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# COMPARATIVE EFFECTS OF BENZTROPINE AND NOMIFENSINE ON DOPAMINE UPTAKE AND RELEASE FROM STRIATAL SYNAPTOSOMES

DAVID MCKILLOP and HENRY F. BRADFORD*
Biochemistry Department, Imperial College of Science & Technology, London SW7 2AZ, U.K.

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**Abstract**—The spontaneous release of $^3$H-dopamine from preloaded superfused rat striatal synaptosomes was markedly increased by benztropine, but to a much lesser degree by nomifensine. Effects of similar magnitude were also observed in static incubations, indicating that superfusion had little inhibitory effect on dopamine re-uptake. However, benztropine and nomifensine were equivalent in their blockade of $^3$H-dopamine uptake into striatal synaptosomes. Fenfluramine produced a significant increase in the release of $^3$H-dopamine from superfused striatal synaptosomes. The fenfluramine-induced increase in $^3$H-dopamine efflux was additive to the benztropine response, but not to the nomifensine effect. The possibility that nomifensine may also act inhibiting the spontaneous release of dopamine by interaction at presynaptic dopamine receptors is discussed.

Dopamine release from central nerve endings has been the subject of much work in recent years, in an effort to elucidate the biochemical mechanisms and the regulatory control aspects of neurotransmitter synthesis and release [1–3]. Many such studies have employed various compounds to alter both the release and the re-uptake of dopamine [4, 5]. From these, it has become increasingly apparent that it is very difficult to distinguish between a releaser and an uptake blocker, both of which effectively increase the neurotransmitter concentration in the medium [6]. This has led to the position where the same drug has been proposed by some workers to raise the concentration of dopamine in the medium by a direct releasing action on the nerve ending, whilst other workers favour uptake inhibition as its mode of action [7, 8]. Although the mechanism of dopamine uptake has been well characterised as a $Na^+$-dependent, temperature sensitive, saturable, carrier-mediated process [9], the various steps involved in the release of this catecholamine from nerve endings, have not been so well defined. Dopamine release is thought to proceed physiologically by two separate mechanisms, one a spontaneous basal efflux of neurotransmitter and the other, a $Ca^{2+}$-dependent stimulated release produced by depolarisation of the neuronal membrane [10, 11]. The release of dopamine can also be stimulated by indirectly acting sympathomimetics, which are actively taken up into the neuronal tissue where they induce dopamine release by an exchange or displacement process of unresolved mechanism [12], although thought by some to involve a reversal of carrier-mediated uptake [13].

A precise determination of the mode of action of these compounds is important, since, although they all effectively raise external dopamine concentrations, they may produce different effects on presynaptic regulation processes. Over the past few years, the role of presynaptic receptors in dopaminergic transmission in the corpus striatum has been extensively studied [14, 15]. This work revealed that the regulation and modulation of dopamine release was influenced by different types of presynaptic receptors, activated by dopamine itself, acetylcholine, $\gamma$-aminobutyric acid, and probably several other neuroactive compounds [16–21]. In the present study, the effects of benztropine and nomifensine, both potent inhibitors of dopamine uptake [22, 23], have been investigated using rat striatal synaptosomes, in an attempt to define their exact mode of action in increasing dopamine overflow into the medium.

## MATERIALS AND METHODS

*Preparations of synaptosomes.* Crude synaptosomal fractions ($P_2$) were prepared from the corpus striatum of female Sprague–Dawley rats (180–220 g) as described previously [24]. The synaptosomal pellets were resuspended in 0.32 M glucose at a protein concentration of about 5 mg/ml, and, for use, diluted 1:10 in Krebs-bicarbonate medium of composition: 123 mM NaCl; 5 mM KCl; 1.2 mM $Na_2HPO_4$; 1.3 mM $MgSO_4$; 0.75 mM $CaCl_2$; 26 mM $NaHCO_3$, pH 7.5 containing 10 mM glucose, 0.5 mM ascorbic acid, 0.1 mM nialamide, and gassed with 95 per cent $O_2$/5 per cent $CO_2$.

*Superfusion procedure.* The synaptosomal suspension was equilibrated at 37° in a shaking waterbath for 10 min and incubated for a further 10 min in the presence of 0.2$\mu$M $^3$H-dopamine (13 Ci/mmol). Aliquots (0.7 mg protein) of the pre-labelled synaptosomes were pipetted onto Millipore filters at the bottom of five parallel superfusion vessels, maintained at 37°. Using suction from a vacuum pump, each tissue fraction was washed free of radioactivity with standard medium (3 × 5 ml) and superfusion begun with a flow rate of 0.5 ml/min. The eluent collected in the first 6 min was routinely discarded,

* Author to whom correspondence should be addressed.

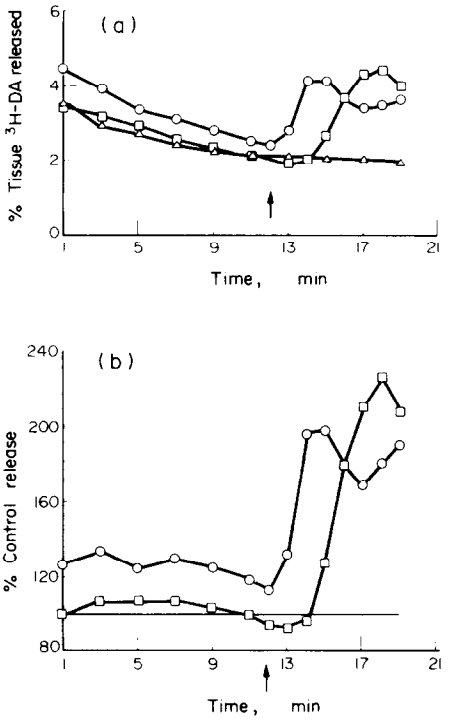


Fig. 1. Effect of KCl and veratrine on the release of $^3$H-dopamine from superfused striatal synaptosomes. (a) The spontaneous release of $^3$H-dopamine (△) in each 1 min fraction was calculated as a percentage of radioactivity in the tissue at that time. KCl (55 mM) (○) and veratrine (10 μM) (□) were added after 12 min as shown. (b) Each fraction was calculated as a percentage of control release at that time. Each curve is the mean of at least six experiments.

but thereafter, eluent was collected every minute, and the radioactivity of each fraction was measured by liquid scintillation spectrometry. Extracts of synaptosomes in 1 M HCl were also counted. Over 90 per cent of the radioactivity was associated with dopamine separated on a cation exchange resin as described earlier [26].

*Expression of results*. Each experiment was repeated at least three times, producing very similar release patterns, although some variation in absolute terms was apparent. The radio-activity in each eluent fraction was calculated as a percentage of that available in the tissue at the time of release [see Fig. 1(a)]. The effect of each condition was then calculated as (a) the maximal response and (b) the total counts effluxed as a percentage of the basal efflux from control synaptosomes over the release period studied, using the period before any drug addition as a control level [Fig. 1(b)]. For purpose of comparison, Fig. 1(a) shows the data of Fig. 1(b) plotted as percentage of total radio-activity in the tissue. Actual S.E.M. values are omitted from the figures for reasons of clarity, but the range for each plot is given in the Tables.

*Uptake experiments*. After equilibration for 10 min in a shaking water bath at 37°, aliquots of synaptosomes were transfered to the superfusion system and allowed to equilibrate for a further 10 min by superfusion with standard medium at 37°. Uptake was begun by addition of $^3$H-dopamine, at a final concentration of 0.2 μM, and superfusion continued for 3 min. The uptake was ended by removal of the radiolabelled medium, and by washing the synaptosomes with standard Krebs (10 ml), using suction. The filter was extracted with 1M HCl for 2 hr and the radioactivity taken up by the tissue was counted as before.

*Static Incubation procedure*. The synaptosomes were equilibrated and prelabelled as detailed above. The suspension was then centrifuged at 5000 *g* for 5 min, washed twice by resuspension in standard medium and centrifuged as before. After the final resuspension at 4°, aliquots (0.5 ml) of the prelabelled synaptosomes were incubated in a shaking water bath at 37° for various periods, and the action terminated by centrifugation for 1 min in an Eppendorf bench ultra centrifuge. Both the supernatant and the extracted pellet were measured for radioactivity by liquid scintillation.

*Materials*. Analar reagents were used throughout. Tetrodotoxin was purchased from Sigma, and (2,5,6-$^3$H)-dopamine obtained from the Radiochemical Centre (Amersham, U.K.). Benztropine was a gift from Merck, Sharp and Dohme, and nomifensine a gift from Hoechst Pharmaceuticals.

## RESULTS

### *Spontaneous and evoked $^3$H-dopamine release*

Using a superfusion system in an effort to minimise the re-uptake of released neurotransmitter [27], the characteristics of the spontaneous release of dopamine from striatal synaptosomes, prelabelled with $^3$H-dopamine, were examined. During the first few minutes of superfusion, efflux of $^3$H-dopamine was generally high and fairly variable. Thereafter, the

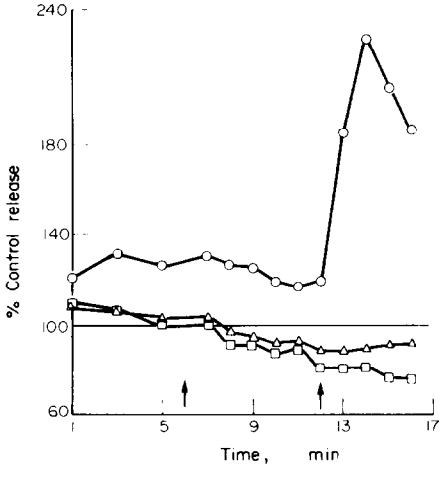


Fig. 2. Effect of tetrodotoxin on KCl- and veratrine-stimulated release of $^3$H-dopamine in superfused striatal synaptosomes. Tetrodotoxin (1 μM) was added to each superfusion after six minutes as shown, and its effect on control (△), $K^+$-stimulated (○), and veratrine-stimulated (□) release is shown as a percentage of basal $^3$H-dopamine efflux. KCl (55 mM) and veratrine (10 mM) were added after 12 min, as indicated. Each curve is the average of four experiments.

Table 1. Effect of various conditions on cumulative release of $^3$H-dopamine from synaptosomes

| | Time of addition (min) | Total released (13–19 min) | Max. effect | $n$ |
|---|---|---|---|---|
| Control | – | 100 ± 36 | 100 | 9 |
| $K^+$ (55mM) | 12 | 144 ± 32*** | 166 ± 44*** | 6 |
| Veratrine (10 μM) | 12 | 162 ± 37*** | 227 ± 37*** | 6 |
| Tetrodotoxin (1 μM) | 6 | 79 ± 34* | 76 ± 9** | 4 |
| Tetrodotoxin (1 μM) + $K^+$ (55mM) | 6, 12 | 157 ± 22*** | 188 ± 14*** | 4 |
| Tetrodotoxin (1 μM) + Veratrine (10μM) | 6, 12 | 92 ± 20 | 94 ± 22 | 4 |
| $Ca^2$ – free medium† | 0 | 75 ± 5** | 39 ± 5*** | 3 |

Results are the mean cumulative release ± standard deviation, expressed as a percentage of control release during the same period (13–19 min). Cumulative release of $^3$H-dopamine from control tissue (13–19 min) was 14.3 ± 5.1 per cent of $^3$H-DA in the tissue at that time.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$.

† $Ca^{2+}$ was omitted at the beginning of superfusion, and the effect compared with control release over the first 14 min.

spontaneous release of $^3$H-dopamine decreased exponentially and was quite reproducible. Under control conditions, $^3$H-dopamine was released at an average rate of 2.4 ± 0.10 per cent/min measured over 19 min [Fig. 1(a)], with 58.3 ± 3.0 per cent of the labelled catecholamine remaining in the tissue. Addition of tetrodotoxin (1 μM) to the superfusion medium significantly decreased the spontaneous $^3$H-dopamine efflux by 24 per cent over a 10 min period (Fig. 2, Table 1). Spontaneous release was also decreased by 25 per cent, on omission of $Ca^{2+}$ from the perfusate (Table 1).

Both KCl (55 mM) and veratrine (10μM) stimulated the total release of $^3$H-dopamine from striatal synaptosomes, by an average of 44 and 62 per cent respectively, during the whole 7 min after application [Fig. 1(b)]. The potassium-induced maximum (66 per cent) occurred after 2 min, whilst the maximum veratrine response (127 per cent) did not occur until 6 min after addition. The presence of tetrodotoxin consistently increased the averaged response to KCl stimulation from 44 to 57 per cent over spontaneous levels of efflux, whereas the averaged response to veratrine was substantially decreased from 62 to 13 per cent stimulation over the same period. (Table 1). These observations illustrated the dependence of the veratrine response on $Na^{\pm}$ channel activation, an unnecessary requirement for KCl depolarisation [28]. Omission of $Ca^{2+}$ decreased the responses to both KCl and veratrine in single experiments.

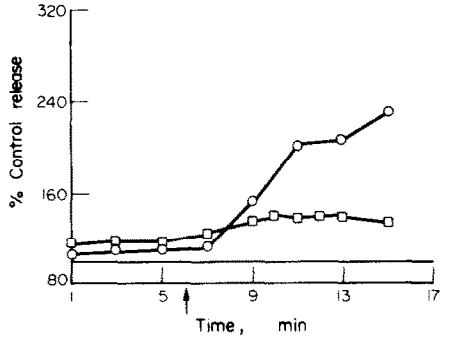


Fig. 3. Effect of benztropine and nomifensine on $^3$H-dopamine release from superfused striatal synaptosomes. Benztropine (10 μM) (○) and nomifensine (10 μM) (□) were added after 12 min as indicated, and their effects shown as a percentage of control release. Each curve is the average of at least six experiments.

*Benztropine and nomifensine on spontaneous and evoked release of $^3$H-dopamine*

Addition of benztropine (10 μM) to the superfusion medium increased the total efflux of $^3$H-dopamine by 86 per cent during the following 10 min, the maximum response being 159 per cent. Under the same conditions, nomifensine (10 μM) produced a significant increase of only 8 per cent over control levels with a maximum response of 16 per cent (Fig 3; Table 2). At the end of superfusion in the presence of benztropine, 40.7 ± 3.4 per cent of $^3$H-dopamine remained in the tissue, whilst, with nomifensine present, 56.4 ± 1.6 per cent remained, an amount very similar to the 58.3 per cent of $^3$H-dopamine normally residual in control tissues. Similar results to these were used by Raiteri *et al.* [13] to illustrate the efficiency of the superfusion system in blocking re-uptake. Thus, in their experiments nomifensine, a known dopamine uptake blocker, produced little or no increase in $^3$H-dopamine overflow, with an increase due to benztropine being explained as a direct effect on release rather than as inhibition of re-uptake.

However, if $^3$H-dopamine re-uptake were not in fact, blocked by the superfusion system, but by an uptake inhibition due to benztropine, (see below) then the low stimulation of overflow produced by nomifensine can be interpreted as the net result of its uptake-blocking ability, and a presynaptic inhibition of the spontaneous release of $^3$H-dopamine.

Both benztropine and nomifensine increased the total potassium-stimulated release of $^3$H-dopamine over 10 min from 42 to 254 and 86 per cent respectively (Table 2). This was presumably due to their capacity to inhibit dopamine re-uptake, and possibly also to cause release directly (benztropine).

*Actions of benztropine and nomifensine on $^3$H-dopamine uptake*

The uptake of $^3$H-dopamine (0.2 μM) into striatal synaptosomes was studied over a 3 min superfusion

Table 2. Effect of drugs on cumulative release of $^3$H-dopamine from synaptosomes

| | Time of addition (min) | Cumulative release (7–16 min) | Cumulative release (13–16 min) | Max. effect | n |
|---|---|---|---|---|---|
| Control | – | 100 ± 23 | 100 ± 30 | 100 | 9 |
| Benztropine (10 μM) | 6 | 186 ± 64*** | 227 ± 76*** | 259 ± 94*** | 10 |
| Nomifensine (10 μM) | 6 | 108 ± 15* | 107 ± 8 | 116 ± 6*** | 10 |
| $K^+$ (55 mM) | 12 | | 142 ± 38*** | 166 ± 44*** | 6 |
| Benztropine + $K^+$ | 6, 12 | | 354 ± 55*** | | 3 |
| Nomifensine + $K^+$ | 6, 12 | | 186 ± 40*** | | 3 |
| Fenfluramine (10 μM) | 6 | 141 ± 14*** | | 156 ± 13*** | 3 |
| Benztropine + fenfluramine | 6, 6 | 242 ± 53***† | | 285 ± 17*** | 3 |
| Nomifensine + fenfluramine | 6, 6 | 144 ± 24*** | | 156 ± 23*** | 3 |

Results are the mean cumulative release ± S.D., expressed as percentage of control release at that time. Control release (7–16 min) was 22.0 ± 5.1 per cent and control (13–16 min) 8.4 ± 2.5 per cent of $^3$H-DA in the tissue at the time of release.

Differences from control significant with: *$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

† Response with both benztropine and fenfluramine significantly different from benztropine alone with $P < 0.01$.

period in the presence and absence of benztropine and nomifensine. At a concentration of 10 μM, the two compounds were equipotent in their inhibition of $^3$H-dopamine uptake, producing almost total blockade. Benztropine caused a 92 per cent and nomifensine 90 per cent inhibition of $^3$H-dopamine uptake during superfusion, and blockade of a similar extent was seen in static incubations (Table 3). This observation fitted well with previously published uptake studies [22, 23], and indicated that the difference in the actions of benztropine and nomifensine on the spontaneous release of $^3$H-dopamine from prelabelled synaptosomes was not due to any difference in their abilities to block the re-uptake of dopamine. Also, benztropine clearly has uptake-blocking actions in this superfusion system in addition to any capacity to cause direct release.

### $^3$H-*dopamine release in static incubations*

The effects of benztropine and nomifensine on the spontaneous efflux of $^3$H-dopamine from prelabelled synaptosomes were re-examined in test-tube incubations, where re-uptake could proceed as normal. After a 16 min incubation, 44.5 per cent of the $^3$H-dopamine had been released into the medium of control incubations. This was increased to 77.7 per cent in the presence of benztropine (10 μM), and to 55.6 per cent in the presence of nomifensine (10 μM) (Fig. 4). The proportional changes produced by these compounds were very similar to those found by the superfusion method, indicating that the superfusion system had little effect on re-uptake of transmitter. Also the $^3$H-dopamine releasing actions of these two compounds were not directly related to their uptake blocking capacities, other mechanisms being involved.

### *Actions of fenfluramine on* $^3$H-*dopamine release*

Fenfluramine is a neuroleptic agent with dopamine receptor blocking properties thought by some to be principally presynaptic in action [29, 7]. The actions of this drug on efflux of $^3$H-dopamine from prelabelled striatal synaptosomes was examined using the superfusion system with and without the uptake

Table 3. Effects of nomifensine and benztropine on $^3$H-dopamine uptake

| Time (min) | Control | $^3$H-Dopamine in tissue (cpm/mg protein) Benztropine | Nomifensine | n |
|---|---|---|---|---|
| (a) Superfusion assay | | | | |
| 3 | 120,328 | 9226 (7.7 per cent) | 11,470 (9.5 per cent) | (2) |
| (b) Static test-tube assay | | | | |
| (1) 2 | 80,220 | 7336 (9.1 per cent) | 0 | (0 per cent) |
| 5 | 145,836 | 0 (0 per cent) | 2,624 | (1.8 per cent) |
| (2) 2 | 109,752 | 8,695 (7.9 per cent) | 13,493 | (12.3 per cent) |
| 5 | 129,850 | 17,314 (13.3 per cent) | 19,750 | (15.2 per cent) |
| (3) 2 | 85,370 | 8,824 (10.3 per cent) | 8,110 | (9.5 per cent) |
| 5 | 83,236 | 17,150 (20.6 per cent) | 14,906 | (17.9 per cent) |

Experimental details are given under materials and methods. Values in parentheses are cpm in drug-treated tissue as percentage of control levels.

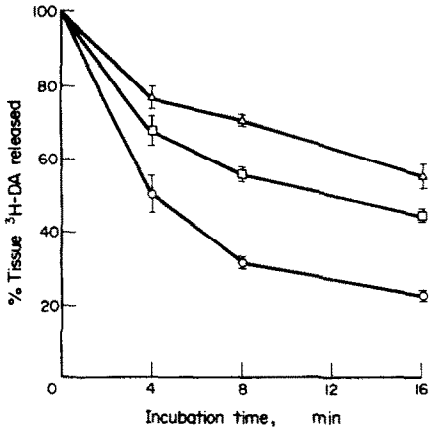


Fig. 4. Effect of benztropine and nomifensine on the release of $^3$H-dopamine from test-tube incubated striatal synaptosomes. Benztropine (10 $\mu$M) (○) and nomifensine (10 $\mu$M) (□) were added at zero time, and their effects are presented as the percentage of tissue $^3$H-dopamine released at each time point. Control levels of $^3$H-dopamine release are indicated (△). Each curve is the mean of three experiments, each run in duplicate.

blockers present (Fig. 5). Fenfluramine (10 $\mu$M), alone, produced a maximum response of 56 per cent and a total increase of 41 per cent over spontaneous efflux levels, when measured for 10 min after addition. (Table 2). This increase proved to be additive to the benztropine effect, since these two compounds in combination increased the total amount of $^3$H-dopamine released over 10 min by an average of 142 per cent, compared with 86 per cent for benztropine (10 $\mu$M) alone, indicating that each drug has a different mode of action. In the presence of nomifensine, however, the fenfluramine response was not significantly altered and any additive effect was not detectable.

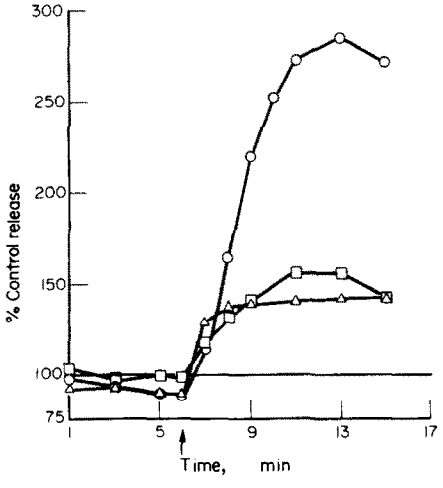


Fig. 5. Combined effects of fenfluramine with benztropine and nomifensine on $^3$H-dopamine release from superfused striatal synaptosomes. The effect of fenfluramine (10 $\mu$M) was examined alone (△), and in combination with benztropine (10 $\mu$M) (○), or with nomifensine (10 $\mu$M) (□) by addition of the drugs to the medium after 6 min. Each curve is the mean of the three experiments in duplicate.

## DISCUSSION

Superfusion of synaptosome preparations, employing apparatus and flow rates identical to those employed in this present study, has been interpreted as entirely preventing the re-uptake of released compounds, thereby allowing the full extent of their release to be measured [27]. This view was supported when nomifensine, a potent inhibitor of dopamine uptake [23], was found to produce no increase in the spontaneous efflux of radio-labelled transmitter to superfusion fluids, and was proposed to have no releasing activity on the basal efflux of dopamine from striatal synaptosomes [13]. Raiteri *et al.* [13] also found that benztropine caused a small but consistent increase in the spontaneous efflux of $^3$H-dopamine, but considered this to be some form of stimulated release.

In our experiments, benztropine produced a steadily increasing stimulation of $^3$H-dopamine release, occupying a much longer time-course than for other agents (e.g. KCl, veratrine, amphetamine, dopamine) thought to have a direct releasing effect [3]. Nomifensine was found to cause a much smaller increase in the spontaneous efflux of $^3$H-dopamine, although it inhibited the uptake of $^3$H-dopamine into striatal synaptosomes to the same extent as benzropine. These combined observations indicate that the different effects on the release of $^3$H-dopamine produced by benztropine and nomifensine could not be explained in terms of uptake blockade since they have similar potencies in this respect. The possibilities are that either benztropine directly stimulated dopamine release, as suggested by Raiteri *et al.* [13], or that nomifensine actually inhibited the increase in spontaneous release of dopamine due to uptake blockade. Recent work has shown that prior exposure of rat striatal slices to nomifensine produced a decrease in both the spontaneous and potassium-stimulated release of prelabelled dopamine [31]. Since this effect was apparent after removal of the drug from the medium, it was unlikely to be due to uptake blockade, but it was suggested that nomifensine might have interacted with a presynaptic receptor to produce inhibition of dopamine release [31].

When these effects of benztropine and nomifensine on the basal efflux of dopamine were compared with their actions in a static incubation system, little difference between the results was apparent. With static incubations, it was found that over 3 min benztropine caused a substantial loss of $^3$H-dopamine from the particulate fraction, and this was also observed to occur in superfusions over 10 min. This finding indicated that the superfusion system did little to prevent re-uptake of neurotransmitter. Previous work by Schacht and Heptner [32] showed that nomifensine had almost no effect on the amount of $^{14}$C-noradrenaline retained in the particulate fraction compared to control tissues, and this was interpreted as showing that nomifensine possessed no direct releasing activity. However, this report also showed that re-uptake could proceed into control tissue but not into nomifensine-treated tissue, indicating that nomifensine must have inhibited the spontaneous release of the catecholamine. The possibility that uptake blockers might also act directly on presyn-

aptic receptors was alluded to recently by Starke [15].

Fenfluramine is structurally related to amphetamine, but is distinct from amphetamine in that it produces none of the locomotor side-effects of amphetamine [33]. It did not inhibit the uptake of dopamine into rat brain synaptosomes and showed only a weak releasing-action on prelabelled $^3$H-dopamine, even at concentrations higher than 100 $\mu$M [34]. Also, its indirect sympathomimetic properties are not detectable until millimolar concentrations are used [35]. At the concentration used the present study (10 $\mu$M), fenfluramine had no indirect sypathomimetic activity [35]. However, it increased the rate of dopamine release above the spontaneous level, in a manner found to be additive to the benztropine-induced release. This observation indicated that the fenfluramine response was not due to inhibition of uptake of spontaneously released $^3$H-dopamine. The recent observation that fenfluramine was able to block dopamine receptors [29] might explain this increase in spontaneous dopamine release. Presynaptic receptors being the site of action, since recent work has indicated that fenfluramine does not cause direct blockade of postsynaptic dopamine receptors, as neuroleptics do [38].

When nomifensine was used in conjunction with fenfluramine no additive effect was observed. If both these drugs were competing antagonistically for some regulatory factor controlling the spontaneous release of $^3$H-dopamine from striatal synaptosomes, possibly a presynaptic dopamine receptor [3] then fenfluramine has by far the greater binding affinity. Nomifensine has been reported to have properties similar to the non-amphetamine, anti-depressant, methylphenidate [36], which was observed to potentiate the effects of endogenous dopamine primarily by blockade of re-uptake. However, methylphenidate and similar compounds, such as phencyclidine, have recently been found to inhibit tyrosine hydroxylase and, consequently, dopamine synthesis, possibly by receptor mediated feedback [37]. This would be a common point of attack for fenfluramine and nomifensine on the regulation of dopamine synthesis and release.

In conclusion, the present investigation has indicated that superfusion of striatal synaptosomes provides little or no inhibition of dopamine re-uptake. Nevertheless, the superfusion system is more satisfactory than incubations for defining the time-course of effects and also allowed each tissue sample to provide its own spontaneous release level for comparison with drug-induced effects. Both benztropine and nomifensine blocked the uptake of $^3$H-dopamine into striatal synaptosomes to the same extent, but nomifensine appeared to *inhibit* the spontaneous release of $^3$H-dopamine, possibly by an agonist effect on presynaptic dopamine receptors.

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