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FATE OF [^{3}H]AMEZINIUM IN SYMPATHETICALLY INNERVATED RABBIT TISSUES

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Abstract—Perfused rabbit hearts accumulated intra-aortically infused [^{3}H]amezinium. At concentrations of 1 or 10 nM, the accumulation proceeded at a constant rate for at least 60 min. After 60 min, tissue medium ratios were between 6 (1 μM) and approx. 100 (1 or 10 nM [^{3}H]amezinium). Cocaine or pretreatment with 6-hydroxydopamine abolished, and pretreatment with reserpine reduced the accumulation of [^{3}H]amezinium (1 nM). Kinetic analysis yielded a K_m value of 0.9 μM and a V_{max} of 1.2 nmole g^{-1} min^{-1}. When hearts had been labelled with 1 or 10 nM [^{3}H]amezinium, the fractional rate of the subsequent efflux was very low (0.001 min^{-1}). It was greatly increased when the animals had been pretreated with reserpine. Electrical stimulation of the sympathetic nerves released [^{3}H]amezinium from pre-labelled rabbit pulmonary artery strips. The electrically evoked overflow was abolished by tetrodotoxin or omission of Ca^{2+}; it was enhanced by cocaine, desipramine and yohimbine and decreased by clonidine. The results show that amezinium, at least at low concentrations, is selectively taken up into postganglionic sympathetic neurones, that it is partly sequestered in the vesicles, and that it is released by action potentials. Amezinium is a structurally novel substrate of both the noradrenaline transport mechanism of the axolemma and the transport mechanism of the noradrenaline-storing synaptic vesicles.

The new indirectly acting sympathomimetic agent amezinium (4-amino-6-methoxy-1-phenylpyridazinium) [1] possesses a novel pattern of effects on postganglionic sympathetic neurones [2, 3]. At low concentrations, it blocks selectively the monoamine oxidase (MAO, EC 1.4.3.4) inside these neurones; the blockade is prevented when amezinium is administered in the presence of cocaine. Moreover, amezinium inhibits the neuronal uptake of noradrenaline, and high concentrations release the transmitter. It has been proposed that this pattern is due to uptake of amezinium by the noradrenaline transport system of the axolemma, followed by intraneuronal accumulation to high concentrations [2, 3]. In contrast to drugs like bretylium that also block intraneuronal MAO, amezinium is not an adrenergic neurone blocking agent [2].

In the present study, tritium-labelled amezinium was used to obtain more direct information on its fate in sympathetically innervated tissues. Some findings have been reported to the German Pharmacological Society [4].

MATERIALS AND METHODS

The experiments were carried out on isolated perfused hearts and superfused strips of the pulmonary artery of male or female rabbits (1.5–3.0 kg). The rabbits were killed by a blow on the head, and tissues were immediately removed. Some animals were pretreated with reserpine or 6-hydroxydopamine as indicated under Results.

Perfused hearts. The coronary vessels were perfused according to the Langendorff technique with Tyrode's solution at a constant rate of 25 ml/min by means of a roller pump. The medium contained (mM): NaCl 137, KCl 2.7, $CaCl_2$ 1.8, $MgCl_2$ 1.1, $NaHCO_3$ 11.9, NaH_2PO_4 0.4, glucose 10.0, ascorbic acid 0.3, disodium EDTA 0.03. It was saturated with 5% CO_2 in O_2 and prewarmed to 35°.

In one series of experiments, [^{3}H]amezinium 1 nM–1 μM (sp. act. 8.2–0.0082 Ci/mmole; 8.2 nCi/ml) was infused into the aortic cannula for 60 min (rate of infusion 64 μl/min), and hearts were then perfused for another 60 min with label-free medium. The venous effluent was collected in 2- and 5-min samples both during and after the infusion of [^{3}H]amezinium in order to determine the removal of the drug as described for noradrenaline by Lindmar and Muscholl [5] and its subsequent efflux, respectively. At the end, hearts were weighed and homogenized in 9 vol. 0.4 M $HClO_4$. In two experiments with [^{3}H]amezinium 10 nM (sp. act 8.2 Ci/mmole instead of the normal 0.82 Ci/mmole), an attempt was made to identify the tritiated material in perfusate and tissue by thin layer chromatography (see Results for details).

In another series of experiments, the kinetics of the uptake of [^{3}H]amezinium were studied as described by Graefe *et al.* [6], with minor modifications. Briefly, the hearts were connected to a perfusion apparatus that allowed a rapid switch from one perfusion fluid to another. They were perfused for 3 min with [^{14}C]sorbitol 0.1 mM (sp. act. 50 mCi/mole; 5 nCi/ml) plus [^{3}H]amezinium 0.1–3 μM (sp. act. 0.18–0.006 Ci/mmole; 18 nCi/ml). During these 3 min, the venous effluent was con-

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tinuously collected in 5-sec samples. At the end, the hearts were weighed. The clearance of [^{14}C]sorbitol and [^{3}H]amezinium from the perfusion fluid was calculated from the arterio–venous difference of ^{14}C and ^{3}H, respectively. Subtraction of the [^{14}C]sorbitol clearance from the [^{3}H]amezinium clearance yielded that part of the clearance of [^{3}H]amezinium that was due to uptake into cellular compartments. The slope of the linear part of the cumulative [^{3}H]amezinium uptake curve represented the initial rate of uptake [6].

Superfused pulmonary arteries. Spirally cut strips of the arteries [7, 8] were incubated with [^{3}H]amezinium 0.1 μM (sp. act. 8.2 Ci/mmole; 820 nCi/ml) for 1 hr and then superfused with [^{3}H]amezinium-free Krebs–Henseleit solution at a rate of 2 ml/min. The medium contained (mM): NaCl 118, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4$ 1.2, $NaHCO_3$ 25.0, KH_2PO_4 1.2, glucose 11.0, ascorbic acid 0.3, disodium EDTA 0.03. It was saturated with 5% CO_2 in O_2 and prewarmed to 37°. Drugs were infused into the superfusion stream at a rate of 16 μl/min. The sympathetic nerves were stimulated electrically with transmural pulses of 0.3 msec width and 200 mA current strength at a frequency of 4 Hz. Four stimulation periods of 3 min each were applied after 132 (S_1), 153 (S_2), 174 (S_3) and 195 min (S_4) of superfusion. The superfusate was collected from 129 to 210 min in 3-min samples. At the end, the tissue was solubilized in Soluene® (Packard Instrument Co., Frankfurt am Main). The stimulation-evoked overflow of tritium was calculated as per cent of the tritium content of the tissue at the onset of stimulation [8].

Determination of radioactivity. ^{3}H and ^{14}C were determined by liquid scintillation counting. [^{3}H]- and [^{14}C]toluene were used as internal standards.

Drugs, statistics. The following drugs were used: Unlabelled amezinium methylsulphate and [^{3}H]amezinium chloride (4-amino-6-^{3}H-methoxy-1-phenylpyridazinium chloride), sp. act. 8.2 Ci/mmole (BASF, Ludwigshafen, F.R.G.); [^{14}C]sorbitol, sp. act. 324 Ci/mole (NEN, Dreieich, F.R.G.); clonidine hydrochloride and yohimbine hydrochloride (Boehringer, Ingelheim, F.R.G.); 6-hydroxydopamine hydrobromide and tetrodotoxin (Calbiochem, Frankfurt, F.R.G.); desipramine hydrochloride and reserpine (Serpasil® ampouls; Ciba–Geigy, Basel, Switzerland); corticosterone (Fluka, Buchs, Switzerland); cocaine hydrochloride and tyramine hydrochloride (Merck, Darmstadt, F.R.G.). 6-Hydroxydopamine hydrobromide was dissolved in 0.9% NaCl containing 1% ascorbic acid, tetrodotoxin in 0.1 M sodium acetate buffer pH 4.85, and corticosterone in ethanol. Stock solutions of other drugs were prepared in water. In control experiments, solvents were administered. Concentrations are final concentrations in the superfusion or perfusion media.

Means ± S.E. are given throughout. Student's *t* test was used to test differences between means for significance. *n*, Number of experiments.

RESULTS

Uptake

When [^{3}H]amezinium was infused into the aortic cannula of perfused rabbit hearts, part of it disappeared from the medium during passage through the coronary vessels. At low concentrations (1 and 10 nM), the ensuing arterio–venous difference was about 35% of the concentration infused, and remained constant for at least 60 min (Fig. 1; the decrease in arterio–venous difference from the first to the second 2-min sample probably reflects equilibration of the cardiac extracellular space). At higher concentrations, the percentage removal declined with time. From the total amount of [^{3}H]amezinium removed by the heart during the 60-min infusion period, tissue/medium ratios of 96 ± 4 (n = 4), 104 ± 10 (n = 3), 47 ± 4 (n = 4) and

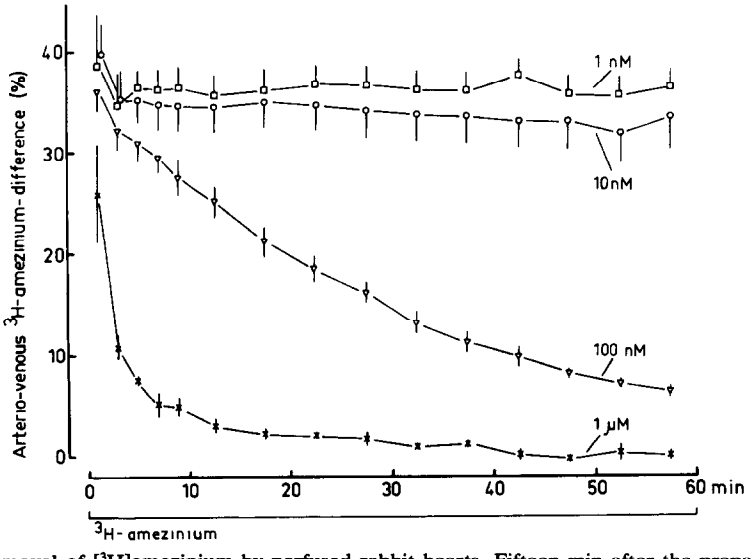

Fig. 1. Removal of [^{3}H]amezinium by perfused rabbit hearts. Fifteen min after the preparations had been set up, [^{3}H]amezinium 1 nM–1 μM was infused into the perfusion stream. Abscissa, time of [^{3}H]amezinium infusion. Ordinate, arterio–venous difference as % of the concentration infused. Means ± S.E. of 3–4 experiments.

6 ± 1 (n = 3) were calculated for concentrations of 1, 10, 100 and 1000 nM, respectively.

The effect of drugs on the removal of [3H]amezinium 1 nM is shown in Fig. 2. Cocaine rapidly diminished the arterio–venous difference. In fact, after the addition of cocaine, the venous effluent tended to contain a slightly *higher* concentration of [3H]amezinium than the arterial inflow. Corticosterone had no effect, independently of whether it was added after cocaine (Fig. 2) or in the absence of cocaine (not shown). Pretreatment with 6-hydroxydopamine almost abolished the ability of the heart to remove [3H]amezinium. In hearts from reserpine-pretreated rabbits, the arterio–venous difference declined with time even when the concentration of [3H]amezinium was only 1 nM.

The initial rate of the cellular uptake of [3H]amezinium was determined with the method of Graefe *et al.* [6], in which distribution into the extracellular space is corrected for by simultaneous perfusion with [14C]sorbitol. Figure 3 shows a typical experiment with [3H]amezinium 0.1 μM. The cumu-

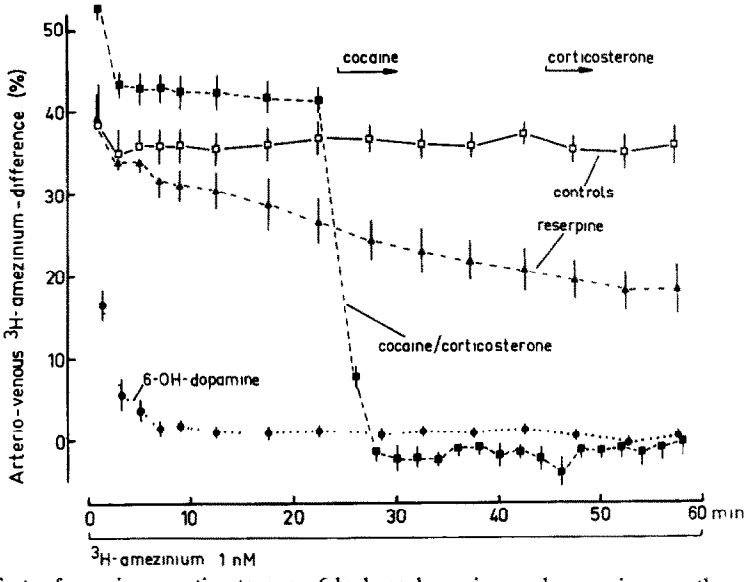

Fig. 2. Effect of cocaine, corticosterone, 6-hydroxydopamine and reserpine on the removal of [3H]amezinium by perfused rabbit hearts. Fifteen min after the preparations had been set up, [3H]-amezinium 1 nM was infused into the perfusion stream. □, Controls (n = 4; experiments identical with those of Fig. 1). ■, cocaine 30 μM and corticosterone 87 μM were infused from 25 and 45 min, respectively, after the beginning of the [3H]amezinium infusion onwards (n = 3). ●, 6-Hydroxydopamine hydrobromide 30 mg/kg was injected i.v. 40, 21 and 16 hr before the experiments (n = 3). ▲, Reserpine 1 mg/kg was injected i.v. 48 and 24 hr before the experiments (n = 3). Abscissa, time of [3H]amezinium infusion. Ordinate, arterio–venous difference as % of the concentration infused. Means ± S.E.

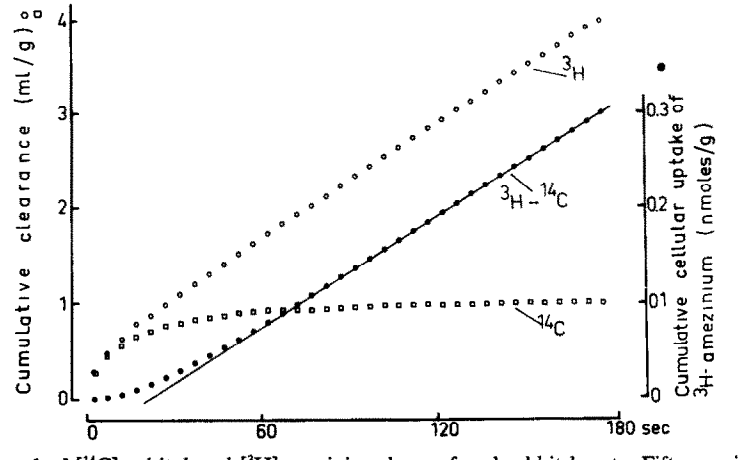

Fig. 3. Removal of [14C]sorbitol and [3H]amezinium by perfused rabbit hearts. Fifteen min after the preparations had been set up, normal medium was switched to medium containing [14C]sorbitol 0.1 mM and [3H]amezinium 0.1 μM for 180 sec. Abscissa, time of perfusion with the labelled compounds. Left ordinate and open symbols, cumulative clearance of 3H and 14C, calculated from the arterio–venous differences. Right ordinate and solid symbols, cumulative cellular uptake of [3H]amezinium. Shown is a typical experiment.

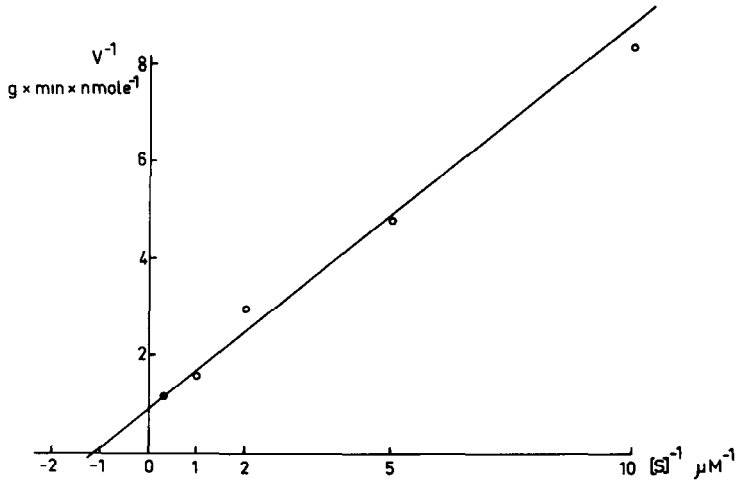

Fig. 4. Uptake of [^{3}H]amezinium into perfused rabbit hearts: double reciprocal plot of initial rate of uptake (V) versus [^{3}H]amezinium concentration ([S]). Data were obtained from experiments like that shown in Fig. 3. Means of 3–4 experiments for each concentration.

lative clearance of [^{14}C]sorbitol rapidly approached a constant value of about 1 ml/g, thus demonstrating rapid equilibration of the extracellular space. The high value of 1 ml/g is due to the fact that [^{14}C]sorbitol also distributes into the dead space of the perfusion system as well as into the fluid of the cavities of the heart [6]. The cumulative clearance of [^{3}H]amezinium increased rapidly at first and more slowly, but constantly from about 20 sec onwards. The calculated difference curve, which reflects the cellular uptake of [^{3}H]amezinium, becomes linear after a short lag period [6]. The slope of the linear part of this difference curve is the initial rate of uptake of [^{3}H]amezinium and was used for the double reciprocal plot shown in Fig. 4, as well as for calculation of the kinetic constants [9]. The K_m value was 0.89 ± 0.14 μM, and the V_{max} 1.17 ± 0.10 nmole g^{-1} min^{-1}.

Efflux

After hearts had been perfused with [^{3}H]amezinium for 60 min, they were perfused for a further 60 min with [^{3}H]amezinium-free medium (experiments of Figs. 1 and 2). In the first few minutes, the fractional rate of outflow (i.e. outflow divided by the [^{3}H]amezinium content of the tissue at the respective time) declined because of washout of dead space and extracellular space. When the concentration of [^{3}H]amezinium had been low (1 or 10 nM), the fractional rate of outflow remained constant afterwards and amounted to about 0.001 min^{-1}. When the labelling concentration had been 0.1 or 1 μM, fractional rates were higher, continued to decline, and amounted to about 0.005 min^{-1} at the end of the experiments. Pretreatment with reserpine increased the fractional rate of outflow which then was, for instance, 0.008 min^{-1} after perfusion with [^{3}H]amezinium 1 nM.

In two experiments, the radioactivity in the venous effluent was analyzed by TLC on alumina and silica gel plates. Figure 5 shows chromatograms with a high and a low R_F-value. Practically all the tritium in the perfusate co-chromatographed with authentic [^{3}H]amezinium and unlabelled amezinium. Similar results were obtained with two other mobile phases, and with aliquots of extracts from the heart (not shown).

The efflux of [^{3}H]amezinium was also studied in superfused strips of the pulmonary artery of the rabbit (Fig. 6). Transmural electrical stimulation which, under the conditions used, selectively excites the sympathetic nerves of the tissue [7, 8], greatly increased the outflow of [^{3}H]amezinium. The overflow evoked by S_1 amounted to 0.57 ± 0.06% of the [^{3}H]amezinium content of the tissue at the onset of S_1 ($n = 22$; all experiments of Fig. 6 pooled). In control experiments, the response was reproducible. Superfusion with Ca^{2+}-free medium or addition of tetrodotoxin abolished the stimulation-evoked overflow of [^{3}H]amezinium. Cocaine and desipramine increased both the basal efflux and the stimulation-evoked overflow. Yohimbine selectively and markedly increased the response to stimulation. Clonidine slightly, but significantly reduced the stimulation-evoked overflow of [^{3}H]amezinium (ratio S_3/S_1 in control experiments 1.18 ± 0.17, $n = 4$, in experiments with clonidine 0.54 ± 0.16, $n = 3$; $P < 0.05$).

DISCUSSION

Thin layer chromatography suggested that both the tritiated material retained in the rabbit heart 60 min after perfusion with [^{3}H]amezinium, and the material found in the venous effluent, was unchanged [^{3}H]amezinium. Hence, the ^{3}H material will be referred to as [^{3}H]amezinium.

Intra-aortically infused [^{3}H]amezinium was partly removed from the perfusion fluid during the passage through the heart and, in the absence of metabolic degradation, accumulated in the tissue. At least at low concentrations, the accumulation was entirely due to uptake into postganglionic sympathetic neurones via the noradrenaline carrier mechanism. This contention is borne out by the observation that cocaine (which blocks the uptake mechanism) or

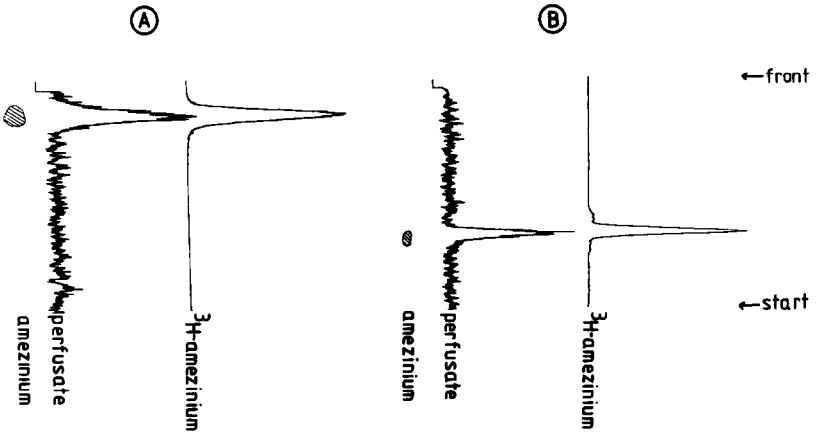

Fig. 5. Thin layer chromatography of the radioactive material released from a rabbit heart after perfusion with [3H]amezinium. [3H]Amezinium 10 nM (sp. act. 8.2 Ci/mmole) was infused into the perfusion stream for 60 min. After washout for 60 min, the rate of perfusion was decreased to 5 ml/min, and tyramine 100 μM was infused in order to increase the concentration of radioactive material. (Tyramine releases [3H]amezinium; not shown.) 100 μl-aliquots of this perfusate were chromatographed. Stationary phase: A, alumina 60 F_{254} (Merck, Darmstadt); B, silica gel 40 F_{254} (Merck). Mobile phase for A and B: butanol/acetic acid/water 80/20/40 (v/v). Detection: Dünnschichtscanner II (Berthold, Wildbad) or u.v.-light. [3H]Amezinium chloride 125 ng (sp. act. 8.2 Ci/mmole) and unlabelled amezinium methylsulphate 5 μg were used as references.

pretreatment with 6-hydroxydopamine (which destroys the neurones) prevented the accumulation of [3H]amezinium. Corticosterone (which blocks the extraneuronal uptake of noradrenaline) caused no change. From the effect of amezinium on the metabolism of noradrenaline we have previously concluded that the drug is selectively transported into noradrenergic varicosities to reach MAO-inhibiting levels [2, 3]. The present results are direct evidence for this view. In fact, neuronal uptake brings about a pronounced accumulation of [3H]amezinium. Since tissue/medium ratios were up to about 100, the concentration gradient between the sympathetic neurones (which are but a small fraction of the tissue) and the medium must have been very large.

The initial rate of uptake was measured in experiments in which the distribution of [3H]amezinium into the extracellular space was corrected for [6]. Kinetic analysis yielded a K_m value of 0.9 μM. In synaptosomes from rat hippocampus, Traut [10] found that the uptake of [3H]amezinium was half-saturated at 29 nM, i.e., at about 1/30 of our K_m. Although this would seem to be a large discrepancy, it should be noted that the uptake of [3H]noradrenaline into hippocampal synaptosomes was also half-maximal at 1/30 of the K_m of [3H]noradrenaline for uptake into the perfused rabbit heart (0.11 μM [10] vs 3.3 μM [6]). The noradrenaline carrier mechanism in the synaptosomal preparation seems to generally display higher substrate affinities than the carrier mechanism in the perfused heart, under the respective experimental conditions.

What is the fate of amezinium after uptake into the neurones? Part of it interacts with mitochondrial MAO. The following findings demonstrate that a second, quantitatively important fraction enters into the transmitter-containing vesicles.

(1) In hearts from un-pretreated animals, the arterio–venous difference of intra-aortically infused [3H]amezinium remained constant for at least 60 min, provided the concentration was low (up to 10 nM). In contrast, when animals had been pretreated with reserpine (which prevents the vesicular storage of noradrenaline), the removal of [3H]amezinium declined even when its concentration was only 1 nM. Obviously, inactivation of the vesicular compartment leads to high axoplasmic levels of [3H]amezinium, marked backflow into the extracellular space and, hence, a decrease of the arterio–venous difference. Normally, vesicular storage seems to be able to cope with virtually all of the [3H]amezinium entering the neurones at extracellular concentrations of up to 10 nM.

(2) When hearts from un-pretreated rabbits had been labelled with amezinium 1 or 10 nM, the subsequent fractional rate of outflow was very low (0.001 min^{-1}, half life about 12 hr). Pretreatment with reserpine increased the fractional rate of outflow, certainly because the efflux then mainly originated not from the vesicles but from a less stable extravesicular pool.

(3) The best evidence is that [3H]amezinium was released by sympathetic nerve stimulation. The release was prevented by tetrodotoxin or omission of Ca^{2+}, thus demonstrating that, like release of noradrenaline, it was due to exocytotic emptying of the vesicles. It might be argued that [3H]amezinium was not released directly by the action potentials but indirectly by noradrenaline after re-uptake of the transmitter ([3H]amezinium is in fact released by tyramine 10 nM or higher concentrations; not shown). If so, blockade of re-uptake should prevent the release of [3H]amezinium. In fact, however, cocaine and desipramine *increased* the stimulation-evoked as well as the basal outflow, probably because

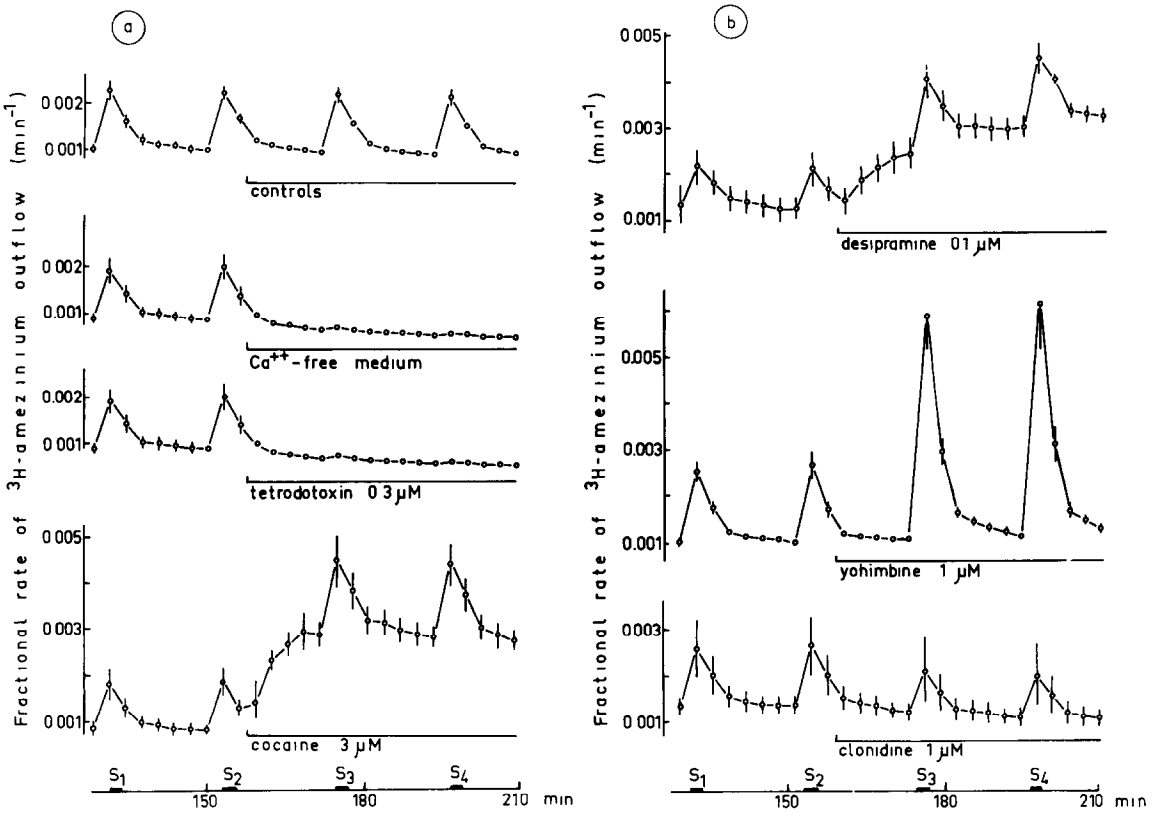

Fig. 6. Outflow of tritium from superfused rabbit pulmonary artery strips preincubated with [^{3}H]amezinium. The tissue was incubated with [^{3}H]amezinium 0.1 µM for 60 min and then superfused with [^{3}H]amezinium-free medium for 210 min. The sympathetic nerves were stimulated 4 times for 3 min each (S_1–S_4). Abscissa, time of superfusion. Ordinate, fractional rate of outflow of [^{3}H]amezinium. Means ± S.E. (a) Control experiments (n = 4) and experiments in which either calcium was omitted (n = 3) or tetrodotoxin (n = 3) or cocaine (n = 3) added 15 min before S_3. (b) Experiments in which either desipramine (n = 3) or yohimbine (n = 3) or clonidine (n = 3) was added 15 min before S_3.

re-uptake of [^{3}H]amezinium was inhibited. Like the exocytotic release of noradrenaline, that of [^{3}H]amezinium could be modified via presynaptic α-adrenoceptors [11] as shown by the facilitatory effect of yohimbine and the inhibitory effect of clonidine.

In conclusion, our experiments show than amezinium, at least at low concentrations, is selectively taken up into postganglionic sympathetic axons, partly sequestered in the vesicles, and released by action potentials. The results extend our knowledge of the mode of action of this new sympathomimetic compound and identify a structurally novel substrate of both the noradrenaline transport mechanism of the axolemma and the transport mechanism of the noradrenaline-storing synaptic vesicles.

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