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Drug-induced changes in the release of [^{3}H]-noradrenaline from field stimulated rat iris

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Summary

1. Isolated rat irides were incubated with [^{3}H]-noradrenaline [^{3}H-NA] (10^{-7}M), superfused with buffer and then stimulated by an electrical field. The effect of desipramine, clonidine, phentolamine, phenoxybenzamine, GD131, normetanephrine and 4-tropolone-acetamide on the stimulation-induced overflow of [^{3}H]-NA was tested by adding the drug to the superfusing buffer. The effect of pretreatment with phentolamine or phenoxybenzamine on the stimulation-induced overflow of [^{3}H]-NA was also studied.

2. The effect of desipramine, clonidine, phentolamine, phenoxybenzamine and GD131 on uptake of [^{3}H]-NA in isolated irides was determined.

3. Desipramine moderately increased the stimulation-induced overflow at concentrations which almost completely inhibited neuronal uptake. It was calculated that in the isolated rat iris 30–40% of the released [^{3}H]-NA is inactivated by reuptake into the nerve terminal. This figure may represent the true reuptake percentage in this preparation. Desipramine-induced inhibition of [^{3}H]-NA release from the nerve terminal, possibly via a negative feed-back mechanism, may also contribute to this low figure.

4. Phentolamine and phenoxybenzamine, in concentrations or doses which did not inhibit neuronal uptake of [^{3}H]-NA, consistently increased the stimulation-induced overflow. This increase was further augmented when neuronal uptake was inhibited.

5. The α-adrenoceptor stimulating drug clonidine decreased the stimulation-induced overflow.

6. GD131, normetanephrine and 4-tropolone-acetamide did not greatly affect the stimulation-induced overflow of [^{3}H-NA].

7. It is concluded that the increased [^{3}H]-NA overflow obtained after α-adrenoceptor blockade is due to an increased [^{3}H]-NA release from the nerve terminals.

Introduction

Electrical stimulation of adrenergic nerves causes release of noradrenaline (NA) from the nerve terminals (Euler, 1956 ; Iversen, 1967). The fate of released NA has not been fully established though several different mechanisms of NA inactivation have been demonstrated (Folkow, Häggendal & Lisander, 1967). A part of the transmitter is taken up again into the nerve terminal by an active uptake mechanism at the level of the cell membrane—the so called membrane pump—and then either reincorporated into the amine storage granules or deaminated by

intraneuronal monoamine oxidase (MAO) (Carlsson, 1966; Hamberger, 1967). One portion of released NA is metabolized extraneuronally by catechol-*o*-methyltransferase (COMT) and/or MAO (Axelrod, 1966). Another fraction of the NA released from the nerves overflows unchanged from the tissue into the circulation. This is also the case for NA metabolites which do not accumulate within the tissue (Jonsson, Hamberger, Malmfors & Sachs, 1969).

Most studies on NA release have been concerned with the compounds overflowing from the tissue upon stimulation. This fraction is not identical with the total release from the nerves and the 'true' release is not easily determined. To obtain a more valid estimation of the 'true' release from the nerve terminals, attempts have been made to obtain a drug-induced increase of the fraction of NA overflowing into the circulation, for example by inhibition of the enzymatic destruction (Brown, 1965; Folkow *et al.*, 1967), inhibition of the membrane pump (Trendelenburg, 1959; Blakeley, Brown & Ferry, 1963; Kirpekar & Cervoni, 1963; Thoenen, Hürlimann & Haefely, 1964a; Geffen, 1965; Boullin, Costa & Brodie, 1967) or α-adrenoceptor blockade (Brown & Gillespie, 1957; Kirpekar & Cervoni, 1963; Rosell, Kopin & Axelrod, 1963; Thoenen, Hürlimann & Haefely, 1964b; Boullin *et al.*, 1967; Kirpekar & Wakade, 1970). However, great care must be taken when evaluating these results as the drugs used may also change the true release.

In this investigation the overflow of previously incorporated [^{3}H]-NA from the isolated rat iris induced by field stimulation was studied. The rat iris, which is densely innervated by adrenergic nerves, is very thin and released NA can thus easily diffuse into the superfusing buffer solution. Circulatory effects of stimulation should not affect the overflow of NA into the buffer solution. The aim was to study the effect of the membrane pump blockade, α-adrenoceptor blockade and α-adrenoceptor stimulating agents on [^{3}H]-NA overflow in this preparation.

Methods

Female albino rats (Sprague-Dawley, 180–200 g) were used. Some rats were pretreated with phentolamine or phenoxybenzamine (20 mg/kg i.p.) 0·5 h or 4 h before killing. The eyes were removed under ether anaesthesia and the irides (including the ciliary bodies) dissected free (Malmfors, 1965). The isolated irides were incubated for 30 min at 37° C with [^{3}H]-NA, 10^{-7}M, in a Krebs-Ringer bicarbonate buffer solution equilibrated with 6·5% CO_2 in O_2 (see Jonsson *et al.*, 1969). After incubation, the irides were transferred to small chambers where they were superfused and stimulated according to Farnebo & Hamberger (1970a). After superfusion for 30 min with buffer solution containing the drug to be tested, the irides were stimulated by an electrical field generated from a Grass S-4 stimulator (biphasic pulses, 12 mA, 2 ms, 10 Hz) for 10 min and then further superfused for 15 minutes. The superfusate (0·5 ml/min) was collected directly in 5 min fractions in the counting vials and the radioactivity determined by counting in a Packard 3320 Tri-Carb Scintillation Spectrometer after addition of 5 ml Insta-Gel scintillation solution. After superfusion the irides were dissolved in 0·5 ml Soluene and radioactivity was determined after addition of 10 ml toluene scintillation solution. Quenching was determined by recounting representative samples after the addition of a standard amount of [^{3}H]-toluene. The tritium

overflow induced by stimulation was calculated by subtracting the estimated spontaneous tritium efflux from the total tritium efflux for each fraction during stimulation, as schematically illustrated in Fig. 1. The stimulation-induced overflow (A) was expressed as per cent of the total tritium present in the tissue at the onset of stimulation (A+B+C).

The effect of desipramine, clonidine, phentolamine, phenoxybenzamine and GD131 on the uptake of [³H]-NA in isolated irides was also studied. Isolated irides from untreated rats were first preincubated for 15 min in a Krebs-Ringer bicarbonate buffer solution in the presence of the drug to be tested. [³H]-NA was then added to give a final concentration of 10^{-7}M and the incubation continued for 30 minutes. After rinsing in drug-free buffer solution for 10 min (also at 37° C) the radioactivity was determined as above. Irides, incubated in drug-free buffer solution, were included in every experiment as controls and the tritium uptake after various concentrations of drugs had been added was expressed as a percentage of the control.

The following substances were used: (±)-noradrenaline-7-[³H] (5–10 Ci/mmol, New England Nuclear), desipramine (Pertofrin, Geigy), phentolamine (Rogitine, Ciba), phenoxybenzamine (Dibenyline, S.K.F. Lab. through A. Johnsson & Co.),

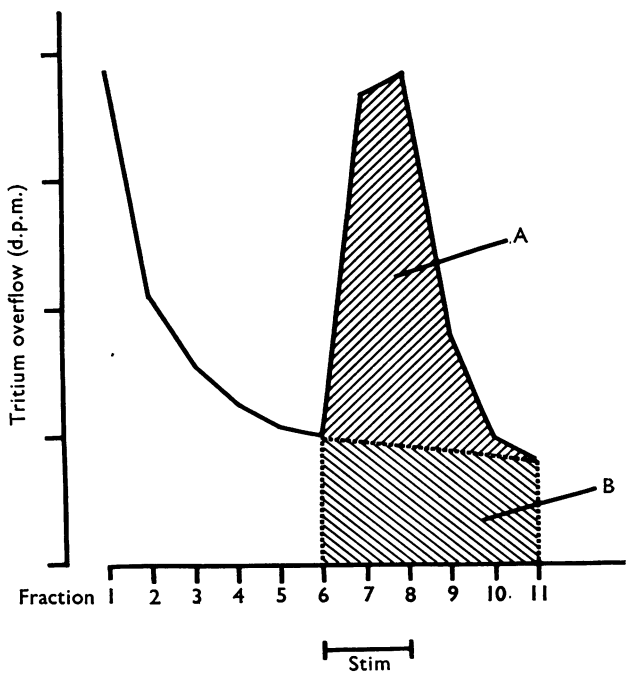

FIG. 1. Schematic illustration of the method used for calculation of stimulation-induced overflow. The tritium overflow (from an iris preincubated with [³H]-NA) in 5 min fractions during superfusion and stimulation is plotted. The tritium overflow induced by stimulation (A) is calculated by subtracting the estimated spontaneous tritium efflux (B) from the total tritium efflux for each fraction. Stimulation-induced overflow is expressed as per cent of the tritium content in the tissue at the onset of stimulation. That is, where the tritium content in the tissue at the end of supervision=C, stimulation-induced overflow $=\frac{A}{A+B+C} \times 100$.

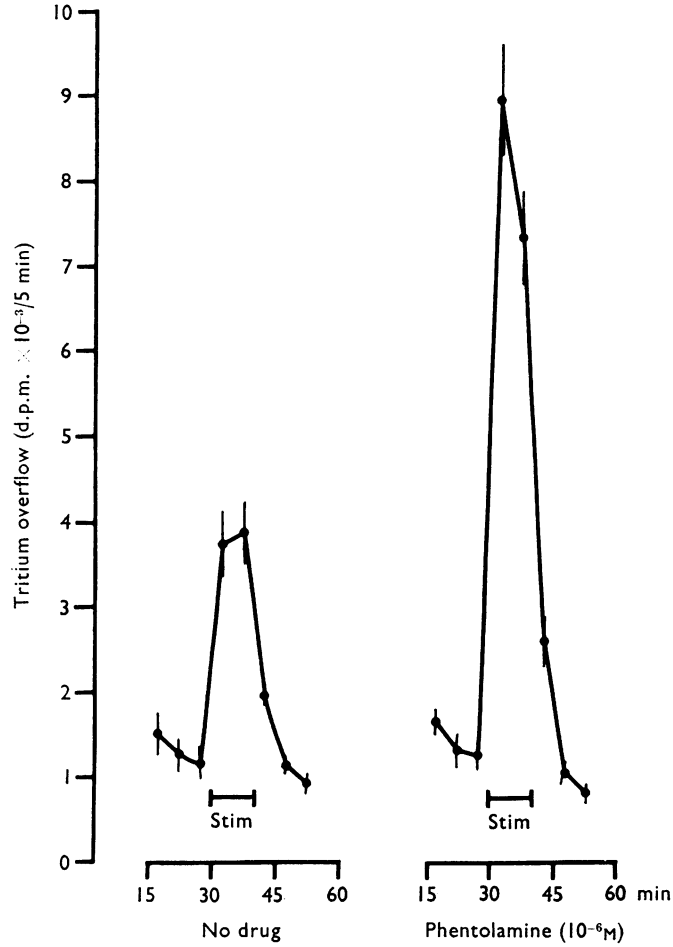

FIG. 2. Efflux of tritium from irides of untreated rats superfused with drug-free buffer solution (left) or buffer solution containing phentolamine 10^{-6}M (right). The irides were preincubated with [^{3}H]-NA, 10^{-7}M, for 30 min before superfusion and stimulation. Each point represents mean ± S.E.M. of four experiments.

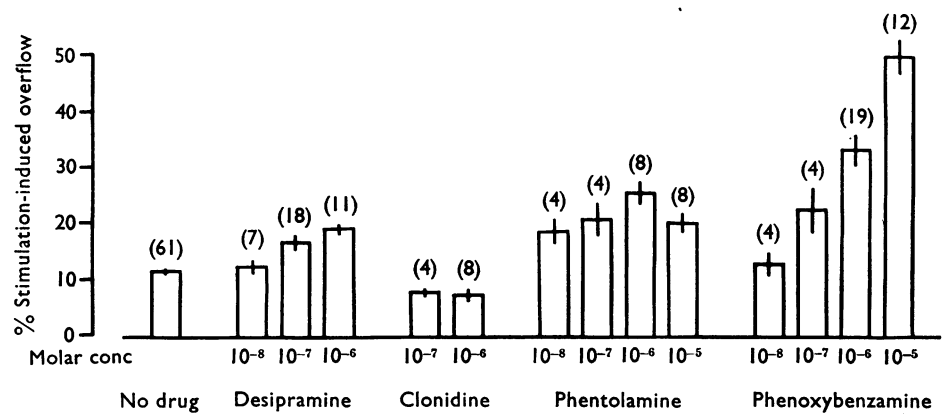

FIG. 3. Effect of desipramine, clonidine, phentolamine and phenoxybenzamine on the stimulation-induced overflow (see Fig. 1). Irides were preincubated with [^{3}H]-NA (10^{-7}M) for 30 min and superfused with buffer solution containing the drug to be tested. The superfusion and stimulation schedule is the same as in Fig. 2. Mean ± S.E.M. Number of experiments within parentheses.

clonidine (Catapresan, Boehringer), 4-tropolone-acetamide (H17/27, Hässle AB), normetanephrine (Sigma), GD131 (N-cyclohexyl-methyl-N-ethyl-β-chloroethylamine, courtesy of Dr. R. F. Furchgott).

Results

Isolated irides from untreated rats were preincubated with [³H]-NA, 10^{-7}M, superfused and stimulated by an electrical field. At the end of the prestimulatory period the tritium overflow per 5 min fraction became fairly stable (Fig. 2). Stimulation caused a large increase of the tritium overflow, which could be modified by the addition to the buffer solution of various drugs, for example phentolamine, which substantially increased the tritium overflow during stimulation (Fig. 2). The stimulation-induced overflow (see Fig. 1) for drug-free controls was 11·5±0·4% (mean±S.E.M. of sixty-one experiments). Desipramine (Fig. 3) caused a moderate increase of the stimulation-induced overflow at 10^{-7}M (16·6%) and 10^{-6}M (18·9%). Desipramine is a potent inhibitor of NA uptake at the cell

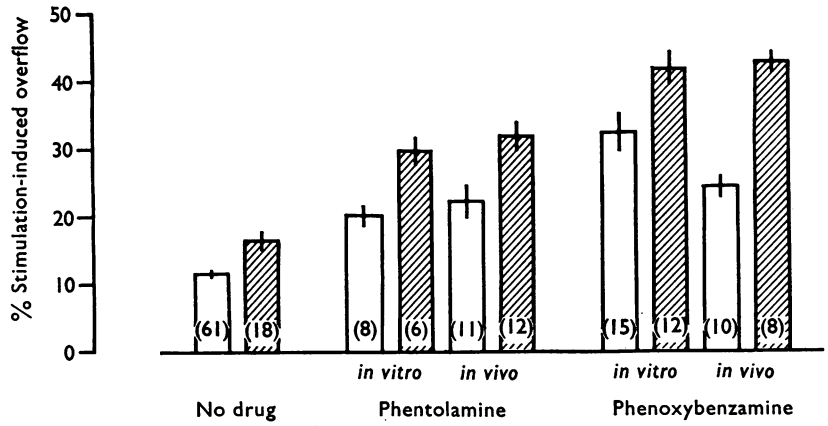

FIG. 4. Effect of desipramine (10^{-7}M) (▩) on the stimulation-induced overflow (see Fig. 1) from isolated irides preincubated with [³H]-NA (10^{-7}M) for 30 minutes. The effect of desipramine was determined on irides from untreated rats superfused with buffer solution containing phentolamine (10^{-5}M) or phenoxybenzamine (10^{-6}M) (*in vitro*). For comparison some results from Fig. 3 are included in the Figure. Futhermore, the effect of desipramine was determined on irides from rats pretreated with phentolamine or phenoxybenzamine (20 mg/kg i.p.) for 0·5 or 4 h respectively (*in vivo*). Pretreatment with phentolamine *per se* did not inhibit the membrane pump (101±4% of untreated control), while phenoxybenzamine reduced the uptake to 69±5%. Mean±S.E.M. Number of experiments within parentheses.

TABLE 1. *Effect of desipramine, clonidine, phentolamine, phenoxybenzamine and GD131 on the uptake of [³H]-NA in isolated rat iris**

Drug	10^{-5}M	10^{-6}M	10^{-7}M	3×10^{-8}M	10^{-8}M	3×10^{-9}M
Desipramine			5·9±2·7 (4)	22±1·1 (4)	39±1·0 (4)	63±3·5 (4)
Clonidine	92±4·2 (4)	87±12·0 (4)				
Phentolamine	67±3·5 (8)	116±5·8 (8)				
Phenoxybenzamine	4·6±0·5 (12)	52±5·1 (12)	75±4·5 (9)		114±12·6 (4)	
GD131	8·0±0·4 (4)	52±2·6 (4)	94±3·7 (4)		115±6·2 (4)	

* The irides were preincubated with drug for 15 min before addition of [³H]-NA to a final concentration of 10^{-7}M and were then incubated for 30 minutes. After rinsing with fresh buffer solution for 10 min the radioactivity was determined. The values are expressed as percentage uptake of [³H]-NA compared with that in drug-free controls run in the same experiment and are mean ± S.E.M. Number of observations within parentheses.

membrane level (the membrane pump) and the concentration of desipramine causing a 50% inhibition of [^{3}H]-NA uptake (at [^{3}H]-NA 10^{-7}M) was below 10^{-8}M in the isolated rat iris (Table 1).

The α-adrenoceptor stimulating drug, clonidine (Hoefke & Kobinger, 1966; Boissier, Guidicelli, Fichelle, Schmitt & Schmitt, 1968) decreased the stimulation-induced overflow to about 8% (Fig. 3) and had almost no influence on the membrane pump (Table 1). The α-adrenoceptor blocking drugs phentolamine and phenoxybenzamine increased the stimulation-induced overflow (Fig. 3). Phenoxybenzamine (10^{-5}M) was especially potent and caused a 4-fold increase of the overflow. Phentolamine (10^{-6}M) did not inhibit the membrane pump (Table 1), while phentolamine (10^{-5}M) reduced the uptake to about 70%. Phenoxybenzamine (10^{-6}M) caused about 50% inhibition of the membrane pump. Also the administration *in vivo* of the α-adrenoceptor blocking drugs increased the stimulation-induced overflow (Fig. 4). Phentolamine did not influence [^{3}H]-NA uptake while phenoxybenzamine decreased [^{3}H]-NA uptake to 70% of the control.

Combination of phentolamine or phenoxybenzamine with desipramine showed that the increase of the stimulation-induced overflow caused by the α-adrenoceptor blocking drugs could be further increased by desipramine (Fig. 4). The additional effect of desipramine was of about the same relative magnitude as the effect of desipramine alone.

GD131, a β-haloalkylamine similar to phenoxybenzamine (Nickerson & Gump, 1949) with very weak α-adrenoceptor blocking activity (Furchgott & Kirpekar, 1963) hardly influenced the stimulation-induced overflow in concentrations of 10^{-8}M to 10^{-6}M (Fig. 5). GD131 (10^{-5}M), greatly increased the spontaneous outflow of radioactivity, but completely inhibited stimulation-induced overflow. The potency of GD131 in inhibiting the membrane pump was similar to that of phenoxybenzamine (Table 1).

Normetanephrine, which inhibits Uptake$_2$ (Iversen, 1965, Eisenfeld, Landsberg & Axelrod, 1967) slightly decreased the stimulation-induced overflow (Fig. 5). Inhibition of COMT by 4-tropolone-acetamide (10^{-5}M) (Ross & Haljasmaa, 1964) failed to influence the stimulation-induced overflow.

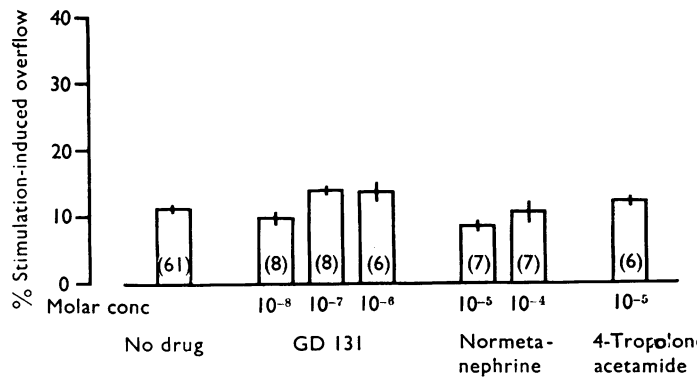

FIG. 5. Effect of GD131, normetanephrine and 4-tropolone-acetamide on the stimulation-induced overflow (see Fig. 1). Irides were preincubated with [^{3}H]-NA (10^{-7}M) for 30 min and superfused with buffer solution containing the drug to be tested. The superfusion and stimulation schedule is the same as in Fig. 2. Mean ± S.E.M. Number of experiments within parentheses.

Discussion

Adrenergic nerves in the isolated rat iris can take up [³H]-NA from the incubation medium by the membrane pump and further incorporate the amine into the amine storage granules (Carlsson, 1966 ; Jonsson *et al.*, 1969). After incubation with low concentrations of [³H]-NA (10^{-6}M or lower) more than 90% of the tritium content is intraneuronally located [³H]-NA (Jonsson *et al.*, 1969). Field stimulation increases tritium overflow from superfused tissue previously incubated with [³H]-NA (Baldessarini & Kopin, 1967 ; Farnebo & Hamberger, 1970a, b ; Häggendal, Johansson, Jonasson & Ljung, 1970 ; Langer, 1970 ; Su & Bevan, 1970). The stimulation-induced tritium overflow from irides of untreated rats consists mainly of unchanged [³H]-NA (Farnebo, 1971). In this investigation no determination of [³H]-NA and its metabolites in the superfusates was performed. Estimation of the total overflow of radioactivity might be more relevant than estimation of unchanged [³H]-NA alone, since the major metabolism is likely to occur after the release from the nerve terminal (*cf.* Langer, 1970).

Addition of the membrane pump blocking drug desipramine to the superfusing medium moderately increases the stimulation-induced overflow. This is in line with several investigations where only a small (Kirpekar & Cervoni, 1963 ; Thoenen *et al.*, 1964a) or even no effect (Trendelenburg, 1959 ; Blakeley *et al.*, 1963 ; Geffen, 1965 ; Boullin *et al.*, 1967) of membrane pump blocking drugs on NA overflow upon stimulation has been found. If reuptake of NA into the nerve terminal is the major inactivation pathway one might expect a substantial increase in transmitter overflow when this mechanism is effectively inhibited.

The moderate increase of [³H]-NA overflow after desipramine can be explained in several ways. Desipramine could have a direct inhibiting effect on the release process proper. That desipramine is a potent inhibitor of NA release seems unlikely, however, since desipramine causes a concentration dependent increase of [³H]-NA overflow and also increases overflow in combination with α-adrenoceptor blocking drugs. Based on the results obtained with desipramine the amount of released [³H]-NA taken up again by the membrane pump could be estimated to be 30–40% of the total amount of [³H]-NA released. This figure may be related to the *in vitro* technique, which presumably permits rapid diffusion of [³H]-NA away from the nerve terminal. However, this figure may also be the true reuptake percentage, as similar results have been obtained using isolated mouse atrium (unpublished results) and slices of rat brain cortex (Farnebo & Hamberger, 1970b).

Another tentative explanation for the results obtained with desipramine is to assume that the release from the nerve terminal is decreased upon membrane pump inhibition via a negative feed-back mechanism operating at the neuro-effector junction (Häggendal, 1969, 1970 ; Farnebo & Hamberger, 1970b ; Hedqvist, 1970).

The existence of a local feed-back mechanism could help to explain not only the failure of many investigators to show an increase in NA overflow upon stimulation in the presence of membrane pump blocking drugs, but also the discrepancy in potentiating effect by these drugs between exogenous NA and NA released from the nerve terminals (Fleckenstein & Bass, 1953 ; Trendelenburg, 1959).

The increased NA overflow upon nerve stimulation caused by α-adrenoceptor blocking agents has been ascribed to the α-adrenoceptor blockade (Brown & Gillespie, 1957 ; Boullin *et al.*, 1967 ; Kirpekar & Wakade, 1970) or the inhibition

of neuronal uptake of NA (Thoenen *et al.*, 1964b) in combination with facilitated overflow due to inhibition of vasoconstriction (Rosell, *et al.*, 1963).

In this investigation a wide range of concentrations was used to find out whether membrane pump blockade was of importance for the increased NA overflow obtained with α-adrenoceptor blocking drugs. Phentolamine, which does not inhibit the membrane pump at 10^{-6}M, at 10^{-8}M causes an increased overflow similar to that caused by desipramine. Furthermore, administration of phentolamine *in vivo*, which in the same experiment does not cause inhibition of the membrane pump, more than doubles [^{3}H]-NA overflow. Phenoxybenzamine (10^{-7}M) or given *in vivo* (4 h before killing) only slightly inhibits the membrane pump compared to desipramine *in vitro*, but increases overflow more than desipramine.

Phenoxybenzamine inhibits extraneuronal uptake of NA (Iversen, 1965; Eisenfeld *et al.*, 1967) and this has been suggested as one explanation for the increased NA overflow using this drug (Iversen & Langer, 1969; Langer, 1970). However, no increase of [^{3}H]-NA overflow is obtained with normetanephrine, which inhibits extraneuronal uptake (Iversen, 1965; Eisenfeld *et al.*, 1967). GD131, a β-haloalkylamine similar to phenoxybenzamine (Nickerson & Gump, 1949), which inhibits neuronal as well as extraneuronal uptake without blocking the α-adrenoceptors (Furchgott & Kirpekar, 1963; Kalsner & Nickerson, 1969) increases only very slightly stimulation-induced [^{3}H]-NA overflow (see also Kirpekar & Wakade, 1970). In high concentration (10^{-5}M) GD131 causes a large increase in spontaneous outflow (Furchgott & Kirpekar, 1963) but the stimulation-induced overflow is inhibited.

The effect of phenoxybenzamine on [^{3}H]-NA overflow does not seem to be due to inhibition of, or prevented access to, COMT (Iversen & Langer, 1969; Kalsner & Nickerson, 1970; Levin & Furchgott, 1970; Langer, 1970) since no effect is obtained with the COMT-inhibitor 4-tropolone-acetamide (Ross & Haljasmaa, 1964). Previous work has shown that inhibition of MAO by nialamide does not increase tritium overflow from field stimulated rat iris (Farnebo, 1971). The negative results obtained with inhibitors of MAO and COMT are in agreement with earlier findings in other preparations (Brown, 1965; Folkow *et al.*, 1967).

From the discussion above it can be concluded that the increased [^{3}H]-NA overflow obtained with phentolamine or phenoxybenzamine (low concentration) is not related to inhibition of neuronal or extraneuronal uptake of [^{3}H]-NA, but is due to an increased release of [^{3}H]-NA from the nerve terminals. Since an increased overflow of NA can be obtained also by using α-adrenoceptor blocking agents chemically different from phenoxybenzamine and phentolamine (for example ergot alkaloids; Hedqvist & Stjärne, 1969; Pacha & Salzmann, 1970), it seems likely that the increased release of NA is closely related to the α-adrenoceptor blocking activity of these drugs.

It has been suggested (Häggendal, 1969, 1970; Farnebo & Hamberger, 1970b) that α-adrenoceptor blockade should abolish a negative feed-back mechanism at the neuro-effector junction leading to an increased NA release. Modulation of NA release could be mediated via some substance, for example a prostaglandin, liberated from the excited effector cell (Hedqvist, 1970) or via changes in Ca^{++} concentration outside the nerve terminal (Häggendal, 1970).

Another possible mechanism is inhibition of NA release via α-adrenoceptors on the nerve terminal. There are reports that NA can modulate the release of acetyl-

choline from postganglionic parasympathetic nerves and from motoneurones, possibly via presynaptic α-adrenoceptors (Paton & Vizi, 1969 ; Kuba, 1970). In line with this is the finding that α-adrenoceptor blocking drugs greatly increase [³H]-NA overflow in the field stimulated mouse atrium, while β-adrenoceptor blocking drugs are without marked effects (unpublished results). The view that NA release can be influenced via α-adrenoceptors is further supported by the results obtained with the α-adrenoceptor stimulating agent clonidine (Hoefke & Kobinger, 1966 ; Boissier *et al.* 1968) which slightly decreases the stimulation-induced NA overflow (Werner, Starke & Schümann, 1970).

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