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Increased release of xylocholine (TM10) from guinea-pig vas deferens treated with amphetamine sulphate

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Although both *NN*-diethyl- and *NN*-dimethyl-, *N*-methyl-[^{14}C]-2-(2,6-xylyloxy)ethylammonium iodide (^{14}C-1DE10 iodide and ^{14}C-TM10 iodide; xylocholine) are taken up by guinea-pig vas deferens to a similar extent *in vitro*, only TM10 reduced the response of the vas deferens to electrical stimulation. Prior incubation of vas deferens with ^{14}C-1DE10 iodide or with ^{14}C-TM10 iodide followed by measurement of the amount of each of these compounds eliminated from the tissue by repeated washing with Krebs solution, shows both compounds to be strongly retained by the tissue. Incorporation of 10^{-5} g/ml amphetamine sulphate in the wash fluid failed to modify the release of ^{14}C-1DE10 iodide but produced a short-lived increase in the amount of ^{14}C-TM10 iodide eliminated which reached a maximum approximately 10 min after administration of amphetamine. The additional ^{14}C-TM10 iodide eliminated by amphetamine was about 5% of the total tissue ^{14}C-TM10 iodide content; the possible role of this fraction is discussed in relation to the adrenergic neuron-blocking action of ^{14}C-TM10 iodide.

Although amphetamine sulphate prevents the onset of adrenergic neuron blockade and also reverses a pre-existing blockade of the guinea-pig vas deferens by TM10 [*NNN*-trimethyl-2-(2,6-xylyloxy)ethylammonium bromide] it does not cause a significant reduction in the tissue content of TM10 (Dean & Hughes, 1971). The possibility that the reversal of a TM10 blockade by amphetamine is due to displacement of only a small proportion of the total TM10 content has been tested in the present experiments by measuring the release of ^{14}C-TM10 iodide from the vas deferens. The uptake, release and blocking ability of 1DE10 [*NN*-diethyl-*N*-methyl-2-(2,6-xylyloxy)ethylammonium bromide] on guinea-pig vas deferens has also been determined; structurally, this compound is closely related to TM10 and has similar local anaesthetic properties but little adrenergic neuron blocking activity (Fielden, Roe & Willey, 1964; Clark & Hughes, 1966).

METHODS

Synthesis of NN-*diethyl*-N-*methyl*-[^{14}*C*]-2-(2,6-*xylyloxy*)*ethylammonium iodide* (^{14}*C*-1*DE*10 *iodide*). Methyl iodide [^{14}C] (1·0 mCi; 0·018 mmol) was condensed *in vacuo* onto *NN*-diethyl-2-(2,6-xylyloxy)ethylamine (0·475 mmol) in dry acetone (5 ml) and the mixture was allowed to stand at room temperature for 5 days. Methyl iodide (0·739 mmol) was then condensed on to the reaction mixture which was allowed to stand at room temperature for a further 5 days. The acetone and excess methyl iodide were removed in a vacuum and a crystalline deposit of ^{14}C-1DE10 iodide remained. Recrystallization from 2:1 dry acetone/light petroleum (bp 60°–80°)

followed by the further addition of light petroleum to the mother liquor yielded 128 mg (0·353 mmol) of material mp 121·5–122·5°. [A sample of material prepared under the same conditions but omitting the addition of methyl iodide [^{14}C] had a mp of 121–122°. Found: C, 49·75; H, 6·95; N, 3·7%. $C_{15}H_{26}ION$ requires C, 49·6; H, 7·2; N, 3·8%.]

Thin-layer chromatography of the ^{14}C-1DE10 iodide followed by scanning of the plates in a Tracerlab Scanner showed a single spot in each of the five thin layer chromatography systems described previously (Dean & Hughes, 1971).

Adrenergic neuron blockade. Vasa deferentia from adult guinea-pigs (400–700 g) were set up for electrical stimulation either transmurally (Birmingham & Wilson, 1963) or through the hypogastric nerve (Huković, 1961) (with the electrodes close to the organ) in Krebs solution (NaCl, 6·9; KCl, 0·35; $CaCl_2 6H_2O$, 0·65; $MgSO_4 7H_2O$, 0·28; K_2HPO_4, 0·16; $NaHCO_3$, 2·1; glucose, 2·0 g/litre) at 37° bubbled with 5% carbon dioxide in oxygen. The parameters for stimulation of the hypogastric nerve (TM10 experiments) were 0·5 ms rectilinear pulses, 20 V, 50 Hz delivered as four trains of pulses at 15 s intervals, each train lasting 5 s; those for transmural stimulation (1DE10 experiments) were 0·5 ms rectilinear pulses, supramaximal voltage (usually 20 V), 50 Hz for 5 s every 15 min. Contractions were recorded using an isotonic transducer (load $\simeq$500 mg) and Heathkit chart recorder. Contractions caused by hypogastric nerve stimulation were expressed as a percentage of the maximal contraction to methacholine (10^{-4} g/ml).

Release of ^{14}C-TM10 iodide. The vas deferens, mounted on its electrode, was incubated for 1·5 h in Krebs solution at 37° containing 2·23 μg/ml *NN*-dimethyl-*N*-methyl-[^{14}C]-2-(2,6-xylyloxy)ethylammonium iodide (^{14}C-TM10 iodide: specific activity 1·31 mCi/mmol). The preparation was then transferred to an organ bath and the bath fluid changed at 2 min intervals with a known volume (usually 4–5 ml) of preheated solution supplied from one of two alternative systems. At 48 min the supply of fluid was changed from the first system containing Krebs solution, to the second system containing Krebs solution alone (6 experiments) or Krebs solution plus 10^{-5} g/ml amphetamine sulphate (6 experiments). The tissue was exposed to methacholine (10^{-4} g/ml) for 60 s beginning at 27 min and the hypogastric nerve was stimulated as described above at 34 and 60 min. A 1·0 ml aliquot of each 2 min washout sample from the organ bath was mixed with 10 ml of a dioxan based scintillator and counted for ^{14}C in a Packard Liquid Scintillation Spectrometer (model 3320). The gross counts were corrected for background, counting efficiency (by an external standard channels ratio method) and ratio of sample volume to organ bath volume and results are expressed as ng ^{14}C-TM10 iodide released by the tissue in each 2 min period. At the end of the experiment the tissue ^{14}C-content was determined by a combustion method described previously (Dean & Hughes, 1971) and was expressed as ng ^{14}C-TM10 iodide per tissue uncorrected for recovery of ^{14}C from the combustion process. For each group of experiments the means and standard errors of the tissue ^{14}C-TM10 iodide content, the amounts of ^{14}C-TM10 iodide released and the mean tissue responses were calculated. Tests for statistical significance were made using Students *t*-test.

Uptake of ^{14}C-1DE10 iodide. Prepared tissues were incubated at 37° in Krebs solution with 2·34 or 7·02 μg/ml ^{14}C-1DE10 iodide and the tissue content of ^{14}C was determined at various times as described previously (Dean & Hughes, 1971). Results are expressed as μg ^{14}C-1DE10 iodide/g tissue (wet weight) and are uncorrected for

recovery of ^{14}C from the combustion process which was 94·1 ± 1·1% (mean ± s.e.).

Release of ^{14}C-1DE10 iodide. After incubation of prepared tissues for 1·5 h with 2·34 μg/ml *NN*-diethyl-*N*-methyl-[^{14}C]-2-(2,6-xylyloxy)-ethylammonium iodide (^{14}C-1DE10 iodide: specific activity 2·10 mCi/mmol) the release of ^{14}C-1DE10 iodide from the tissue was determined as described above except that electrical stimulation and administration of methacholine were omitted. Results were expressed in terms of ^{14}C-1DE10 iodide.

Drugs used were: (±)-amphetamine sulphate, methacholine chloride and *NN*-dimethyl-, and *NN*-diethyl-, *N*-methyl-2-(2,6-xylyloxy)-ethylammonium (TM10 and 1DE10 respectively) as the bromides or iodides (labelled with *N*-methyl-[^{14}C] where appropriate). All concentrations are expressed in terms of these salts.

RESULTS

Release and effectiveness of ^{14}C-TM10 iodide

Adrenergic neuron blockade. Each vas deferens responded to methacholine (10^{-4} g/ml) with a large contraction. The first period of stimulation of the hypogastric nerve produced a response approximately 30% of that shown to methacholine and a similar response (32%) was obtained to the second period of stimulation when Krebs solution alone was used throughout the washout procedure. In those tissues where Krebs solution plus 10^{-5} g/ml amphetamine sulphate was used during the second part of the washout procedure the response to the second period of electrical

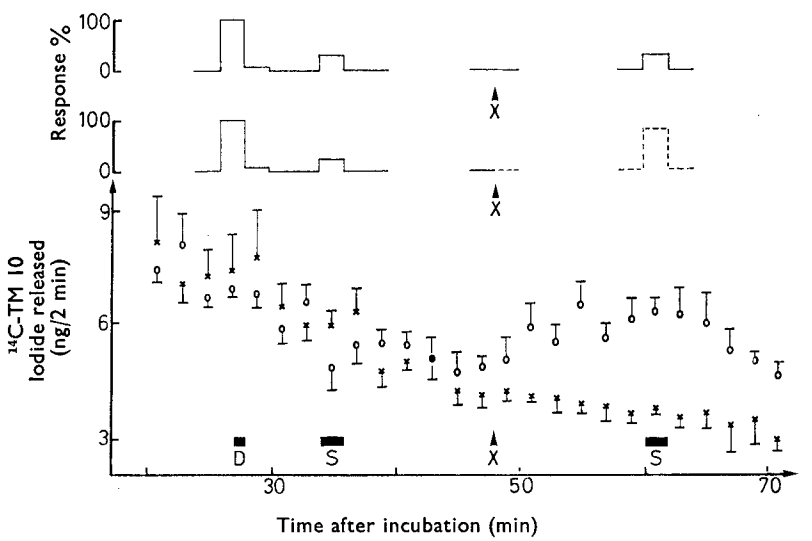

FIG. 1. *Upper records:* Reversal by amphetamine of a ^{14}C-TM10 iodide blockade of the effects of electrical stimulation of the hypogastric nerve. Mean responses of tissues expressed as a percentage of the response to methacholine. *Lower records:* Effect of amphetamine on the mean (plus or minus standard error) amounts of ^{14}C-TM10 iodide released from the vas deferens (ng/2 min). Tissue incubated at 37° in Krebs solution containing 2·23 μg/ml ^{14}C-TM10 iodide for 1·5 h and then washed by drainage every 2 min. Abscissa shows time (min) after completion of incubation. D—methacholine 10^{-4} g/ml, S—4 trains of pulses at 15 s intervals each train lasting 5 s and consisting of 0·5 ms duration; 20 V pulses delivered at 50 Hz. At X the wash solution was changed from wash solution supply system 1 (Krebs solution alone) to wash supply system 2 which contained either Krebs solution alone (Rt.H. top solid line) (6 expts: × and ——) or Krebs solution plus 10^{-5} g/ml amphetamine sulphate (Rt.H. broken line) (6 expts: ○ and – – –). Coincident point is shown as ●. In these experiments the bath volume averaged 4·4 ml.

stimulation was much greater (85%) than that obtained from those tissues washed with Krebs solution alone (Fig. 1: upper records).

Release. The amounts of ^{14}C-TM10 iodide eliminated from the tissue in the wash fluid were high initially ($\simeq$40 ng/2 min) but fell to low levels within 6–10 min of the start of the washout procedure. Electrical stimulation of the hypogastric nerve and application of methacholine both failed to produce any significant increase in the amount of ^{14}C-TM10 iodide released by the tissue either in the 2 min period during which these treatments were applied or in the succeeding 2 min period ($P > 0{\cdot}3$ in all cases). During the first washout procedure with Krebs solution alone, there was no significant difference in the amounts of ^{14}C-TM10 iodide eliminated from the two groups of tissues in any corresponding 2 min period ($P > 0{\cdot}2$ in all cases) (Fig. 1). During the second washout procedure however, those tissues washed with Krebs solution plus amphetamine released significantly more ^{14}C-TM10 iodide than those washed with Krebs solution alone. Although there was no significant difference in the amounts eliminated between minutes 48 and 50 ($P < 0{\cdot}1$ but $> 0{\cdot}05$) (i.e., the first period after the application of amphetamine), the amounts eliminated between minutes 50 and 52 ($P < 0{\cdot}02$) and in all 2 min periods from minute 52 to the end of the experiment ($P < 0{\cdot}01$) were significantly greater for those tissues washed in Krebs solution plus amphetamine (Fig. 1). Integration of the areas under the mean points on the two curves shows that in the 24 min from the first application of amphetamine, 63·8 ng ^{14}C-TM10 iodide were eliminated, while the corresponding figure for each tissue washed in Krebs solution alone was 41·4 ng.

At the end of the experiment the ^{14}C-TM10 iodide content of the group of tissues maintained in Krebs solution alone throughout the washout procedure was 583·3 $\pm$ 47·4 ng/tissue while those treated with Krebs solution plus amphetamine during the second part of the washout procedure contained 545·7 $\pm$ 59·4 ng/tissue (mean $\pm$ s.e.). These values are not significantly different ($P > 0{\cdot}5$). The mean weights of the tissues in these two groups were 92·0 $\pm$ 3·3 and 90·4 $\pm$ 6·2 mg respectively and were not significantly different ($P > 0{\cdot}8$).

Effect of 1DE10 bromide on the response of the vas deferens to transmural stimulation. At concentrations of 2·04 and 6·12 μg/ml (equivalent to 2·34 and 7·02 μg/ml 1DE10 iodide) no reduction in the response to transmural stimulation was produced during the 1·5 h period in which the tissue was exposed to the drug (5 expts. at each concentration).

Uptake of ^{14}C-1DE10 iodide. Incubation of vas deferens with 2·34 or 7·02 μg/ml ^{14}C-1DE10 iodide produced a steady increase in the amount of compound in the tissue and the tissue concentrations attained after various times of incubation are shown in Fig. 2. When tissues which had been previously incubated for 1·5 h in 2·34 μg/ml ^{14}C-1DE10 iodide were washed and allowed to remain in drug-free Krebs solution for 30 or 60 min, there was little fall in the tissue content of ^{14}C-1DE10 iodide (Fig. 2).

Release of ^{14}C-1DE10 iodide. The amounts of ^{14}C-1DE10 iodide eliminated from the tissues were high and variable initially but fell quickly (8–12 min) to low concentrations and then declined slowly throughout the remainder of the experiment. At no time was there any significant difference ($P > 0{\cdot}1$ in all cases) in the amounts of ^{14}C-1DE10 iodide eliminated in corresponding 2 min periods between those tissues

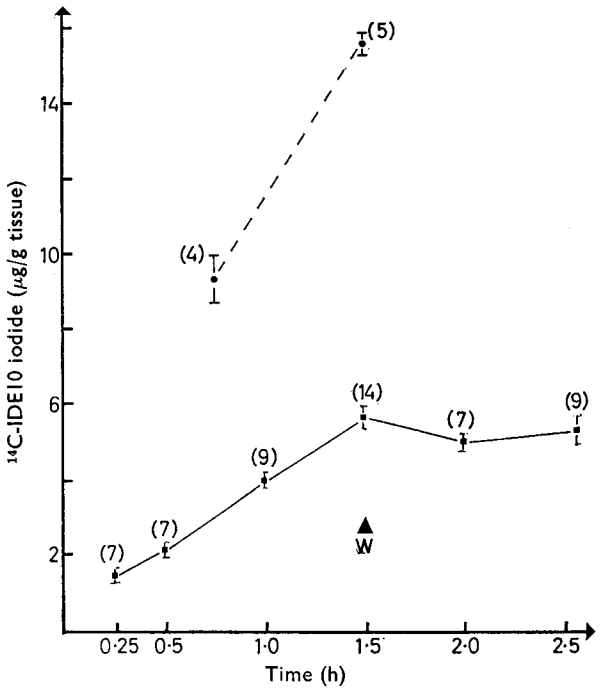

FIG. 2. Means and standard errors of the tissue concentrations of ^{14}C-1DE10 iodide attained in guinea-pig vas deferens (μg/g) on incubation at 37° in Krebs solution with 2·34 (■—■) or 7·02 (●- - -●) μg/ml ^{14}C-1DE10 iodide for various times. At W, some tissues were transferred to a tissue bath containing fresh drug-free Krebs which was renewed every 15 min. The figures in parentheses indicate the number of tissue estimations contributing to each point.

washed with Krebs solution alone throughout the experiment and those washed with Krebs solution initially and then with Krebs solution plus 10^{-5} g/ml amphetamine (Fig. 3).

At the end of the experiment there was no significant difference ($P > 0{\cdot}3$) in the

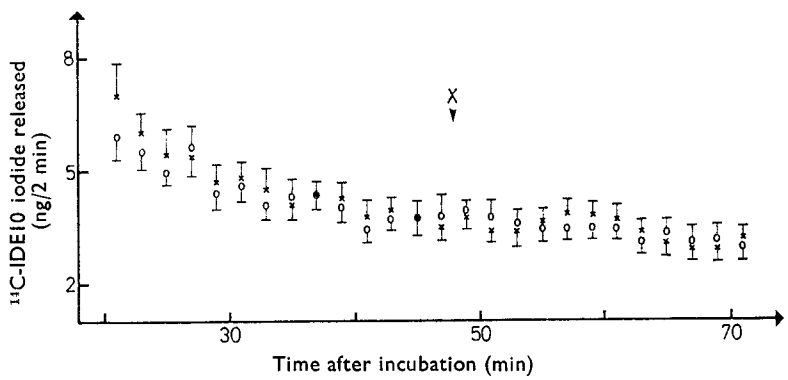

FIG. 3. Effect of amphetamine on the release of ^{14}C-1DE10 iodide. Vas deferens, incubated for 1·5 h in Krebs solution at 37° containing 2·34 μg/ml ^{14}C-1DE10 iodide and then washed by drainage every 2 min. Abscissa shows time (min) after completion of incubation. Ordinate shows mean (plotted plus or minus standard error) amounts of ^{14}C-1DE10 iodide released (ng) per 2 min period. At X, the wash solution was changed from wash solution supply system 1 (Krebs solution alone) to wash supply system 2 which contained Krebs solution alone (6 expts: +) or Krebs solution plus 10 μg/ml amphetamine sulphate (6 expts: ○). (Coincident points are shown as ●). (In these experiments the bath volume averaged 4·3 ml.)

^{14}C-1DE10 iodide content of those tissues washed in Krebs solution alone (372·6 ± 43·8 ng/tissue) and those washed in Krebs solution plus amphetamine (427·0 ± 24·3 ng/tissue: neither was there any significant difference ($P > 0·9$) between the mean weights of the tissues which were 79·6 ± 11·3 and 89·3 ± 7·2 mg respectively.

DISCUSSION

It has been assumed that all ^{14}C is present as ^{14}C-TM10 or ^{14}C-1DE10 as appropriate.

Both TM10 iodide (Dean & Hughes, 1971) and 1DE10 iodide accumulate in vas deferens and are strongly retained by this tissue since prolonged washing leads to little fall in the gross tissue content and the amounts of the compounds eliminated in the wash fluids are small (the initial high concentrations are probably due to carry-over of the incubation fluid in the electrode assembly and tissue holder).

Although TM10 and 1DE10 are very similar structurally, and have similar local anaesthetic potencies (Clark & Hughes, 1966), only TM10 is effective in reducing the response of the vas deferens to electrical stimulation at low concentrations in spite of the fact that gross tissue concentrations of 1DE10 can be much higher than those attained with TM10. It would appear therefore that gross tissue concentrations and local anaesthetic potency cannot be correlated with adrenergic neuron-blocking activity in these two compounds.

At a concentration of 10^{-5} g/ml amphetamine antagonized the adrenergic neuron-blocking action of TM10 and increased the amounts of ^{14}C-TM10 released but failed to modify the rate of release of 1DE10. The additional TM10 eliminated by washing with amphetamine-containing solutions was about 22 ng per tissue and represents less than 5% of the tissue content of TM10.

As a partial reversal of the adrenergic neuron-blocking action of TM10 occurs within 30 s of the administration of amphetamine (Dean & Hughes, 1971) but increased release of TM10 does not occur until after 2 min, it is possible that the two effects of amphetamine are unrelated. However, this difference can be explained by TM10 taking some time to diffuse out of the tissue from its site of action.

It is also possible that the increased release of TM10 by amphetamine may be due to reduced uptake of TM10. However, even if the vas deferens were able to take up appreciable quantities of TM10 at the low concentrations present in the tissue bath during washout, it might be expected that blockade of a significant reuptake would lead to a sustained rise in the amounts of TM10 eliminated from the tissue. Fig. 1 shows that the amounts of TM10 eliminated towards the end of the experiment drop sharply and that the higher rate of release seen shortly after the administration of amphetamine is not maintained.

The results may indicate that only a small proportion of the total tissue content of TM10 is involved in the production of adrenergic neuron-blockade and that the ability of amphetamine to displace this small amount may account for its ability to reverse the blockade.

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