the beginning of superfusion or added after 75 min. Shown is the ratio of the fractional rates of outflow from 125 – 130 min of superfusion and from 70 – 75 min of superfusion. Means ± S.E.

Drug present throughout	Drug added at 75 min	Fractional rate of outflow 125 – 130 min / Fractional rate of outflow 70 – 75 min					n
		^{3}H-Total	^{3}H-Dopamine	^{3}H-DOPAC	^{3}H-MTA	^{3}H-OMDA	
–	–	0.86 ± 0.01	0.89 ± 0.04	0.87 ± 0.01	0.56 ± 0.03	0.78 ± 0.02	17
–	Amezinium 10^{-8} M	0.69 ± 0.05**	1.07 ± 0.23	0.67 ± 0.04**	0.69 ± 0.11	0.65 ± 0.04*	3
–	Amezinium 10^{-7} M	0.46 ± 0.03**	1.53 ± 0.11**	0.33 ± 0.03**	0.87 ± 0.13*	0.42 ± 0.03**	4
–	Amezinium 10^{-6} M	0.45 ± 0.04**	3.01 ± 0.38**	0.19 ± 0.01**	1.19 ± 0.04**	0.35 ± 0.02**	3
–	Amezinium 10^{-5} M	1.63 ± 0.17**	15.51 ± 0.60**	0.40 ± 0.03**	4.44 ± 0.69**	0.68 ± 0.02	3
–	Nomifensine 10^{-5} M	1.18 ± 0.08**	5.46 ± 0.60**	0.82 ± 0.02	1.34 ± 0.09**	0.99 ± 0.04**	3
–	Apomorphine 10^{-7} M	0.72 ± 0.02**	0.77 ± 0.02	0.73 ± 0.02**	0.55 ± 0.01	0.71 ± 0.03	4
–	Apomorphine 10^{-6} M	0.74 ± 0.01**	0.72 ± 0.07	0.72 ± 0.02**	0.57 ± 0.02	0.73 ± 0.04	4
–	Apomorphine 10^{-5} M	0.83 ± 0.03	1.00 ± 0.11	0.89 ± 0.03	0.58 ± 0.03	0.74 ± 0.02	4
–	Haloperidol 10^{-5} M	9.73 ± 0.56**	2.41 ± 0.11**	11.22 ± 0.56**	1.56 ± 0.19**	8.14 ± 0.54**	4
Nomifensine 10^{-5} M	–	0.87 ± 0.01	0.81 ± 0.03	0.91 ± 0.02	0.74 ± 0.04	0.83 ± 0.05	5
Nomifensine 10^{-5} M	Amezinium 10^{-7} M	0.78 ± 0.01****	0.87 ± 0.04	0.76 ± 0.05***	0.84 ± 0.08	0.77 ± 0.06	3
Nomifensine 10^{-5} M	Haloperidol 10^{-5} M	7.64 ± 0.56****	3.09 ± 0.20****	10.57 ± 0.67****	4.46 ± 0.35****	7.75 ± 0.15****	3
Haloperidol 10^{-8} M	–	0.91 ± 0.03	0.79 ± 0.06	0.96 ± 0.04	0.61 ± 0.05	0.80 ± 0.02	4
Haloperidol 10^{-8} M	Apomorphine 10^{-7} M	0.86 ± 0.03	0.82 ± 0.04	0.91 ± 0.02	0.57 ± 0.01	0.85 ± 0.06	4

* Significantly different from experiments without drugs ($P < 0.02$)
** Significantly different from experiments without drugs ($P < 0.001$)
*** Significantly different from experiments with nomifensine alone throughout ($P < 0.02$)
**** Significantly different from experiments with nomifensine alone throughout ($P < 0.001$)

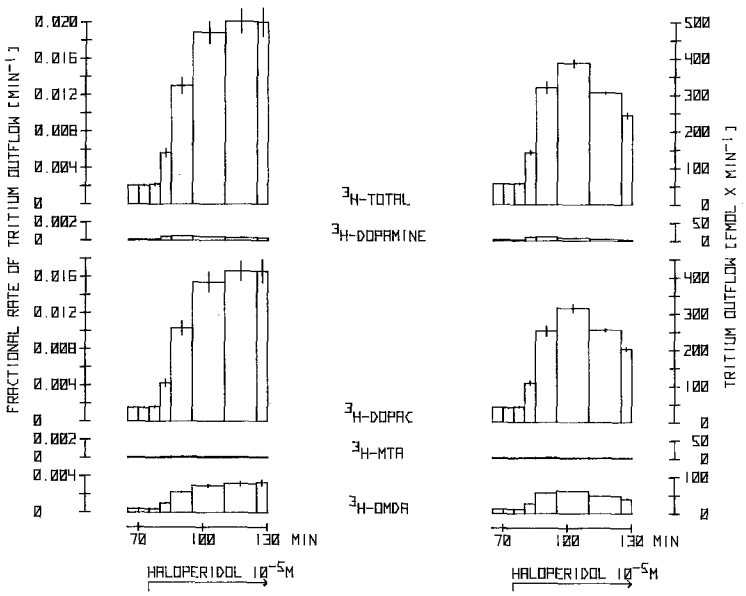

Fig. 4
Effect of haloperidol on the basal outflow of ^{3}H-compounds from caudate slices preincubated with ^{3}H-dopamine. The superfusate was collected from 65 – 130 min of superfusion (*abscissa*). Haloperidol was added after 75 min of superfusion. On the left-hand side the outflow is plotted as fractional rate; on the right-hand side it is plotted as fmol per minute. Means ± S.E. of 4 experiments, which are identical with those of Fig. 3

OMDA metabolites was increased more than tenfold and the outflow of ^{3}H-dopamine and ^{3}H-MTA slightly less than threefold. Nomifensine 10^{-5} M enhanced the haloperidol-induced increase in ^{3}H-dopamine and ^{3}H-MTA outflow. Even in the presence of nomifensine, however, by far the largest portion of the haloperidol-induced increase was ^{3}H-DOPAC (Fig. 3, Table 1).

Figure 4 illustrates an advantage of calculating the outflow of ^{3}H-compounds as a fractional rate. When outflow is expressed as fmol × min^{-1} (right-hand panel), the effect of haloperidol 10^{-5} M seems to be transient. This, however, is due to a depletion of the tritium in the tissue. When the depletion is corrected for (as by calculation of the fractional rate), the effect of haloperidol is seen to be persistent (left-hand panel).

Apparent rate constants for the efflux of ^{3}H-DOPAC and ^{3}H-OMDA metabolites were calculated from experiments with haloperidol 10^{-5} M and haloperidol 10^{-5} M + nomifensine 10^{-5} M. These experiments were chosen because the tissue content of the acids, which enters into the calculation as the denominator, was higher than in controls and, therefore, could be measured more reliably (see below). The constants were 0.068 ± 0.001 min^{-1} for ^{3}H-DOPAC and 0.101 ± 0.002 min^{-1} for ^{3}H-OMDA metabolites ($n = 7$).

Table 2. Effects of various drugs on the basal outflow of ^{3}H-compounds from caudate slices preincubated with ^{3}H-dopamine (experiments with electrical stimulation at 3 Hz). The superfusate was collected from 65–130 min of superfusion. Drugs were present from the beginning of superfusion. Shown are the fractional rates of outflow of total tritium and individual ^{3}H-compounds in the sample collected from 70–75 min; values have been multiplied by 1000. Means ± S.E.

Drugs	Fractional rate of tritium outflow (min^{-1}) × 1000					n
	^{3}H-Total	^{3}H-Dopamine	^{3}H-DOPAC	^{3}H-MTA	^{3}H-OMDA	
–	1.79 ± 0.05	0.109 ± 0.006	1.26 ± 0.04	0.069 ± 0.003	0.340 ± 0.009	27
Nomifensine 10^{-7} M	1.67 ± 0.06	0.145 ± 0.002	1.15 ± 0.04	0.065 ± 0.004	0.319 ± 0.017	3
Nomifensine 10^{-6} M	2.00 ± 0.08	0.392 ± 0.039**	1.15 ± 0.04	0.072 ± 0.004	0.306 ± 0.014	6
Nomifensine 10^{-5} M	2.42 ± 0.10**	0.796 ± 0.047**	1.06 ± 0.06*	0.121 ± 0.004**	0.423 ± 0.018**	8
Cocaine 10^{-5} M	2.46 ± 0.07**	0.642 ± 0.009**	1.25 ± 0.04	0.079 ± 0.011	0.379 ± 0.042	3
Apomorphine 10^{-6} M	1.16 ± 0.05**	0.081 ± 0.005	0.80 ± 0.05**	0.059 ± 0.004	0.235 ± 0.007**	4
Bromocriptine 10^{-6} M	1.79 ± 0.15	0.078 ± 0.009	1.27 ± 0.08	0.064 ± 0.007	0.310 ± 0.035	3
Haloperidol 10^{-8} M	1.80 ± 0.06	0.101 ± 0.013	1.27 ± 0.03	0.065 ± 0.006	0.351 ± 0.028	3
Haloperidol 10^{-7} M	2.26 ± 0.17*	0.114 ± 0.015	1.65 ± 0.14*	0.071 ± 0.003	0.424 ± 0.034*	3
Pargyline 10^{-5} M	0.48 ± 0.05**	0.137 ± 0.008*	0.14 ± 0.03**	0.107 ± 0.006**	0.064 ± 0.009**	6
Pargyline 10^{-5} M + nomifensine 10^{-6} M	0.73 ± 0.03***	0.431 ± 0.011****	0.08 ± 0.01	0.118 ± 0.004	0.098 ± 0.015	4
Pargyline 10^{-5} M + apomorphine 10^{-6} M	0.42 ± 0.04	0.118 ± 0.014	0.14 ± 0.02	0.099 ± 0.013	0.061 ± 0.019	3

* Significantly different from experiments without drugs ($P < 0.05$)
** Significantly different from experiments without drugs ($P < 0.001$)
*** Significantly different from experiments with pargyline alone ($P < 0.01$)
**** Significantly different from experiments with pargyline alone ($P < 0.001$)

In the experiments with electrical stimulation described below, drugs, if added, were always present from the beginning. Since two 5-min-samples of superfusate were collected before stimulation, drug effects on the basal outflow of tritium could also be detected from an analysis of these pre-stimulation samples. The fractional rates of outflow of ^{3}H-compounds in the sample collected from 70–75 min of superfusion are summarized in Table 2. The Table confirms that nomifensine 10^{-5} M accelerated the outflow of total tritium and that this was mainly due to an increase in ^{3}H-dopamine; the outflow of ^{3}H-MTA and ^{3}H-OMDA metabolites was also increased, whereas the outflow of ^{3}H-DOPAC was slightly decreased. Lower concentrations of nomifensine as well as cocaine 10^{-5} M increased the outflow of ^{3}H-dopamine, but did not significantly change the outflow of the other ^{3}H-compounds. Table 2 also confirms the inhibition by apomorphine. Bromocriptine did not share this effect with apomorphine. Haloperidol caused no change at 10^{-8} M, but a slight increase in the outflow of total tritium, ^{3}H-DOPAC and ^{3}H-OMDA metabolites at 10^{-7} M. Pargyline greatly reduced the outflow of total tritium by a decrease of ^{3}H-DOPAC and ^{3}H-OMDA metabolites; the outflow of ^{3}H-dopamine and ^{3}H-MTA was increased.

Electrically Evoked Overflow

Caudate nucleus slices were stimulated electrically for 2 min after 75 min of superfusion. Unless stated otherwise, the frequency of stimulation was 3 Hz. Time courses of the outflow of tritium are shown in Figs. 5–7. Values for the electrically evoked overflow of total tritium and the percentage composition of the evoked overflow are presented in Table 3.

Control experiments with stimulation at 3 Hz are shown in the left-hand panels of Figs. 5–7. Stimulation greatly increased the outflow of total tritium. The outflow peaked not in the sample that included the stimulation period, but in the next sample. This time course reflected the delayed outflow of ^{3}H-DOPAC and ^{3}H-OMDA metabolites which, as for basal outflow, were the major components of the evoked overflow of total tritium (74.2 and 15.1 %, respectively; Table 3). The outflow of ^{3}H-dopamine culminated earlier, but the amine represented only 10 % of the evoked overflow of total tritium. The percentage recovered as ^{3}H-MTA was only 0.4.

Figure 5 shows the effect of nomifensine. Nomifensine 10^{-6} and 10^{-5} M markedly changed the time course of the outflow of total tritium. It now peaked in the sample collected during and up to 3 min after stimulation and no longer reflected the outflow of deaminated metabolites, but that of ^{3}H-dopamine. A tendency towards this was already apparent at nomifensine 10^{-7} M (Fig. 5). As documented in Table 3, nomifensine 10^{-7} and 10^{-6} M slightly reduced, whereas nomifensine 10^{-5} M slightly enhanced the stimulation-evoked overflow of total tritium. Nomifensine produced a concentration-dependent increase in the percentage consisting of ^{3}H-dopamine and ^{3}H-MTA and a decrease in the percentage consisting of ^{3}H-DOPAC. Nomifensine also reduced the percentage of ^{3}H-OMDA metabolites; this reduction was less marked at 10^{-5} M than at 10^{-6} M.

Cocaine, like nomifensine, is a potent inhibitor of the high affinity uptake of dopamine (Heikkila et al. 1975; Reimann et al. 1979). When it was used at a concentration of 10^{-5} M, the time course of the outflow of ^{3}H-compounds (not shown) and the composition (Table 3) were similar to what was observed with nomifensine 10^{-6} and 10^{-5} M.

As expected, the evoked overflow of total tritium became very small in the presence of apomorphine or bromocriptine (Fig. 6, Table 3). This was mainly due to a decrease of ^{3}H-DOPAC. The evoked overflow of ^{3}H-dopamine was much less decreased and, hence, the percentage recovered as ^{3}H-dopamine was augmented. The time course of the outflow of total tritium was correspondingly changed. Haloperidol

Table 3. Effects of various drugs on the electrically evoked overflow of ^{3}H-compounds from caudate slices preincubated with ^{3}H-dopamine. The slices were stimulated electrically for 2 min, starting after 75 min of superfusion. Drugs were present from the beginning of superfusion. Shown are the stimulation-evoked overflow of total tritium, expressed as percent of the tritium content of the slices at the onset of stimulation, and the percentage composition of this evoked overflow. Means ± S.E.

Frequency (Hz)	Drugs	Stimulation-evoked overflow of tritium (% of tissue tritium)	Percentage composition of the evoked overflow				*n*
			^{3}H-Dopamine	^{3}H-DOPAC	^{3}H-MTA	^{3}H-OMDA	
3	–	5.2±0.2	10.0±0.4	74.2±0.6	0.4±0.1	15.1±0.5	27
3	Nomifensine 10^{-7} M	4.3±0.5	24.9±1.3**	62.4±0.6**	0.7±0.1	11.6±1.2*	3
3	Nomifensine 10^{-6} M	3.4±0.5**	70.4±3.2**	19.2±3.0**	3.0±0.4**	7.0±0.9**	6
3	Nomifensine 10^{-5} M	6.7±0.6*	74.9±1.0**	8.0±0.5**	7.0±0.7**	9.8±0.7**	8
3	Cocaine 10^{-5} M	4.3±0.2	79.0±2.0**	8.6±1.6**	4.5±0.2**	7.7±0.4**	3
3	Apomorphine 10^{-6} M	0.6±0.1**	48.9±6.5**	40.7±3.5**	0.7±0.2	8.5±3.9*	4
3	Bromocriptine 10^{-6} M	1.0±0.1**	27.3±1.3**	58.7±3.4**	1.0±0.6*	12.3±2.1	3
3	Haloperidol 10^{-8} M	7.9±1.0**	9.1±0.7	72.4±1.5	0.5±0.1	16.8±0.5	3
3	Haloperidol 10^{-7} M	6.4±0.3	9.7±0.6	73.1±0.8	0.5±0.1	16.4±0.3	3
3	Pargyline 10^{-5} M	0.8±0.1**	84.9±3.6**	7.3±2.4**	3.4±0.6**	2.6±1.2**	6
3	Pargyline 10^{-5} M + Nomifensine 10^{-6} M	2.0±0.1****	89.7±0.1	0.2±0.1***	9.0±0.2****	0.7±0.1	4
3	Pargyline 10^{-5} M + Apomorphine 10^{-6} M	0.07±0.03****	96.4±1.0	0.0±0.0	0.5±0.5***	1.7±1.7	3
30	–	9.9±0.3	12.8±1.4	70.9±0.9	0.7±0.1	15.2±0.6	3
30	Apomorphine 10^{-6} M	6.3±0.2**	18.6±0.9*	65.5±1.3*	0.8±0.1	14.9±1.2	3
30	Pargyline 10^{-5} M	2.3±0.5**	80.6±0.9**	8.1±1.0**	8.6±0.4**	2.0±0.2**	3
30	Pargyline 10^{-5} M + Apomorphine 10^{-6} M	1.6±0.3	88.1±1.8***	3.5±1.0***	6.7±0.5***	1.5±0.8	3
0.5	–	1.6±0.1	16.6±1.3	70.4±1.4	0.4±0.1	11.5±0.8	9
0.25	–	0.7±0.1	26.9±1.5	57.6±0.9	0.6±0.3	14.6±1.8	4
0.1	–	0.5±0.1	49.2±5.5	37.8±8.1	1.3±0.6	9.5±4.4	5

* Significantly different from experiments (same frequency) without drugs ($P < 0.05$)
** Significantly different from experiments (same frequency) without drugs ($P < 0.001$)
*** Significantly different from experiments (same frequency) with pargyline alone ($P < 0.05$)
**** Significantly different from experiments (same frequency) with pargyline alone ($P < 0.001$)

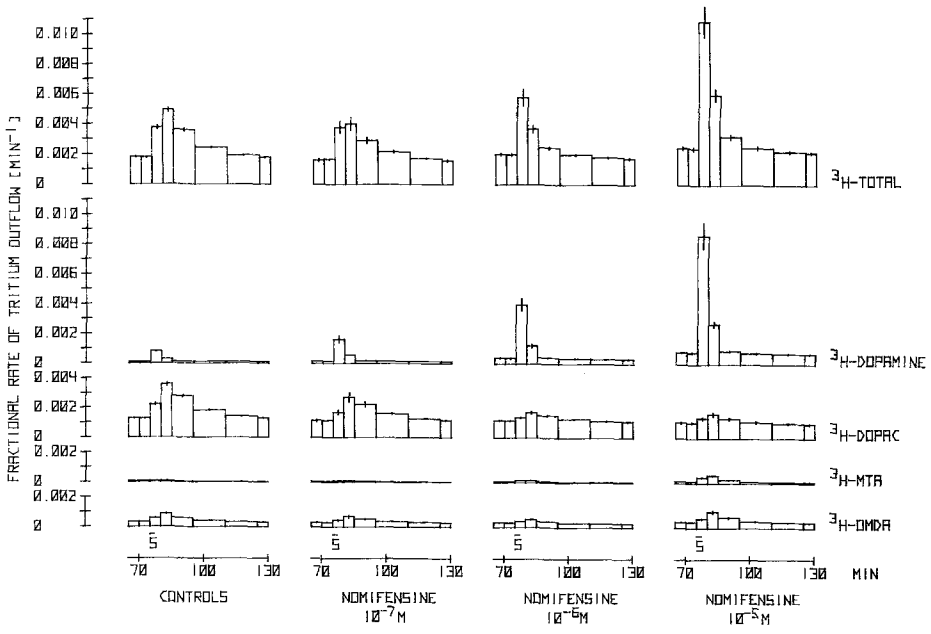

Fig. 5
Effect of nomifensine on the basal and the electrically (3 Hz) evoked overflow of ^{3}H-compounds from caudate slices preincubated with ^{3}H-dopamine. The superfusate was collected from 65–130 min of superfusion (*abscissa*). The slices were stimulated electrically for 2 min (*S*). Nomifensine was present from the beginning of superfusion. Means ± S.E. of 27 (controls) or 3–6 experiments

increased the evoked overflow of total tritium (not significantly for 10^{-7} M; Fig. 6, Table 3). As shown by the unchanged percent values, the evoked overflow of the various metabolite fractions was increased proportionately.

Pargyline greatly reduced the stimulation-evoked overflow of total tritium (Fig. 7, Table 3). The remaining overflow was mainly ^{3}H-dopamine and, hence, peaked already in the sample collected during stimulation. Nomifensine 10^{-6} M,

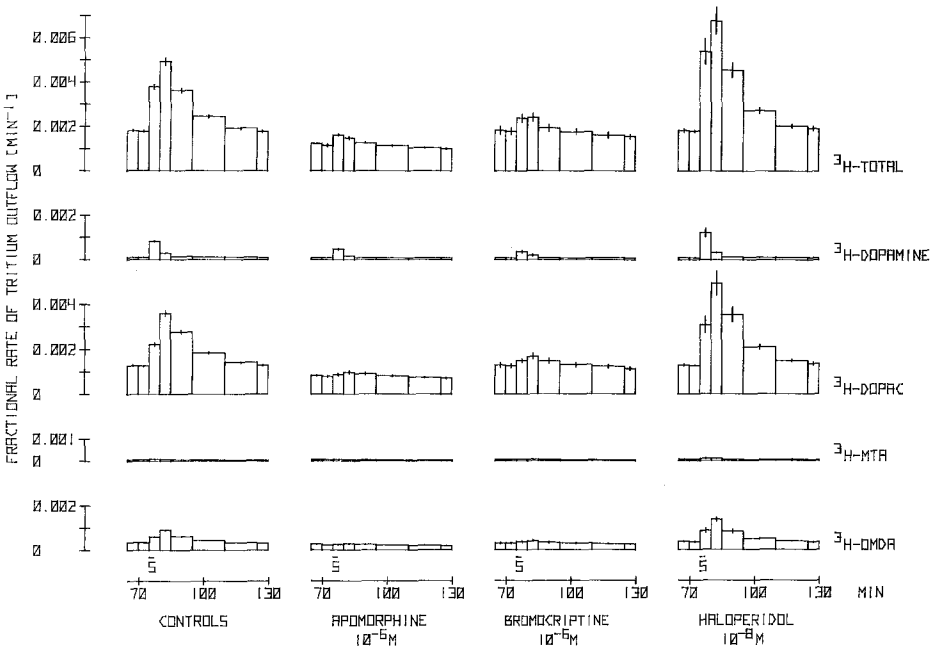

Fig. 6
Effects of apomorphine, bromocriptine and haloperidol on the basal and the electrically (3 Hz) evoked overflow of ^{3}H-compounds from caudate slices preincubated with ^{3}H-dopamine. The superfusate was collected from 65–130 min of superfusion (*abscissa*). The slices were stimulated electrically for 2 min (*S*). Apomorphine, bromocriptine or haloperidol was present from the beginning of superfusion. Means ± S.E. of 27 (controls) or 3–4 experiments. Controls are identical with those of Fig. 5

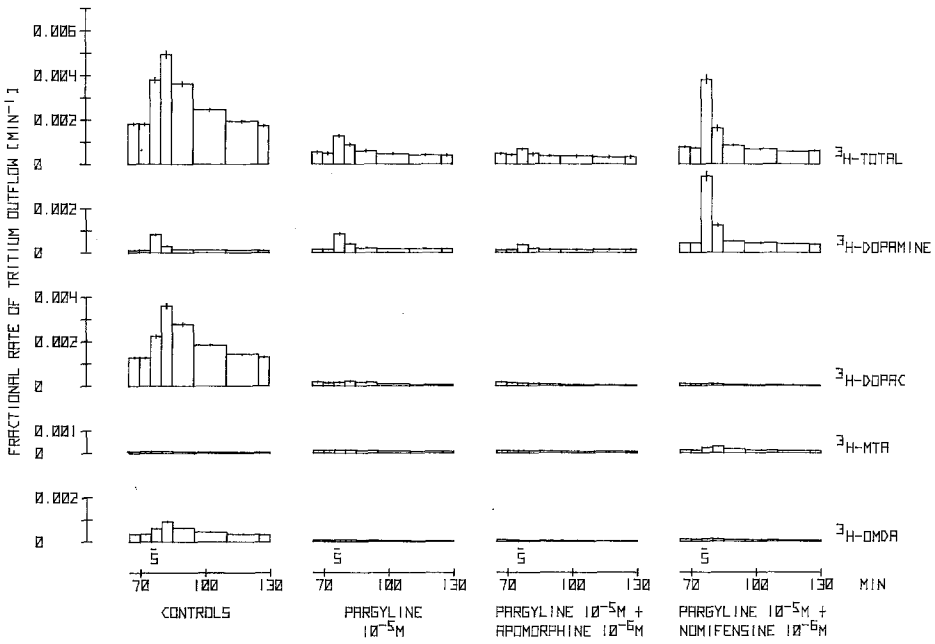

Fig. 7
Effects of pargyline, apomorphine and nomifensine on the basal and the electrically (3 Hz) evoked overflow of ^{3}H-compounds from caudate slices preincubated with ^{3}H-dopamine. The superfusate was collected from 65–130 min of superfusion (*abscissa*). The slices were stimulated electrically for 2 min (*S*). Pargyline, apomorphine and nomifensine were present from the beginning of superfusion. Means ± S.E. of 27 (controls) or 3–6 experiments. Controls are identical with those of Fig. 5

added simultaneously with pargyline, increased the evoked overflow of total tritium beyond the level obtained with pargyline alone and almost to that obtained with nomifensine 10^{-6} M alone. In the experiments with pargyline + nomifensine (Fig. 7) as well as in those with nomifensine 10^{-5} M alone (Fig. 5), the evoked overflow of ^{3}H-MTA was sufficiently enhanced to show that it peaked 3–8 min after stimulation.

Apomorphine was also tested in slices treated with pargyline. It caused a further decrease in the evoked overflow of total tritium which was now almost entirely ^{3}H-dopamine (Fig. 7, Table 3).

A tenfold increase in stimulation frequency to 30 Hz (and tenfold increase in number of pulses) approximately doubled the evoked overflow of total tritium (Table 3). The time course of the outflow of ^{3}H-compounds (not shown) and the chemical composition (Table 3) were practically unchanged. Apomorphine reduced the evoked overflow considerably less at 30 Hz (by 36%) than at 3 Hz (by 88%). As found at 3 Hz, ^{3}H-DOPAC was more decreased than ^{3}H-dopamine, so that the percentage recovered as ^{3}H-dopamine was increased. Pargyline produced changes similar to those observed at 3 Hz. Apomorphine reduced only slightly and not significantly the evoked overflow of tritium in slices treated with pargyline (Table 3).

When the frequency of stimulation was lowered to 0.5, 0.25 and 0.1 Hz, the evoked overflow of total tritium decreased (Table 3). Simultaneously, there were changes in the pattern of ^{3}H-compounds. The smaller the evoked overflow of total tritium, the higher was the percentage recovered as ^{3}H-dopamine and the lower the percentage of ^{3}H-DOPAC

(Table 3); accordingly, the outflow of total tritium peaked earlier (not shown).

Tritium in the Tissue

At the end of all experiments, the slices were homogenized, and their tritium content was analyzed. In the 17 control experiments without electrical stimulation, 94.1 ± 0.7% of the tritium in the tissue consisted of ^{3}H-dopamine, 4.3 ± 0.6% of ^{3}H-DOPAC, 0.36 ± 0.03% of ^{3}H-MTA and 1.0 ± 0.1% of ^{3}H-OMDA metabolites (experiments of Table 1). At the end of the 48 control experiments with electrical stimulation at 0.1 – 30 Hz, the composition was similar. Most drugs did not change this pattern. Exceptions were haloperidol 10^{-5} M, which reduced ^{3}H-dopamine to 81% and increased ^{3}H-DOPAC to 16% and ^{3}H-OMDA metabolites to 2%; pargyline, which increased ^{3}H-dopamine to approximately 97% and decreased ^{3}H-DOPAC to approximately 2.5%; and apomorphine, which reduced the percentage of ^{3}H-DOPAC. The values should be regarded with some caution, since tissue contents of metabolites were determined in the presence of a large excess of ^{3}H-dopamine.

Discussion

At the low concentration that we used for preincubation (10^{-7} M), ^{3}H-dopamine is selectively taken up into dopaminergic terminal axons. Some properties of the subsequent outflow of total tritium have been described previously (Starke et al. 1978). No significant formation of ^{3}H-noradrenaline was detected (cf. Jonason and Rutledge 1968). The basal outflow was only marginally decreased by tetrodotoxin and, hence, largely independent of action potentials. In contrast, the electrically evoked overflow was entirely tetrodotoxin-sensitive and, hence, reflected action potential-evoked ^{3}H-dopamine release (Starke et al. 1978). Of which compounds do the basal outflow and the electrically evoked overflow of tritium consist? Where are the compounds formed? How is the outflow modified by various drugs? We shall try to answer these questions making two main assumptions, namely, that the diffusion of the ^{3}H-compounds into the superfusion fluid (outflow, evoked overflow) was approximately proportionate to their average extracellular concentration within the tissue; and that nomifensine and cocaine acted mainly by inhibiting the uptake of dopamine into dopaminergic terminal axons (Heikkila et al. 1975; Reimann et al. 1979).

Basal Outflow

Composition of the ^{3}H-Material. By far the major component of the basal outflow of tritium was ^{3}H-DOPAC (about 68%). This confirms the original observation, in the same tissue, of Jonason and Rutledge (1968). Whereas the aldehyde formed by MAO from dopamine is mainly oxidized by AD to the acid (DOPAC), the aldehyde formed from noradrenaline is mainly reduced to the alcohol (DOPEG; Jonason 1969; Bönisch 1980; see review by Tipton et al. 1977). ^{3}H-OMDA metabolites, ^{3}H-dopamine and ^{3}H-MTA were minor components. ^{3}H-DOPET was not identified with certainty. This pattern agrees well with that found by Cubeddu et al. (1979a, b) for rat striatal slices.

Our apparent rate constants for the efflux of ^{3}H-DOPAC (0.068 min^{-1}; half time 10.2 min) and ^{3}H-OMDA metabolites (0.101 ± 0.002 min^{-1}; half time 6.9 min) compare well with those reported for rat striatal slices and the perfused rat heart (Cubeddu et al. 1979b; Bönisch 1980). The constants reflect the lipophilicity of the metabolites (see Trendelenburg et al. 1980). Apparent rate constants for the efflux of a metabolite can also be derived from the decline of efflux after sudden inhibition of the metabolizing enzyme. If we assume that amezinium 10^{-7} or 10^{-6} M inhibited the formation of ^{3}H-DOPAC very rapidly (see below), inspection of Figs. 1 and 2 suggests half times for the efflux of ^{3}H-DOPAC and ^{3}H-OMDA metabolites similar to those calculated from efflux in the last sample and tissue content.

Origin of ^{3}H-Compounds. Cubeddu et al. (1979b) adduced two arguments for the view that *^{3}H-DOPAC* is mainly formed intraneuronally from ^{3}H-dopamine that leaks from the storage granules into the axoplasm. First, the outflow of ^{3}H-DOPAC was only marginally diminished by cocaine and, hence, only to a small extent due to re-uptake of ^{3}H-dopamine from the extracellular fluid. Second, the outflow of ^{3}H-DOPAC was selectively increased by Ro 4-1284 which, like reserpine, interferes with the granular storage of dopamine. Our results with nomifensine and cocaine (Table 2) support the first argument. Moreover, we can add a third one. Amezinium potently and selectively inhibits the MAO inside noradrenergic neurones, because it is accumulated in these neurones by the noradrenaline uptake mechanism (Steppeler et al. 1980; Steppeler and Starke 1980). In the present experiments, amezinium markedly reduced the outflow of ^{3}H-DOPAC (by 78% at 10^{-6} M). In all likelihood, this was due to an analogous accumulation inside dopaminergic terminal axons and selective inhibition of their MAO, since the effect of amezinium was counteracted by nomifensine (Figs. 1, 2; Table 1).

Amezinium 10^{-5} M reduced the outflow of ^{3}H-DOPAC less than amezinium 10^{-6} M. Simultaneously, this concentration released considerable amounts of ^{3}H-dopamine. High concentrations of amezinium also release noradrenaline from sympathetic neurones (Steppeler et al. 1980). In both cases, amezinium probably displaces the catecholamines from the storage granules. Amezinium 10^{-5} M may have increased the oxidative deamination of ^{3}H-dopamine beyond the level observed with amezinium 10^{-6} M because of the enhanced supply of ^{3}H-dopamine to intraneuronal MAO. An alternative is that the marked release of ^{3}H-dopamine into the extracellular space led to some extraneuronal ^{3}H-DOPAC formation.

In contrast to Cubeddu et al. (1979b), we can draw some conclusions concerning the origin of the other ^{3}H-dopamine metabolites as well. *^{3}H-MTA* is an extraneuronal metabolite. Its outflow approximately paralleled the outflow of ^{3}H-dopamine and, hence, the supply of ^{3}H-dopamine to extraneuronal cells. This is well seen in the experiments with various concentrations of amezinium (Table 1). Moreover, haloperidol 10^{-7} M increased the outflow of ^{3}H-DOPAC, presumably by a reserpine-like mechanism, but not the outflow of ^{3}H-MTA (Table 2). Although haloperidol 10^{-5} M did increase the outflow of ^{3}H-MTA, this was not due to intraneuronal O-methylation following leakage of ^{3}H-dopamine into the axoplasm. If it were so, nomifensine should have produced no change. In fact, however, nomifensine further augmented the haloperidol-induced outflow of ^{3}H-MTA, undoubtedly because the simultaneous increase in ^{3}H-

dopamine led to increased extraneuronal methylation (Fig. 3, Table 1).

Our findings indicate that the predominant pathway to *^{3}H-HVA*, the main component of the ^{3}H-OMDA metabolites, is intraneuronal formation of ^{3}H-DOPAC followed by extraneuronal methylation. One argument is that amezinium reduced not only the outflow of ^{3}H-DOPAC but also, in parallel, that of ^{3}H-OMDA metabolites (by 55% at 10^{-6} M). This was due to inhibition of intraneuronal MAO, since nomifensine counteracted the effect of amezinium on ^{3}H-OMDA metabolites as well as on ^{3}H-DOPAC (Table 1). A second argument is that haloperidol 10^{-7} M increased the outflow of both ^{3}H-DOPAC and ^{3}H-OMDA metabolites, without a change in ^{3}H-dopamine and ^{3}H-MTA (Table 2). More strikingly, haloperidol 10^{-5} M caused a more than tenfold increase in the outflow of ^{3}H-DOPAC and ^{3}H-OMDA metabolites, with a less than threefold increase in ^{3}H-dopamine (Table 1). It seems very likely that the intraneuronally formed (see below) ^{3}H-DOPAC served as a substrate for extraneuronal O-methylation. Nomifensine, added together with haloperidol 10^{-5} M, caused a further pronounced increase in ^{3}H-dopamine and its extraneuronal metabolite ^{3}H-MTA, but only a marginal, if any, further increase in ^{3}H-OMDA compounds (Fig. 3).

Other Drug Effects. Haloperidol accelerates the outflow of tritium from rat caudate synaptosomes preincubated with ^{3}H-dopamine (in the presence of nialamide; Seeman and Lee 1974). The effect has been explained by an exocytotic release of ^{3}H-dopamine following either membrane fluidization or displacement of membrane calcium (Seeman and Lee 1974). Although our results confirm the increase in tritium outflow caused by haloperidol (in the absence of MAO-inhibitors), they suggest another mechanism. By far the largest part of the increase was due to deaminated metabolites. Only at the high concentration of haloperidol (10^{-5} M) did small increases in ^{3}H-dopamine and ^{3}H-MTA outflow accompany the increases in ^{3}H-DOPAC and ^{3}H-OMDA metabolites. A very high concentration of nomifensine (10^{-5} M) only slightly diminished the haloperidol-evoked outflow of deaminated ^{3}H-compounds (Fig. 3; Table 1). This pattern is in marked contrast to that found after electrical stimulation, i.e., after what is probably true exocytosis (Fig. 5). Haloperidol probably enters into the dopaminergic terminal axons and labilizes transmitter stores. Most of the dopamine which then leaks into the axoplasm is immediately deaminated, and only a small part escapes into the extracellular space. Hence, haloperidol does not elicit exocytosis but acts like reserpine, as do many α-adrenoceptor antagonists at high concentrations (Borowski et al. 1977).

Our experiments confirm the previous finding (Starke et al. 1978) that apomorphine at low concentrations slightly reduces the basal outflow of tritium. The effect was mainly due to a decrease in ^{3}H-DOPAC (Tables 1 and 2). It seems unlikely that apomorphine inhibited MAO directly. The K_i value of apomorphine for inhibition of mitochondrial MAO with dopamine as substrate is very high, namely 1.4×10^{-4} M (Di Chiara et al. 1974). Moreover, an increase in concentration from 10^{-7} to 10^{-6} M did not decrease ^{3}H-DOPAC further (Table 1). When we consider that a small part of the basal outflow is due to action potential-mediated ^{3}H-dopamine release (see above); that apomorphine also slightly reduced the outflow of ^{3}H-dopamine (although not significantly so); and that the effect was antagonized by haloperidol – it seems possible that apomorphine acted primarily on presynaptic autoreceptors and inhibited the release of ^{3}H-dopamine. The decrease in ^{3}H-DOPAC was then secondary to diminished transmitter release, as was also observed in experiments with electrical stimulation (see below). Apomorphine 10^{-5} M and bromocriptine 10^{-6} M may have failed to reduce the outflow of tritium (Table 1) because they exerted a reserpine-like effect in addition to activating autoreceptors (cf. Table 2 of Starke et al. 1978).

Electrically Evoked Overflow

Composition of the ^{3}H-Material. When, in previous experiments, we used 20 mM potassium to release ^{3}H-dopamine, the evoked overflow of total tritium was 4.29% of tissue tritium, and contained 51% ^{3}H-dopamine and 33% ^{3}H-DOPAC (Jackisch et al. 1980). Although in the present experiments with electrical stimulation (3 Hz) the evoked overflow was similar (5.2% of tissue tritium), it was chemically different, with only 10% ^{3}H-dopamine, but 74% ^{3}H-DOPAC (and some ^{3}H-MTA and ^{3}H-OMDA metabolites). Possibly, re-uptake of ^{3}H-dopamine which is a prerequisite for the formation of ^{3}H-DOPAC (see below) is impaired during continuous depolarization by potassium, but little changed during discontinuous depolarization by electrical pulses. Because of the predominance of ^{3}H-DOPAC, the time course of the outflow of total tritium mirrored the time course of the outflow of this metabolite. Westfall et al. (1976) reported a different pattern of ^{3}H-compounds in the electrically (50 Hz) evoked overflow of tritium from ^{3}H-dopamine-loaded rat striatal slices (for instance, only 14% DOPAC); species and frequency were different and, as mentioned in the Introduction, incomplete recoveries were not corrected for.

Origin of ^{3}H-Compounds. Both dopamine re-uptake inhibitors, nomifensine and cocaine, markedly reduced the stimulation-evoked overflow of *^{3}H-DOPAC*. Simultaneously, the evoked overflow of ^{3}H-dopamine was enhanced, and with increasing concentrations of nomifensine the time course of the outflow of total tritium more and more resembled the outflow of ^{3}H-dopamine (Fig. 5). These results demonstrate that by far the major source of the stimulation-evoked overflow of ^{3}H-DOPAC is intraneuronal deamination after re-uptake of released ^{3}H-dopamine. The small evoked overflow of ^{3}H-DOPAC that persisted in the presence of high concentrations of nomifensine or cocaine (when extracellular ^{3}H-dopamine was greatly increased) may have been extraneuronal in origin.

As in the absence of stimulation, the formation of *^{3}H-MTA* from ^{3}H-dopamine released by electrical pulses took place extraneuronally, since nomifensine and cocaine markedly increased the evoked overflow of ^{3}H-MTA (Fig. 5, Table 3). Nomifensine also strongly enhanced the evoked overflow of ^{3}H-MTA after MAO had been blocked by pargyline (Fig. 7, Table 3).

The bulk of the *^{3}H-OMDA* metabolites (mostly ^{3}H-HVA) in the evoked overflow of tritium was formed by a complex series of steps, namely re-uptake of released ^{3}H-dopamine, intraneuronal deamination and oxidation of the aldehyde, and extraneuronal methylation of the ^{3}H-DOPAC thus formed. Evidence for this pathway is the marked inhibition of the evoked overflow of ^{3}H-OMDA metabolites caused by nomifensine or cocaine (Fig. 5, Table 3). The inhibition paralleled the reduction of the evoked overflow of ^{3}H-DOPAC.

Note that the evoked overflow of ^{3}H-dopamine and ^{3}H-MTA was greatly increased by the re-uptake inhibitors. Hence, if extraneuronal deamination of ^{3}H-dopamine (or ^{3}H-MTA) prevailed in the formation of ^{3}H-OMDA metabolites, the re-uptake inhibitors should have increased these metabolites, which is the contrary of what was found. There is only one indication of some extraneuronal deamination of ^{3}H-dopamine leading to ^{3}H-OMDA metabolites. When the concentration of nomifensine was increased from 10^{-6} to 10^{-5} M, the percentage of ^{3}H-DOPAC in the evoked overflow (and, presumably, the extracellular concentration of ^{3}H-DOPAC) was reduced, yet the percentage of ^{3}H-OMDA metabolites was increased.

Other Drug Effects. Pargyline 10^{-5} M greatly diminished the basal as well as the electrically-evoked overflow of total tritium (Fig. 7; Tables 2 and 3). The decrease was not due to a higher tritium content of the tissue, leading to lower fractional rates. Tissue total tritium was raised by pargyline only slightly, and basal and evoked overflow were almost as markedly reduced by pargyline when expressed in absolute terms (e.g. fmol/min; data not shown). The decrease seems plausible since, after blockade of MAO, ^{3}H-dopamine leaking into the axoplasm or re-captured after exocytosis should be protected from degradation and largely re-stored. Yet, in previous experiments a decrease has not been found. When, in rat striatal slices, pargyline 10^{-5} M was present throughout preincubation with 5×10^{-7} M ^{3}H-dopamine and subsequent superfusion, the potassium-evoked overflow of tritium, expressed as % of tissue radioactivity, was *in*creased (Arbilla and Langer 1980). In our experiments, pargyline, like all other drugs, was added only during superfusion. Its presence during preincubation will allow the accumulation of high levels of ^{3}H-dopamine in the axoplasm, and this may distort results, as explained by Arbilla and Langer (1980).

As shown previously (Starke et al. 1978), apomorphine and bromocriptine markedly reduced and haloperidol slightly increased the stimulation-evoked overflow of tritium. Haloperidol did not change the pattern of metabolites. The agonists, however, raised the percentage of ^{3}H-dopamine and ^{3}H-MTA and simultaneously reduced the percentage of ^{3}H-DOPAC and ^{3}H-OMDA metabolites (Fig. 6, Table 3). These changes were unexpected, since activation of presynaptic α-autoreceptors of noradrenaline neurones produces a proportionate decrease in the evoked overflow of total tritium, ^{3}H-noradrenaline and its major metabolite ^{3}H-DOPEG (Taube et al. 1977). An explanation may come from the finding that a decrease in stimulation frequency from 3 Hz to 0.5, 0.25 and 0.1 Hz reduced the evoked overflow of total tritium and changed its time course and composition in a manner very similar to that observed with apomorphine and bromocriptine (Table 3). These results suggest that, when little ^{3}H-dopamine is released and then re-captured, as in the presence of apomorphine or bromocriptine or at low frequency, the storage vesicles re-incorporate most of the transmitter, so that only a small fraction is metabolized. When, on the other hand, dopamine release is high, re-uptake across the axolemma may exceed the maximal rate of vesicular uptake, so that a larger fraction is deaminated. As discussed above, it seems unlikely that apomorphine inhibited MAO directly in our experiments. Apomorphine reduced the evoked overflow of tritium even in slices treated with pargyline. Although this was partly due to a further decrease in the evoked overflow of ^{3}H-DOPAC, the evoked overflow of ^{3}H-dopamine was also reduced, as can be clearly seen, for instance, from a comparison of the pargyline experiments with the pargyline + apomorphine experiments in Fig. 7.

Conclusions

The fate of dopamine under the conditions of our control (drug-free) experiments is illustrated in Fig. 8. When the neurones are at rest, dopamine metabolism is initiated by leakage of the amine into the axoplasm, where it is degraded through the aldehyde to DOPAC. Part of the DOPAC is methylated extraneuronally to HVA. Traces of MTA are formed by extraneuronal methylation of dopamine. When action potentials arrive, dopamine metabolism is initiated by exocytosis. The bulk of the extracellular dopamine is taken up back into the neurones. When the stimulation frequency is 3 Hz, most of the axoplasmic dopamine is subsequently transformed to DOPAC; little seems to be re-stored. HVA and MTA are generated essentially as during neuronal rest.

It is emphasized that Fig. 8 depicts *major* pathways *under our conditions* and *in control experiments*. It goes without saying that drugs may bring about profound changes. As mentioned above, we have some evidence that extraneuronal deamination may also give rise to DOPAC which then diffuses into the extracellular space or is O-methylated. Furthermore, MTA may be deaminated. A small part of our OMDA metabolites seems to be MOPET. However, these pathways are of little importance under our conditions.

There is a gratifying analogy between noradrenaline and dopamine neurones. Both at rest and when the neurones fire, the formation of DOPEG, the main metabolite of noradrenaline (see Langer 1974; Farah et al. 1977; Taube et al. 1977), corresponds to the formation of DOPAC, the main metabolite of dopamine. In other respects, there may be differences which can be seen from a comparison of our Fig. 8 with Langer's (1974) Fig. 2. For instance, in dopaminergic neurones little, if any, O-methylation seems to occur intraneuronally.

The pathways to DOPAC and HVA shown in Fig. 8 were already proposed, at least basically, by Carlsson and Hillarp (1962; see also the review by Sharman 1973). More recently, Westerink and Korf (1976) and Westerink (1979) have presented in vivo evidence that most if not all HVA is formed from DOPAC, although Westerink (1979) writes that "the exact site of formation of DOPAC" (intra- or extraneuronal) "has not yet been identified". In comparison with these investigations, our study suffers from the disadvantage of artificial in vitro conditions. The advantage is, on the other hand, that it has been possible to identify metabolic pathways more directly.

Tissue levels of DOPAC and HVA are often used to indicate action potential-evoked release of dopamine or,

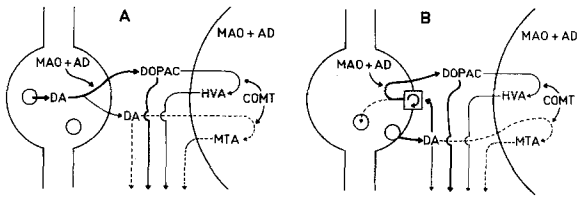

Fig. 8. Major metabolic pathways of dopamine in the rabbit caudate nucleus in vitro. **(A)** During neuronal rest. **(B)** Upon dopamine release by electrical stimulation at 3 Hz

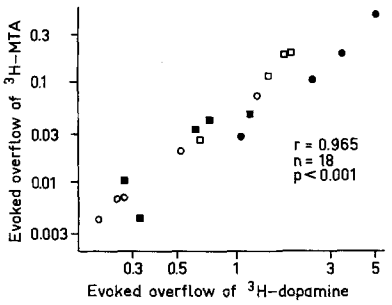

Fig. 9. Correlation between the electrically evoked overflow of ^{3}H-dopamine and of ^{3}H-MTA. Values were calculated from the evoked overflow of total tritium (as % of tissue tritium) and the percentage contribution to this overflow by ^{3}H-dopamine and ^{3}H-MTA (Table 3). The values thus represent the evoked overflow of the two compounds, expressed as % of total tissue tritium at the onset of stimulation. (○), Experiments without drugs; (●), with nomifensine or cocaine; (□), with pargyline or pargyline plus other drugs; (■), with apomorphine, bromocriptine or haloperidol. The point for pargyline + apomorphine, 3 Hz, is not included (its abscissa was 0.07, its ordinate 0.0004)

more precisely, the extracellular concentration of the transmitter. Yet, the fact that substantial amounts of the acids are formed in the absence of nerve impulses questions this approach. When impulse flow in the nigrostriatal fibres is abolished by γ-butyrolactone, striatal DOPAC levels fall by only 25 % (Roth et al. 1976), indicating formation of DOPAC in silent dopamine neurones in vivo as well. Moreover, dopamine re-uptake inhibitors had little effect on dopamine release and actually increased the extracellular concentration of the transmitter, yet reduced the formation of DOPAC and HVA (Fig. 5). Reserpine-like agents lead to rapid formation of DOPAC and HVA with only a minimal increase in extracellular dopamine. In our experiments, only the formation of MTA reflected the extracellular concentration of dopamine. Figure 9 shows a close correlation between the electrically evoked overflow of MTA and of dopamine. Correlations were also found when the outflow of MTA in experiments without electrical stimulation was plotted against the outflow of dopamine (not shown). Factors apart from rate of formation determine the tissue level of a compound. Yet, our experiments support the view that in vivo, of the three major dopamine metabolites, MTA is the best index of the extracellular dopamine concentration in the corpus striatum (Kehr 1976; Di Giulio et al. 1978).

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References

Andén NE, Roos BE, Werdinius B (1963) 3,4-Dihydroxyphenylacetic acid in rabbit corpus striatum normally and after reserpine treatment. Life Sci 5:319–325

Arbilla S, Langer SZ (1980) Influence of monoamine oxidase inhibition on the release of ^{3}H-dopamine elicited by potassium and by amphetamine from the rat substantia nigra and corpus striatum. Naunyn-Schmiedeberg's Arch Pharmacol 311:45–52

Bönisch H (1980) The rate constants for the efflux of deaminated metabolites of ^{3}H-dopamine from the perfused rat heart. Naunyn-Schmiedeberg's Arch Pharmacol 314:231–235

Borowski E, Starke K, Ehrl H, Endo T (1977) A comparison of pre- and postsynaptic effects of α-adrenolytic drugs in the pulmonary artery of the rabbit. Neuroscience 2:285–296

Carlsson A, Hillarp NA (1962) Formation of phenolic acids in brain after administration of 3,4-dihydroxyphenylalanine. Acta Physiol Scand 55:95–100

Carlsson A, Waldeck B (1964) A method for the fluorimetric determination of 3-methoxytyramine in tissues and the occurrence of this amine in brain. Scand J Clin Lab Investigation 16:133–138

Cubeddu LX, Hoffmann IS, Paris VB (1979a) Effects of papaverine on the release and metabolism of dopamine in rat striatum. J Pharmacol Exp Ther 209:73–78

Cubeddu LX, Hoffmann IS, Ferrari GB (1979b) Metabolism and efflux of [^{3}H]dopamine in rat neostriatum: presynaptic origin of 3,4-[^{3}H]dihydroxyphenylacetic acid. J Pharmacol Exp Ther 209:165–175

Di Chiara G, Balakleevsky A, Proceddu ML, Tagliamonte A, Gessa GL (1974) Inhibition by apomorphine of dopamine deamination in the rat brain. J Neurochem 23:1105–1108

Di Giulio AM, Groppetti A, Cattabeni F, Galli CL, Maggi A, Algeri S, Ponzio F (1978) Significance of dopamine metabolites in the evaluation of drugs acting on dopaminergic neurons. Eur J Pharmacol 52:201–207

Farah MB, Adler-Graschinsky E, Langer SZ (1977) Possible physiological significance of the initial step in the catabolism of noradrenaline in the central nervous system of the rat. Nauny-Schmiedeberg's Arch Pharmacol 297:119–131

Galli CL, Cattabeni F, Eros T, Spano PF, Algeri S, Di Giulio A, Groppetti A (1976) A mass fragmentographic assay of 3-methoxytyramine in rat brain. J Neurochem 27:795–798

Gordon EK, Markey SP, Sherman RL, Kopin IJ (1976) Conjugated 3,4 dihydroxy phenyl acetic acid (DOPAC) in human and monkey cerebrospinal fluid and rat brain and the effects of probenecid treatment. Life Sci 18:1285–1292

Graefe KH, Stefano FJE, Langer SZ (1973) Preferential metabolism of (–)-^{3}H-norepinephrine through the deaminated glycol in the rat vas deferens. Biochem Pharmacol 22:1147–1160

Heikkila RE, Orlansky H, Cohen G (1975) Studies on the distinction between uptake inhibition and release of [^{3}H]dopamine in rat brain tissue slices. Biochem Pharmacol 24:847–852

Hoffmann IS, Cubeddu LX (1978) Metabolism of spontaneous and neurally released ^{3}H-dopamine (DA) from rat neostriatal tissue. In: Abstracts of the 7th International Congress of Pharmacology, p 593

Hunt P, Kannengiesser MH, Raynaud JP (1974) Nomifensine: a new potent inhibitor of dopamine uptake into synaptosomes from rat brain corpus striatum. J Pharm Pharmcol 26:370–371

Jackisch R, Zumstein A, Hertting G, Starke K (1980) Interneurones are probably not involved in the presynaptic dopaminergic control of dopamine release in rabbit caudate nucleus. Naunyn-Schmiedeberg's Arch Pharmacol 314:129–133

Jonason J (1969) Metabolism of catecholamines in the central and peripheral nervous system. Acta Physiol Scand Suppl 320

Jonason J, Rutledge CO (1968) Metabolism of dopamine and noradrenaline in rabbit caudate nucleus in vitro. Acta Physiol Scand 73:411–417

Kaplan GP, Hartman BK, Creveling CR (1979) Immunohistochemical demonstration of catechol-O-methyltransferase in mammalian brain. Brain Res 167:241–250

Kehr W (1976) 3-Methoxytyramine as an indicator of impulse-induced dopamine release in rat brain in vivo. Naunyn-Schmiedeberg's Arch Pharmacol 293:209–215

Korf J, Grasdijk L, Westerink BHC (1976) Effects of electrical stimulation of the nigrostriatal pathway of the rat on dopamine metabolism. J Neurochem 26:579–584

Langer SZ (1974) Selective metabolic pathways for noradrenaline in the peripheral and in the central nervous system. Med Biol 52:372–383

Murrin LC, Roth RH (1976) Dopaminergic neurons: reversal of effects elicited by γ-butyrolactone by stimulation of the nigro-neostriatal pathway. Naunyn-Schmiedeberg's Arch Pharmacol 295:15–20

Reimann W, Zumstein A, Jackisch R, Starke K, Hertting G (1979) Effect of extracellular dopamine on the release of dopamine in the rabbit

caudate nucleus: evidence for a dopaminergic feedback inhibition. Naunyn-Schmiedeberg's Arch Pharmacol 306:53–60

Roffler-Tarlov S, Sharman DF, Tegerdine P (1971) 3,4-Dihydroxyphenylacetic acid and 4-hydroxy-3-methoxyphenylacetic acid in the mouse striatum: a reflection of intra- and extraneuronal metabolism of dopamine? Br J Pharmacol 42:343–351

Rosengren E (1960) On the role of monoamine oxidase for the inactivation of dopamine in brain. Acta Physiol Scand 49:370–375

Roth RH, Murrin LC, Walters JR (1976) Central dopaminergic neurons: effects of alterations in impulse flow on the accumulation of dihydroxyphenylacetic acid. Eur J Pharmacol 36:163–171

Seeman P, Lee T (1974) The dopamine-releasing actions of neuroleptics and ethanol. J Pharmacol Exp Ther 190:131–140

Sharman DF (1963) A fluorimetric method for the estimation of 4-hydroxy-3-methoxyphenylacetic acid (homovanillic acid) and its identification in brain tissue. Br J Pharmacol 20:204–213

Sharman DF (1973) The catabolism of catecholamines. Br Med Bull 29:110–115

Starke K, Reimann W, Zumstein A, Hertting G (1978) Effect of dopamine receptor agonists and antagonists on release of dopamine in the rabbit caudate nucleus in vitro. Naunyn-Schmiedeberg's Arch Pharmacol 305:27–36

Starke K, Steppeler A, Zumstein A, Henseling M, Trendelenburg U (1980) False labelling of commercially available ^{3}H-catecholamines? Naunyn-Schmiedeberg's Arch Pharmacol 311:109–112

Steppeler A, Starke K (1980) Selective inhibition by amezinium of intraneuronal monoamine oxidase. Naunyn-Schmiedeberg's Arch Pharmacol 314:13–16

Steppeler A, Pfändler R, Hedler L, Starke K (1980) An analysis of the effects of amezinium on postganglionic sympathetic neurones. Naunyn-Schmiedeberg's Arch Pharmacol 314:1–11

Sugden RF, Eccleston D (1971) Glycol sulphate ester formation from [^{14}C]noradrenaline in brain and the influence of a COMT inhibitor. J Neurochem 18:2461–2468

Swahn CG, Wiesel FA (1976) Determination of conjugated monoamine metabolites in brain tissue. J Neural Transm 39:281–290

Taube HD, Starke K, Borowski E (1977) Presynaptic receptor systems on the noradrenergic neurones of rat brain. Naunyn-Schmiedeberg's Arch Pharmacol 299:123–141

Tipton KF, Houslay MD, Turner AJ (1977) Metabolism of aldehydes in brain. Essays Neurochem Neuropharmacol 1:103–138

Trendelenburg U, Bönisch H, Graefe KH, Henseling M (1980) The rate constants for the efflux of metabolites of catecholamines and phenethylamines. Pharmacol Rev 31:179–203

Westerink BHC (1979) Further studies on the sequence of dopamine metabolism in the rat brain. Eur J Pharmacol 56:313–322

Westerink BHC, Korf J (1976) Turnover of acid dopamine metabolites in striatal and mesolimbic tissue of the rat brain. Eur J Pharmacol 37:249–255

Westfall TC, Kitay D, Wahl G (1976) The effect of cyclic nucleotides on the release of ^{3}H-dopamine from rat striatal slices. J Pharmacol Exp Ther 199:149–157

Wilk S, Zimmerberg B (1973) Absence of 3-methoxy-4-hydroxyphenylethanol in brain. Biochem Pharmacol 22:623–628

Zumstein A, Karduck W, Starke K (1979) Metabolism of dopamine in rabbit caudate nucleus in vitro. Naunyn-Schmiedeberg's Arch Pharmacol 308:R 15

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